UNIVERSIDADE DE TRÁS-OS-MONTES E ALTO DOURO

Aluminum tolerance in *Secale* species: genetic diversity, molecular mechanisms and gene characterization

Tese de Doutoramento em Genética Molecular Comparativa e Tecnológica

Elisabete Cristina Jesus dos Santos

Orientadora: Professora Doutora Maria Manuela de Outeiro Correia de Matos

Coorientador: Professor Doutor César Benito Jiménez

VILA REAL, 2018

Tese elaborada com vista à obtenção de grau de Doutor em Genética Molecular Comparativa e Tecnológica pela Universidade de Trás-os-Montes e Alto Douro, realizada sob a orientação científica da Professora Doutora Maria Manuela de Outeiro Correia de Matos, Professora auxiliar do Departamento de Genética e Biotecnologia da Universidade de Trás-os-Montes e Alto Douro e coorientação do Professor Doutor César Benito Jiménez, Professor Catedrático do Departamento de Genética da Universidade Complutense de Madrid.

VILA REAL, 2018

Declaro para os devidos fins que a Tese de Doutoramento atende as normas técnicas e científicas exigidas pelos regulamentos em vigor da Universidade de Trás-os-Montes e Alto Douro. Declaro, ainda, que as doutrinas apresentadas nesta Tese de Doutoramento são da inteira responsabilidade do autor.

Tese de doutoramento financiada pela Fundação para a Ciência e Tecnologia, "Programa Operacional Potencial Humano – POPH", cofinanciado pelo Fundo Social Europeu (POPH/FSE) e por fundos nacionais (POPH-QREN).

utad Universidade de Trás-os-Montes e Alto Douro

EDITAL

Doutor Artur Agostinho de Abreu e Sá, Presidente da Escola de Ciências da Vida e do Ambiente da Universidade de Trás-os-Montes e Alto Douro, Presidente do Júri, por delegação de competências, das Provas de Doutoramento em Genética Molecular Comparativa e Tecnológica, requeridas por **Elisabete Cristina Jesus dos Santos**, faz saber que:

1. O júri das referidas provas é constituído pelos seguintes vogais:

   a) Doutora Maria Susana Jorge Pereira, Professora Associada da Universidade do Porto (*arguente*);
   b) Doutor Francisco Javier Gallego Rodríguez, Professor Titular da Universidad Complutense de Madrid (*arguente*);
   c) Doutor José Eduardo Lima Brito, Professor Associado da Universidade de Trás-os-Montes e Alto Douro (*vogal*);
   d) Doutora Fernanda Maria Madaleno Rei Tomás Leal Santos, Professora Auxiliar da Universidade de Trás-os-Montes e Alto Douro (*vogal*);
   e) Doutor César Benito Jiménez, da Universidad Complutense de Madrid (*orientador*);
   f) Doutora Maria Manuela do Outeiro Correia de Matos, Professora Auxiliar da Universidade de Trás-os-Montes e Alto Douro (*orientadora*).

2. A tese apresentada tem por título "***Aluminum tolerance in Secale species: genetic diversity, molecular mechanisms and gene characterization***".

3. O ato público de defesa da tese realiza-se no dia 4 de outubro de 2018, pelas 9.30 horas, no Auditório de Geociências da Universidade de Trás-os-Montes e Alto Douro.

4. Concluídas as provas, o júri reúne para proceder à apreciação e respetiva qualificação, por votação nominal fundamentada, cujo resultado constará de ata.

Universidade de Trás-os-Montes e Alto Douro, 14 de setembro de 2018.

O Presidente da Escola,

Artur Agostinho de Abreu e Sá

Escola de Ciências da Vida e do Ambiente
Quinta de Prados 5000-801 Vila Real

To my beloved ones

"Our greatest glory is not in never falling
but in rising every time we fall"

Confucius

AGRADECIMENTOS

À Universidade de Trás-os-Montes e Alto Douro, na pessoa do Magnífico Reitor Professor Doutor António Augusto Fontainhas Fernandes assim como do anterior Reitor Professor Doutor Carlos Alberto Sequeira, agradeço a disponibilização dos meios físicos para a realização desta tese.

À Escola de Ciências da Vida e do Ambiente, na pessoa do atual Professor Doutor António Luís Herculano Melo de Carvalho, agradeço a disponibilização dos meios físicos da Escola para a realização deste trabalho.

À Comissão do curso de Doutoramento em Genética Molecular Comparativa e Tecnológica (3º Ciclo), atualmente na pessoa da Professora Doutora Fernanda Leal e a todos os anteriores Diretores, agradeço a admissão no curso permitindo-me a realização deste doutoramento. Agradeço também aos vários professores pelos ensinamentos prestados.

Ao Instituto de Bioengenharia e Biotecnologia, Centro de Genómica e Biotecnologia da Universidade de Trás-os-Montes e Alto Douro (IBB/CGB-UTAD), nas pessoas dos seus antigos Diretores, os Professores Doutores Arlete Mendes-Faia e Henrique Guedes-Pinto, agradeço os meios para a realização desta tese e a oportunidade de ter feito parte do grupo como bolseira de doutoramento.

Ao BioISI – Biosystems and Integrative Sciences Institute, em particular à Diretora Professora Doutora Margarida Amaral e ainda ao Professor Doutor Rui Malhó, líder do grupo PFG – "Plant Functional Genomics", agradeço por nos receber no seu grupo a fim de desenvolver a nossa atividade científica.

À Fundação para a Ciência e a Tecnologia (FCT), agradeço a concessão da bolsa mista de doutoramento (SFRH/BD/65040/2009) que permitiu a realização deste trabalho.

À minha orientadora, a Professora Doutora Manuela Matos, agradeço por confiar nas minhas capacidades e me dar a oportunidade de elaborar esta tese, dando-me autonomia no laboratório. Agradeço os muitos ensinamentos, sugestões e correções que me permitiram evoluir profissionalmente. Agradeço todo o tempo dedicado a mim e a este projeto. Agradeço ainda a amizade mas acima de tudo a paciência e compreensão quando tudo se tornou mais difícil e por nunca deixar de acreditar em mim.

Ao meu coorientador, o Professor Doutor César Benito, agradeço o voto de confiança para me acolher no seu laboratório a fim de realizar grande parte dos trabalhos laboratoriais desta tese, agradeço o facto de me ter recebido sempre de braços abertos e da total entrega neste projeto. Agradeço os conselhos sábios que me ajudaram a ultrapassar cada dificuldade que ia surgindo e as muitas horas dedicadas a esclarecimento de dúvidas. Agradeço os muitos ensinamentos assim como as correções minuciosas desta tese. Agradeço ainda a amizade, a motivação, o apoio constante nas minhas estadias por Madrid tanto a nível profissional como pessoal e por me ter feito sempre sentir em casa.

À Professora Doutora Olinda Pinto-Carnide, agradeço os ensinamentos ao longo deste percurso e a oportunidade de dar aulas na área da Genética, permitindo-me alargar a minha expertise e incentivando-me para seguir para doutoramento. Agradeço a ajuda na obtenção da bolsa de doutoramento e também as correções dos artigos da tese.

Ao Departamento de Genética da Universidade Complutense de Madrid (UCM), agradeço por me terem acolhido e permitido desenvolver os trabalhos laboratoriais desta tese nas suas instalações. A todos os docentes integrantes agradeço a hospitalidade e todo o apoio prestado sempre que necessário, em particular à Professora Doutora Ana Figueiras e ao Professor Doutor Javier Gallego. Um especial agradecimento aos técnicos Mari Carmen e José que são de facto incansáveis e sempre prestáveis, obrigada acima de tudo pelo carinho. Aos meus colegas de laboratório Ana Luísa, Roberto, Javier Silva, Diaa, Bea, Raquel, Gulce, Naike, Andrea e Marta, agradeço os bons momentos passados no laboratório, o companheirismo, a entreajuda e a amizade, obrigada por tornar a minha estadia muito mais agradável e por tornar este doutoramento menos custoso.

Ao Departamento de Genética e Biotecnologia (DGB), agradeço por me terem recebido tão bem e me terem permitido fazer parte desta pequena família desde 2006, agradeço o carinho e a amizade dos vários professores integrantes.

Ao Professor Doutor José Eduardo Lima-Brito, anterior Diretor do DGB, agradeço a amizade e também os conselhos sábios mas sobretudo o apoio nas fases mais difíceis, não esquecerei as palavras de força e incentivo quando mais precisei.

Agradeço com carinho a todos os funcionários não docentes que pertenceram ou ainda pertencem ao DGB pela ajuda no desenvolvimento dos trabalhos de campo, pela boa disposição, tornando tudo mais fácil e agradável recebendo-me sempre com um sorriso, obrigada em especial à Paula, à Alexandrina, ao Sr. Ilídio, ao Sr. Alcídio e à Dona Cecília.

Agradeço a todos os colegas dos vários laboratórios do DGB que se cruzaram no meu caminho ao longo deste percurso e que de algum modo contribuíram para a elaboração deste trabalho, agradeço o companheirismo, a entreajuda, o convívio, a amizade, a motivação, os bons momentos vividos no laboratório, tornando tudo mais fácil ajudando-me a chegar ao fim desta meta.

À Ana Isabel um especial agradecimento pela amizade e apoio e por me ajudar sempre que necessário, pelos vários conselhos e ensinamentos e por me conseguir motivar sobretudo na fase final.

À Márcia agradeço ter estado presente nos momentos mais difíceis sem pedir nada em troca, agradeço a amizade e apoio constantes ao longo de todo este percurso. Agradeço a preocupação e a motivação sempre que as forças faltavam. Obrigada por sempre teres um gesto e uma palavra amiga e por todos os convívios que me proporcionaste ajudando-me a ultrapassar os momentos mais difíceis.

À Ana Luzio agradeço o positivismo, as boas energias transmitidas que me ajudavam a erguer sempre que tudo se tornava mais difícil. Agradeço sobretudo a força e a disponibilidade aquando da nossa estadia em Madrid quando o meu mundo desmoronou, foste incansável. Obrigada por todo o tempo que me dedicaste, por nunca me deixares baixar os braços e por estares lá sempre para mim.

À Maria João agradeço os conselhos sábios e acima de tudo por se ter mantido na minha vida, mesmo depois de estarmos fisicamente mais afastadas. Obrigada pela amizade e pelo apoio constantes, pela preocupação de mãe e por todas as palavras de motivação e incentivo.

Às minhas meninas, Cátia, Nat, Raq e Patrícia agradeço o apoio incondicional e por terem sido o meu pilar ao longo de grande parte deste percurso, a meio desta viagem a vida levou-vos para longe de mim cada uma a seu tempo mas os nossos laços mantiveram-se, obrigada por poder contar sempre convosco mesmo longe.

À Bela e ao Federico agradeço a hospitalidade, a bonita amizade e por me terem feito sempre sentir em casa facilitando a minha estadia por Madrid. Obrigada por toda a força e palavras de incentivo quando os dias corriam menos bem.

À Dra. Susana Mendonça Costa, ao Dr. Carlos Ferreira e ao Dr. Jorge Furtado que infelizmente já não se encontra entre nós, agradeço tudo o que fizeram por mim permitindo-me chegar ao fim deste doutoramento.

Aos muitos amigos de Vale das Fontes agradeço a lufada de ar fresco que me foram facultando ao longo destes anos, permitindo-me restabelecer energias para prosseguir com esta luta, obrigada por todos os bons momentos vividos ao longo desta jornada.

Ao meu Vi, a minha cara-metade, que foi a luz que iluminou o meu caminho quando a escuridão era palavra de ordem e me ajudou a erguer e a encontrar de novo forças para prosseguir com a luta. Agradeço a paciência, bem sei que nem sempre é fácil lidar com o meu stress e a minha ansiedade. Obrigada por seres o melhor ouvinte e fazeres um esforço por entender os meus dramas mesmo não sendo fácil para ti. Obrigada por me apoiares sempre, obrigada pelo teu positivismo e por acreditares sempre em mim e nas minhas capacidades. Obrigada acima de tudo pelo teu amor e compreensão e por seres o meu antidepressivo natural "*moon of my life*".

Aos meus pais, agradeço por apoiarem sempre as minhas decisões e confiar nas minhas capacidades, agradeço a preocupação e por proporcionarem o seguimento da minha carreira científica. Obrigada pela oportunidade de chegar mais longe e ter uma vida melhor. Obrigada também pela paciência nestes anos difíceis, sei que nem sempre foi fácil, obrigada por me darem a mão sempre que necessário sem questionar nada e por nunca deixarem de acreditar em mim.

À minha grande família, tios, primos, irmão, afilhado, agradeço cada momento vivido, cada palavra de amizade, de reconforto e de carinho, cada gesto de motivação. Obrigada por me ajudarem a ultrapassar as fases mais difíceis e conseguir chegar ao fim desta árdua meta. Obrigada acima de tudo por ouvirem os meus desabafos e por terem sempre um ombro e um abraço amigo.

Às estrelinhas que partiram antes de esta jornada chegar ao fim, obrigada, espero que tenham orgulho em mim e que me continuem a guiar.

MUITO OBRIGADA!!!

RESUMO

O alumínio (Al) é um dos principais constituintes do solo que apenas solubiliza quando o pH se torna ácido (≤ 5.5), convertendo-se numa das formas mais fitotóxicas (Al^{3+}). A toxicidade deste metal, nos abundantes solos ácidos, é uma das principais limitações da produtividade agrícola em grande parte do mundo. O centeio (*Secale cereale* L.) é uma cultura importante pela sua capacidade de resistir a stresses bióticos e abióticos. Dentro dos cereais, o centeio é o que mais tolera a exposição ao Al tendo, por isso, um fundo genético valioso para estudos de melhoramento genético. Pouco ou nada se sabe sobre o comportamento das espécies silvestres de centeio e, torna-se importante o seu estudo a fim de garantir novos recursos genéticos para contornar esta restrição no desenvolvimento das plantas, respondendo assim às necessidades crescentes da alimentação Mundial. Neste trabalho, foram estudadas seis espécies/subespécies silvestres assim como sete cultivares de centeio em diferentes contextos.

Numa primeira abordagem (Capítulo II), usaram-se os marcadores moleculares dominantes *RAPDs* (*Random Amplified Polymorphic DNA*) e *ISSRs* (*Inter Simple Sequence Repeats*) para estudos de diversidade molecular, de relações genéticas e de associação quanto à característica da tolerância ao Al. Estes marcadores revelaram-se bastante informativos, obtendo um elevado grau de polimorfismo tanto no estudo de *bulks* como de genótipos individuais, o que permitiu aferir com sucesso a vasta variabilidade genética existente nos centeios em estudo. Foi possível determinar relações genéticas fiáveis com o recurso destes marcadores, uma vez que se obtiveram um grande número de fragmentos de DNA. A taxonomia do género *Secale* ainda é alvo de muita controvérsia e com estes dados contribuímos para aumentar o conhecimento adquirido e melhorar a classificação, uma vez que é o primeiro estudo publicado de relações genéticas dentro do género *Secale* com o uso de ambos os marcadores (*RAPDs* e *ISSRs*). Também se fizeram estudos de filogenias com os genes *ScMATE1* e *ScMATE3* (*Multidrug and Toxic Compound Extrusion*) que se mostraram similares. Todos estes dados levaram-nos a reconhecer três espécies de centeio (*S. cereale*, *S. strictum* e *S. sylvestre*) sendo possivelmente os ancestrais o *S. strictum* e o *S. sylvestre*.

Os centeios foram caracterizados quanto à sua tolerância ao Al através do método de hidroponia, que se revelou bem-sucedido. A maioria dos centeios (cultivados e silvestres) testados mostrou uma grande capacidade de resistir ao choque provocado pelo Al e de

recuperar a elevadas concentrações deste metal tóxico. Para além disso, encontrou-se uma vasta variabilidade quanto ao comportamento das plantas ao stress do Al quer intra- e interespecífica como intra- e intercultivar. A característica altamente polimórfica dos *RAPDs* e dos *ISSRs* atribui-lhes potencial na identificação de marcadores genéticos ligados à tolerância ao Al. De facto, foram encontrados 34 fragmentos de DNA com ambas as técnicas com associação a este traço genético que podem no futuro ser convertidos em *SCARs* (*Sequence-characterized amplified region*). Ambos os sistemas de marcadores se revelaram eficazes em todas as análises efetuadas, com especial destaque para os *ISSRs*, fornecendo recursos importantes para a seleção assistida por marcadores moleculares (MAS) quanto à tolerância ao Al.

Numa segunda abordagem (Capítulo III), estudaram-se genes possivelmente envolvidos na tolerância ao Al assim como distúrbios relacionados com a toxicidade do Al, de modo a melhor entender o controlo genético e os mecanismos de resistência ao Al envolvidos em cada centeio. Pela primeira vez em centeios silvestres, foram estudados oito genes candidatos (*ScALMT1*, *ScMATE1*, *ScMATE2*, *ScSTOP1*, *ScMDH1*, *ScMDH2*, *ScCu/ZnSOD* e *ScMnSOD*) possivelmente envolvidos na tolerância ao Al. Todos eles parecem contribuir com o aumento da tolerância ao Al de, pelo menos, um centeio silvestre, com os genes *ScALMT1* e *ScMATE2* a ter um papel fundamental. Foi observado na maioria das raízes de centeio um padrão de exsudação de ácidos orgânicos induzível, com dois tempos distintos de libertação, enquanto uma minoria apresentou um padrão constitutivo, mas em quantidade abundante. Quatro perturbações celulares (acúmulo de Al, deteção de H_2O_2 e visualização de peroxidação lipídica e de morte celular) relacionadas com a toxicidade do Al em raízes mostraram estar correlacionados entre si e também, com os recrescimentos radiculares. As estratégias dos centeios tolerantes ao Al parecem basear-se nos mecanismos de exclusão e de desintoxicação.

Encontrámos um gene candidato para controlar o *locus Alt1* situado no cromossoma *6RS*, que denominámos de *ScMATE3*. Os estudos de expressão e de filogenias, tal como, a existência de três *SNPs* (*single-nucleotide polymorphism*) exclusivos a centeios tolerantes, sugerem que este gene está relacionado com a tolerância ao Al. A localização subcelular prevista indica que pode ser um *MATE* tonoplástico e que podemos estar perante um mecanismo interno de tolerância.

Obtiveram-se várias sequências codificantes dos genes *ScMATE1* e *ScMATE3*, publicadas no GenBank, que mostraram variabilidade genética tanto dentro como entre

as espécies e cultivares de centeio. Encontraram-se duas cópias do gene *ScMATE1* associadas à tolerância/sensibilidade ao Al.

Pela primeira vez, os mecanismos de resistência ao Al foram elucidados nos centeios silvestres. No género *Secale*, a tolerância ao Al parece ser um traço evolutivo e geneticamente complexo onde mecanismos diferentes parecem coexistir. Vários genes aparentam estar implicados na resposta ao stress provocado pelo Al, cujo efeito cumulativo poderá ser a chave para a elevada capacidade que os centeios possuem para resistir à toxicidade do Al.

Palavras-chave: *Secale* spp.; solos ácidos; alumínio (Al); fitotoxicidade; tolerância/sensibilidade ao Al; marcadores genéticos; diversidade genética; relações filogenéticas; mecanismos de resistência; expressão de genes

ABSTRACT

Aluminum (Al) is one of the main constituents of the soil that only solubilizes when the pH becomes acidic (≤ 5.5), being converted into its most phytotoxic form (Al^{3+}). Acid soils are abundant and Al toxicity is the major limitation for plant productivity worldwide. Rye (*Secale cereale* L.) is an important crop due to its ability to resist biotic and abiotic stresses. Within cereals, rye is the one that most tolerates Al exposure and, therefore, has a valuable genetic background for Al tolerance genetic improvement. Little or nothing is known about the behavior of wild rye relatives, and its study is crucial in order to guarantee new genetic resources to circumvent this constraint on plant development, thus keeping up with the rising world food demands. In this work, six wild species/subspecies and seven rye cultivars were studied in different contexts.

In a first approach (Chapter II), the dominant molecular markers RAPDs (Random Amplified Polymorphic DNAs) and ISSRs (Inter Simple Sequence Repeats) were used for molecular diversity, genetic relationships and Al tolerance association studies. These markers were very informative, reaching high polymorphism values with both bulks and individual genotypes studies. Moreover, they successfully revealed the vast genetic variability present in the ryes at study. Since a large number of DNA fragments were obtained, reliable genetic relationships were determined with both markers. The taxonomy of the genus *Secale* is still a matter of much controversy. This is the first published work in genetic relationships within this genus with both RAPD and ISSR markers, which allows us to contribute for the increment of the acquired knowledge improving the classification of the *Secale* genus. Similar phylogenetic relationships were obtained with the genes *ScMATE1* and *ScMATE3* (Multidrug and Toxic Compound Extrusion). All data led us to recognize three rye species (*S. cereale*, *S. strictum* and *S. sylvestre*) with *S. strictum* and *S. sylvestre* being possibly the ancestors.

Screening of Al tolerance was carried out through hydroponic methodologies which proved to be successful. Most of the cultivated and wild ryes showed great skills to resist and to recover Al shock at huge concentrations. In addition, a wide intra- and interspecific as intra- and intercultivar variability for Al tolerance was found. The highly polymorphic feature of ISSRs and RAPDs gives them potential for the identification of genetic markers linked to Al tolerance. Indeed, 34 loci associated with this genetic trait were found with both techniques that may be converted into SCARs (Sequence-characterized amplified

region) in the future. Both marker systems proved to be effective in all analyzes with particular emphasis on ISSRs, so providing important resources for molecular marker-assisted selection (MAS) of Al tolerance.

In a second approach (Chapter III), genes possibly involved in Al tolerance as well as cell disturbances related to Al toxicity were studied in order to better understand the genetic control and the Al resistance mechanisms involved in each rye. Eight candidate genes (*ScALMT1*, *ScMATE1*, *ScMATE2*, *ScSTOP1*, *ScMDH1*, *ScMDH2*, *ScCu/ZnSOD* and *ScMnSOD*) possibly involved in Al tolerance were studied for the first time in wild ryes. All candidate genes seem to have an active contribution on enhanced Al-tolerance of, at least, one wild rye where *ScALMT1* and *ScMATE2* have a key role. An inducible organic acid exudation pattern was observed in most rye roots with two distinct release time whereas a minority presented a constitutive pattern but in abundant quantity. Four cell disorders (Al accumulation, H_2O_2 detection and visualization of lipid peroxidation and cell death) related to Al toxicity in roots showed a positive correlation with each other's and with root regrowth measurement. Al-tolerant ryes strategies seem to be based on Al-exclusion and Al-detoxification mechanisms.

We found a candidate gene to control the *Altl* locus located on chromosome *6RS* that we named *ScMATE3*. Expression and phylogeny studies suggest that this gene is related to Al tolerance, such as the existence of three single-nucleotide polymorphisms (SNPs) exclusive to tolerant ryes. The predicted subcellular localization indicates a possible tonoplast MATE suggesting an internal Al tolerance mechanism.

Several coding sequences of *ScMATE1* and *ScMATE3* genes were obtained and published on GenBank who's showed a great genetic variability both within and between rye species/subspecies and cultivars. Two copies of *ScMATE1* gene associated with Al tolerance/sensitivity were found in the species *S. sylvestre* and *S. vavilovii*.

Al-resistance mechanisms were clarified for the first time in wild ryes. In *Secale* genus, Al tolerance appears to be an evolutionary and genetically complex trait where different mechanisms coexist. Several genes seem to be implicated in the Al-stress response of rye, whose cumulative effect may be the key to its high ability to resist Al toxicity.

Keywords: *Secale* spp.; acid soils; aluminum (Al); phytotoxicity; Al tolerance/sensitivity; genetic markers; genetic diversity; phylogenetic relationships; Al resistance mechanisms; gene expression

ABBREVIATIONS

AACT – Al-activated Citrate Transporter

ABC – ATP-binding cassette

AFLP – Amplified Fragment Length Polymorphism

Al – Aluminum

$AlCl_3$ – Al Chloride

$AlK(SO_4)_2$ – Al Potassium Sulfate

ALMT – Al-activated Malate Transporter

ALS – Al Sensitive

ALT, *Alt – Al Tolerance*

ANOVA – Analysis of variance

ART – Al-responsive Transcription Factor

At – *Arabidopsis thaliana*

Bd – *Brachypodium distachyon*

Bs – *Brachypodium stacei*

BLAST – Basic Local Alignment Search Tool

Bn – *Brassica napus*

bp, Gbp, Kb – base pair, Giga base pair, Kilobase

C_2H_2 – Acetylene

Ca – Calcium

$CaCl_2$ – Calcium Chloride

cDNA – complementary DNA

cm, mm – centimeter, millimeter

cM – centimorgan

CS – Citrate synthase

Cu – Copper

cv. – cultivar

DArT – Diversity Arrays Technology

DCF-DA – 2', 7'-dichlorofluorescein diacetate

DNA – Deoxyribonucleic Acid

DOI – Digital Object Identifier

DTZ – Distal transition zone

EDS5 – Enhanced Disease Susceptibility5

EDS5H – Enhanced Disease Susceptibility5 Homologue

e.g. – *Exempli gratia*

FAO – Food and Agriculture Organization

fd – fold difference

Fe – Iron

FH – Fumarate Hydratase

FRDL – Ferric Reductase Defective-like

FW – Fresh weight

g, mg, µg, ng – gram, milligram, microgram, nanogram

gDNA – genomic DNA

GFP – Green Fluorescence Protein

Gm – *Glycine max*

GRIN – Germplasm Resources Information Network

GST – Glutathione S-transferase

H_2O – water

H_2O_2 – Hydrogen Peroxide

HCl – Hydrochloric acid

Hd – Haplotype diversity

$HgCl_2$ – Mercuric Chloride

Hl – *Holcus lanatus*

Hv – *Hordeum vulgare*

Ib – band informativeness

i.e. – *id est*

INDEL – insertion/deletion

IPK – Institute of Plant Genetic and Crop Plant Research

ISSR – Inter Simple Sequence Repeat

k – Number of nucleotide differences

$K_2S_2O_5$ – Potassium Disulfite

kDa – kilodalton

KNO_3 – Potassium Nitrate

L, ml, µl – Litre, millilitre, microliter

LOFT – Levels of Orthology through Phylogenetic Trees

M, mM, µM – molar, millimolar, micromolar

MAS – Marker-Assisted Selection

MATE – Multidrug and Toxic Compound Extrusion

MDH – Malate dehydrogenase

MI – Marker Index

MITE – Miniature Inverted Repeat Transposable Element

$MgCl_2$ – Magnesium Chloride

Mn – Manganese

mRNA – messenger RNA

m/v – mass per volume

NaClO – Sodium Hypoclorite

NCBI – National Center for Biotechnology Information

ND – Nucleotide Diversity

NH_4NO_3 – Ammonium Nitrate

NH_4OAc – Ammonium Acetate

$(NH_4)_2SO_4$ – Ammonium Sulfate

NIP – Nodulin 26-like Intrinsic Protein

Nramp – Natural resistance-associated macrophage protein

Nrat – Nramp Aluminum Transporter

NSGC – National Small Grains Collection

O_2^- – Singlet Oxygen

OA – Organic Acids

OH – hydroxyl

OP – Operon

ORF – Open Reading Frame

Os – *Oryza sativa*

PALT – Plasma membrane-localized Aluminum Transporter

Pi – nucleotide diversity

PIC – Polymorphic Information Content

PCR – Polymerase chain reaction

PME – Pectin Methylesterase

pmol, µmol – picomole, micromole

POX – Peroxidase

ppm – parts per million

qRT-PCR – Quantitative RT-PCR

QTL – Quantitative Trait Locus

RAPD – Random Amplification of Polymorphic DNA

RFLP – Restriction Fragment Length Polymorphism

RNA – Ribonucleic acid

ROS – Reactive Oxygen Species

Rp – Resolving power

rpm – revolutions per minute

RT – Reverse transcriptase

SA – Salicylic Acid

Sb – *Sorghum bicolor*

Sc – *Secale cereale*

SC – Sequence Conservation

SCAR – Sequence-characterized amplified region

SCIM – *S. cereale* inter-microsatellite

SCM – *S. cereale* microsatellite

SD – Standard Deviation

SE – Standard Error

SOD – Superoxide Dismutase

SM – Simple Matching

SNP – Single-Nucleotide Polymorphism

spp. – Species

sqRT-PCR – Semi-quantitative RT-PCR

SSR – Simple Sequence Repeats

STAR – Sensitive to Aluminum Rhizotoxicity

STOP – Sensitive to Proton Rhizotoxicity

ssp. – Subspecies

syn. – synonym

TAE – Tris-Acetate-EDTA

Ta – *Triticum aestivum*

Taq – *Thermus aquaticus* (DNA polymerase)

TBE – Tris-Borate-EDTA

TCA – Tricarboxylic Acid

TMH – Transmembrane Helix

TT12 – TRANSPARENT TESTA12

Tu – *Triticum urartu*

UBC – University of British Columbia

UDP – Uridine diphosphate

UPGMA – Unweighed Pairwise Group Method with Arithmetic Average

USDA-ARS – United States Department of Agriculture - Agricultural Research Service

VALT – Vacuolar Aluminum Transporter

vs – versus

Vu – *Vigna umbellata*

w/v – weight per volume

XTH31 – Xyloglucan Endotransglucosylase-hydrolase31

Zn – Zinc

Zm – *Zea mays*

INDEX

INDEX OF FIGURES

CHAPTER I

CHAPTER II

SUBCHAPTER II-1

SUBCHAPTER II-2

CHAPTER III

SUBCHAPTER III-1

SUBCHAPTER III-2

SUBCHAPTER III-3

CHAPTER IV

INDEX OF TABLES

CHAPTER II

SUBCHAPTER II-1

SUBCHAPTER II-2

CHAPTER III

SUBCHAPTER III-1

SUBCHAPTER III-2

SUBCHAPTER III-3

PREFACE

The origin of agriculture was one of the fundamental achievements for the mankind survival and development, being nowadays the main source livelihood of many people. Agriculture has been providing food, fiber and raw materials allowing the emergence of industries and increasing the economic impact worldwide. A sustainable agriculture is necessary to guarantee greater food security and to meet the increasing demand for plant-derived products.

Biotic and abiotic stresses are major constraints to crop yield and quality, being aluminum phytotoxicity (Al) the main limitation in acid soils all over the world. In Portugal, acid soils are predominant and are located mostly in the Northern region. Rye is an economically important cereal with valuable traits because of its robustness under adverse conditions. Compared to other cereals, rye has been less studied even being one of the most Al tolerant. Rye owns a valuable genetic background for breeding purposes and the wild relatives have important and fruitful resources to improve the domestic crops. Several physiological and molecular approaches have been adopted to understand the Al stress adaptation in plants. However, few data are found in the literature about the genus *Secale*, with no data concerning wild ryes.

This thesis was carried out taking advantage of the follow-up of research projects in this field linking the University of Trás-os-Montes and Alto Douro (UTAD) in Vila Real and the University Complutense of Madrid (UCM) in Madrid. Therefore, experimental works of this doctorate thesis were achieved in these two institutions.

This thesis is divided into four main chapters (I, II, III and IV). In Chapter I, a general introduction on the subjects involved as well as the major aims are presented. In turn, Chapter II and Chapter III comprises two (II-1 and II-2) and three subchapters (III-1, III-2 and III-3), respectively. In Chapter II, molecular markers are used to study different approaches in *Secale* such as genetic diversity, phylogenetic relationships and association studies whereas in Chapter III Al-resistance mechanisms in *Secale* are highlighted being candidate genes characterized. Finally, the general discussion and concluding remarks of this thesis are revealed in Chapter IV.

CHAPTER I

General Introduction and Aims

I-1. General Introduction

1. Acid Soils: distribution, causes and consequences

Soils with a pH value below 5.5 are considered strongly acidic (FAO, 2018). These soils are widespread and are the main limiting factor to agricultural production worldwide, especially grain crops (Kochian et al., 2004). Acid soils occur mainly in two global belts: a northern one with cold and humid temperate climate composed, predominantly, of organic acid soils and another southern with warmer and wetter conditions covered by mineral acid soils (Von Uexkull and Mutert, 1995; Samac and Tesfaye, 2003) (Fig. 1). Two decades ago, Von Uexkull and Mutert (1995) estimated that globally around 30% of the total ice-free land and up to 75% of potentially arable soils (24%) were acidic. The acid soils area have been increasing over years and, certainly, will continue to grow throughout the world.

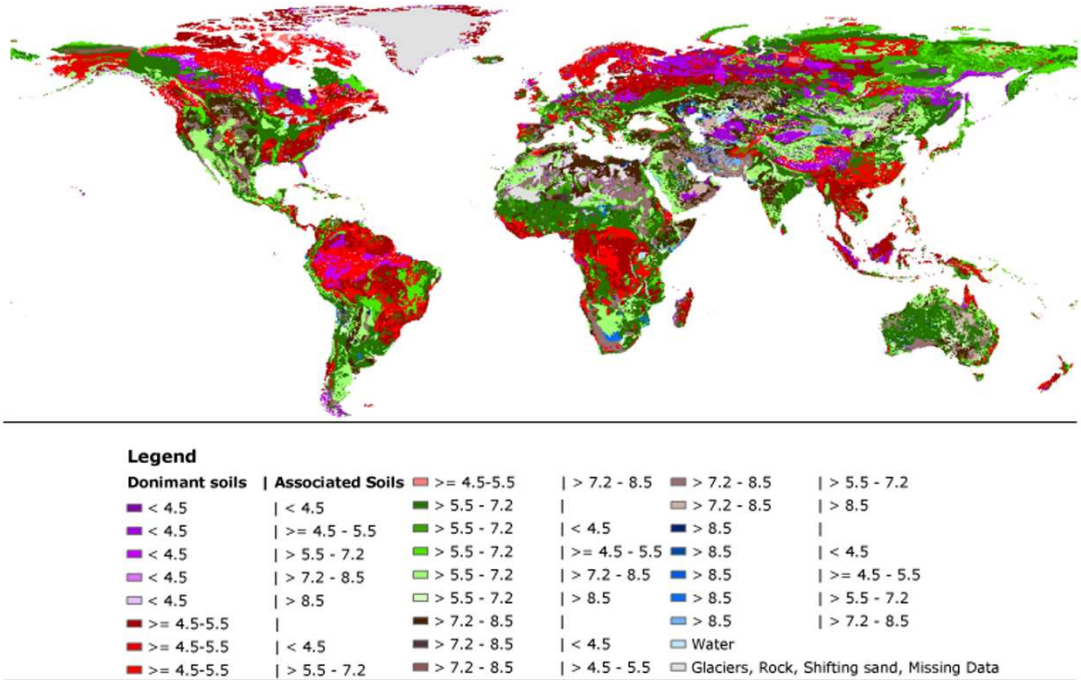


Figure 1. Map exhibiting the topsoil pH values throughout the world. Source: FAO, 2018.

Soil acidity is influenced by edaphic (parent materials), climatic (high rainfall) and biological factors (organic matter decay) (Hede et al., 2001). Many soils become acidic naturally due to the normal wheatering of rocks and leaching of basic minerals (Ma and Ryan, 2010). In addition to natural soil acidity, many farming and industrial activities

accelerated soil acidification including long-term cultivation of legume-based pastures (Bolan et al., 1991), acid precipitation (Ulrich et al., 1980) and fertilizer use, especially ammonium-based fertilizers (nitrification) (Guo et al., 2010).

Acid soils are unproductive to agriculture because of their poor fertility which is due to a combination of mineral toxicities (aluminum, manganese, iron, zinc and copper), essential nutrients deficiencies (phosphorous, calcium, magnesium, potassium, sodium and molybdenum) and acidity per se (hydrogen activity) (Hede et al., 2001; Ryan and Delhaize, 2012; Bian et al., 2013). Low pH decreases the availability of soil nutrients and, increases the solubility of toxic metal elements and the amount of H^+ ions leading to phytotoxicity (Bian et al., 2013).

2. Aluminum Toxicity

Aluminum (Al) toxicity is the major constraint for crop performance on acidic soils, being exceeded only by drought among the abiotic limitations (Barceló and Poschenrieder, 2002; Kochian et al., 2015). This is the third most abundant element, after oxygen and silicon, and the most abundant metal in the Earth's crust, comprising about 7% of its mass, which means plants grown in a soil environment where the roots are exposed to potentially high levels of Al (Delhaize and Ryan, 1995; Kochian et al., 2002; Ma and Furukawa, 2003). Fortunately, most of this Al occurs as harmless oxides and aluminosilicates, and moreover, Al phytotoxic forms are relatively insoluble at alkaline, neutral or slightly acidic soil pH values. However, when acidity in soil raises, the rhizotoxic Al species, Al^{3+}, is solubilized into the soil solution, inhibiting root growth and function and thus, reducing crop yields (Ma et al., 2001; Kochian et al., 2002). The trivalent Al cation is toxic, coping with plants on acid soils, damaging the root system rapidly at micromolar concentrations (Ryan et al., 2011; Delhaize et al., 2012).

The root apical meristem is the primary target of Al accumulation and toxicity, being the inhibition and reduction of the root elongation the first and main morphological symptom of Al toxicity (Ryan et al., 1993; Delhaize and Ryan, 1995; Kochian et al., 2005; Bian et al., 2013). As consequence, Al limits the ability of roots to scavenge for water and nutrients, which seriously compromises the development and productivity of crops (Foy, 1988; Ma and Furukawa, 2003). Al accumulation and subsequent toxicity triggers several physiological symptoms including disjunction of cell walls (Ma et al., 2004a), disorder of plasma membrane integrity (Horst et al., 1997; Kinraide et al., 1998),

interactions on signal transduction pathways as disruption of cytosolic Ca^{2+} (Rengel and Zhang, 2003) and oxidative stress induction (Yamamoto et al., 2001), blockage of cell division (Merino-Gergichevich et al., 2010), changes in cytoskeleton dynamics (Sivaguru et al., 2003) and severe inhibition of DNA synthesis (Silva et al., 2000) (Fig. 2). It was observed in sensitive rye lines that even short-term Al exposure leads to cell cycle delay and changes in root anatomy such as thickness, resulting in irreversible root growth inhibition with a decrease in water and organic matter content but an increase in Ca levels (Silva et al., 2012). In summary, the understanding of the Al toxicity mechanisms is mainly circumstantial, furthermore, the primary target site of Al phytotoxicity leading to inhibition of root elongation is still not well defined (Horst et al., 2010).

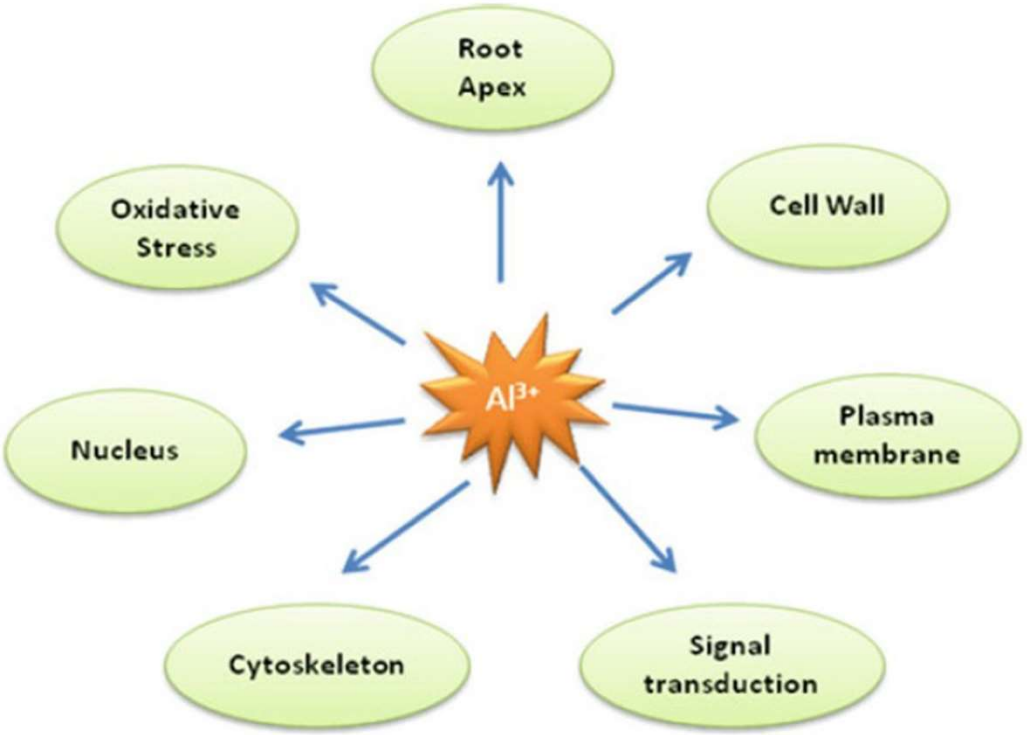


Figure 2. Multiple target sites of the phytotoxic Al^{3+} within a root. Adapted from Aggarwal et al. (2015).

2.1. Solutions to overcome acid soil toxicity

Several strategies have been pursued to overcome acid soil toxicity. The application of lime (calcium carbonate) has been the most common practice for many years, being the most efficient way of ameliorating soil acidity (Ryan and Delhaize, 2012; Bian et al., 2013). This methodology allows the increment of soil pH value and cause Al conversion to less toxic forms relieving Al toxicity in plants (Samac and Tesfaye, 2003; Zhou et al.,

2007). However, these soil amendments are not physically feasible in many locations and the transportation and material costs are often prohibitive, farmers with small subsistence farms rarely have the resources to apply lime (Hede et al., 2001; Samac and Tesfaye, 2003; Zhou et al., 2007; Ryan and Delhaize, 2012). Other disadvantages of this practice is the delay in reducing the subsoil acidity becoming ineffective 25 cm below the ground and the deficiency of Zn and Mn (Pinto-Carnide et al., 1990; Rao et al., 1993; Fontecha et al., 2007). Thus, the best solution to improve crop productivity on acid soils worldwide seems to be the development of highly Al tolerant plants which can be reached by traditional breeding strategies for some species (backcrossing, intercrossing, single seed descent and topcrossing) or by advanced molecular breeding techniques such as marker-assisted selection (MAS) and genetic engineering for others with insufficient natural variation in Al tolerance (Ma et al., 2001; Simões et al., 2012; Bian et al., 2013). Complementation between molecular technologies and conventional breeding is a crucial step for developing research strategies towards more efficient crop improvement in the near future (Inostroza-Blancheteau et al., 2010).

Because many developing countries have acid soils, Al toxicity limits crop performance in those parts of the world where food production is critical (Kochian et al., 2004, 2015). To meet the demands of a growing population, crop yields need to increase significantly all over the world. Since only 4.5% of the acid soils is used for arable crops (Von Uexkull and Mutert, 1995), the near future challenge is the cultivation of all the important economic plant species in soils with low pH values.

3. Mechanisms of alleviating Al toxicity in plants

Many plant species have mechanisms to improve their survival on acid soils (Ryan et al., 2010b). A thorough understanding of both the genetics and physiology of these resistance mechanisms is fundamental to develop crops better adapted to acidic environments. Unfortunately, Al chemistry is quite complex which difficult the study of the Al-related processes in plants (Delhaize and Ryan, 1995; Panda and Matsumoto, 2007). The mechanisms that crops have evolved to cope with Al^{3+} stress can be broadly divided into two main strategies: tolerance (internal) and exclusion (external) mechanisms which can operate individually or in parallel (Ryan et al., 2010; Ryan and Delhaize, 2010) (Fig. 3). Very recently, a coordinated operation between Al exclusion and Al internal tolerance mechanisms was discovered (Wang et al., 2017).

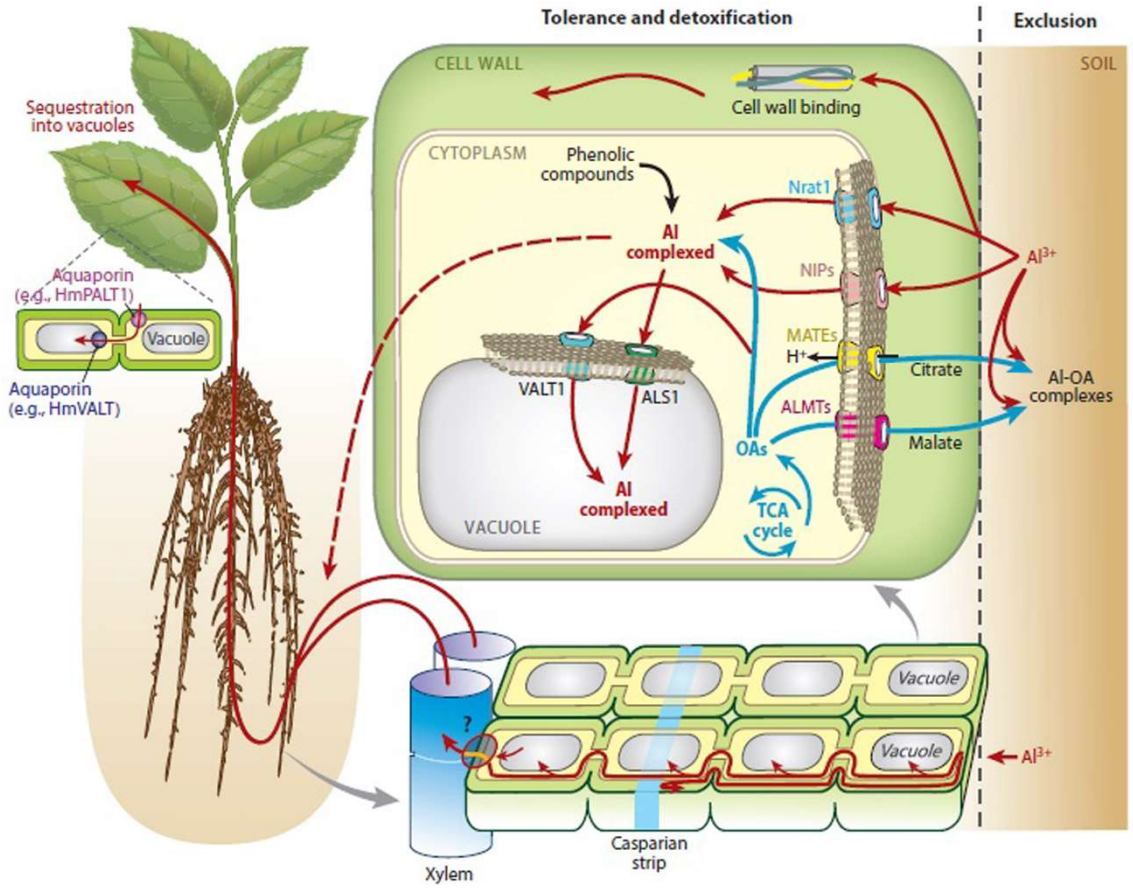


Figure 3. General model illustrating Al resistance mechanisms: external (Al exclusion) and internal (Al tolerance/detoxification). Abbreviations: ABC – ATP-binding cassette; Al – aluminum; ALMT – Al-activated malate transporter; ALS1 – Al-sensitive 1; MATE – multidrug and toxic compound extrusion; NIP – nodulin 26–like intrinsic protein; Nramp – natural resistance-associated macrophage protein; Nrat1 – Nramp Al transporter 1; OA – organic acid; PALT1 – plasma membrane Al transporter 1; TCA – tricarboxylic acid; VALT – vacuolar Al transporter. Adapted from Kochian et al. (2015).

3.1. Internal Al resistance mechanisms

Tolerance mechanisms enable plants to safely accommodate Al^{3+} once it go into the cytosol either by chelating it to form harmless complexes, by storing it in less sensitive organelles where it cannot disrupt metabolism or by rapidly repairing damage incurred (Al detoxification) (Ryan and Delhaize, 2010, 2012). Al can be chelated by organic acids (citrate, malate and oxalate), phenolic compounds (e.g. tannins, flavonols and anthocyanidins) and silicon and the stable non-toxic metallic anion components formed can be translocated around the plant and/or be sequestered thus, reducing Al toxicity in the cell (Barceló and Poschenrieder, 2002; Inostroza-Blancheteau et al., 2008, 2010). The majority of the plants growing on acidic soils accumulate large amounts of Al at the roots, but only a few species translocate the metal to the shoots (Grevenstuk and Romano, 2013). Al accumulator plant species such as hydrangea (*Hydrangea macrophylla*; Ma et

al., 1997), buckwheat (*Fagopyrum esculentum*; Ma and Hiradate, 2000) and tea (*Camellia sinensis*; Matsumoto et al., 1976) are highly tolerant being able to accumulate high Al quantities in their aboveground parts without adverse symptoms and their growth can even be stimulated by Al^{3+} (Inostroza-Blancheteau et al., 2012; Ryan and Delhaize, 2012; Kochian et al., 2015). The formation of less toxic Al complexes seems to be a prerequisite of Al accumulator plants for tolerating the high concentrations of this metal inside the cells, being an important feature of internal Al detoxification mechanisms (Ma et al., 2001; Barceló and Poschenrieder, 2002). In maize (*Zea mays*), the antioxidant activity of phenolic compounds and their ability to bind with Al^{3+} could contribute to the detoxification of the low Al amounts that were able to surpass the typical exclusion barrier (Tolrà et al., 2009).

Four transport pathways may be involved in Al mobilization: apoplastic, symplastic, xylem and phloem transport (Horst et al., 2010; Klug and Horst, 2010; Grevenstuk and Romano, 2013; Zeng et al., 2013). However, little is known about the dynamics of Al translocation from the roots to the upper organs. It seems that Al is either sequestered in the vacuole (e.g. tea; Tolrà et al., 2011) or the cell wall (apoplast) (e.g. buckwheat; Shen et al., 2004) depending on the plant species. Both strategies appear to be effective to prevent contact between Al and essential metabolic processes occurring in the cytoplasm, thereby, not compromising the normal development of the plants (Grevenstuk and Romano, 2013).

Another Al detoxification mechanism is associated to the modification of the properties of the root cell wall, which forms a physical barrier between the rhizosphere and the internal content of plant cells and constitutes the next site of Al-plant interaction after Al^{3+} ions have been able to cross the organic acid barrier (see later) (Kochian et al., 2015). Cell wall polysaccharide contents as pectins and hemicelluloses, have been related to Al resistance in several plant species. For instance, pectin methylesterases (PME) are responsible for the demethylation of pectin in the apoplast and the overexpression of *PME* gene in roots of Al-sensitive cultivars of maize (Maron et al., 2008), rice (*Oryza sativa*) (Yang et al., 2013b) and rye (*Secale cereale*) (El-Moneim et al., 2014b) resulted in higher accumulation and binding of Al^{3+} ions in the root tip cell wall, and increased Al sensitivity. Moreover, Yang et al. (2013b) provided molecular evidence that specific *PME* genes were associated with Al-induced inhibition of root growth. On the other hand, *XTH31* (xyloglucan endotransglucosylase-hydrolase) affects Al sensitivity in

Arabidopsis thaliana by modulating cell wall xyloglucan content and Al binding capacity (Zhu et al., 2012).

Recently, it was discovered that Al uptake is mediated by specific Al transporters operating at the plasma membrane and tonoplast which have a key role in plant Al tolerance mechanisms. In hydrangea, two gene members of the aquaporin family, the plasma membrane (*HmPALT1*) and the vacuolar aluminum transporter (*HmVALT*), have been suggested to facilitate Al transport across the cell membrane into the cytosol and across the tonoplast into the vacuoles of sepal cells, respectively (Negishi et al., 2012). In rice, a plasma membrane-localized natural resistance-associated macrophage protein (Nramp) Al transporter (OsNrat1) may operate together with a vacuolar ABC Al transporter (OsALS1- Aluminum Sensitive 1) by lowering Al levels in the root cell wall moving it into the cell and sequestrate it within the root vacuole, which is a crucial strategy for Al resistance (Huang et al., 2012; Xia et al., 2013; Li et al., 2014). Also in rice, it was identified an ABC transporter complex encoded by sensitive to Al rhizotoxicity 1 and 2 (*STAR1* and *STAR2*) that appears to mediate the efflux of UDP-glucose into the cell wall, which could possibly alter the cell wall composition, leading to a reduction in Al-binding capacity and, subsequently, in Al phytotoxicity (Huang et al., 2009). In *Arabidopsis*, *ALS3* (Aluminum Sensitive 3) encodes a phloem-localized ABC transporter that is required for Al tolerance and is probably involved in the redistribution of Al within the plant away from the sensitive root apex (Larsen et al., 2005). AtALS3 is homologous of OsSTAR2 and may be the functional partner of AtSTAR1, which is homologous of OsSTAR1 (Huang et al., 2009, 2010).

Tolerance mechanisms can also minimize oxidative stress and repair Al^{3+}-induced damage (Ryan and Delhaize, 2012). Reactive Oxygen Species (ROS), including hydrogen peroxide (H_2O_2), superoxide, and hydroxyl radicals, adversely affect root cells (e.g. apoptosis and damages such as cell membrane lipid peroxidation) and influence signaling pathways for essential processes such as growth and stress adaptations (e.g. activation of Al-tolerance gene expression) (Daspute et al., 2017). Al induces a series of physiological events that increase ROS production but, also upregulate the expression of several genes that help plants survive the effects of the Al-induced ROS stress/damages. ROS-scavenging genes have an active role in Al tolerance mechanisms in particular recovery from oxidative damage (Matsumoto and Motoda, 2012; Daspute et al., 2017). Genes involved in cell wall structure and function such as glutathione S-transferase (*GST*), peroxidase (*POX*) and superoxide dismutase (*SOD*) are Al-inducible and could detoxify

ROS components (Ezaki et al., 2000; Basu et al., 2001; Maron et al., 2008; Sánchez-Parra et al., 2015).

3.2. External Al resistance mechanisms

Exclusion mechanisms are based on exudation of Al-chelating organic compounds, such as organic acids or phenolics, from the root apex into the rhizosphere, that can prevent toxic Al species from entering and accumulating in root cells (both apoplasm and symplasm) (Ryan and Delhaize, 2012; Kochian et al., 2015). As occur in tolerance mechanisms these released ligands can effectively chelate Al^{3+} forming harmless complexes thus alleviating Al rhizotoxicity.

3.2.1. Al exclusion via root organic acid exudation

This is by far the most well-characterized Al exclusion mechanism and is one of the most widely used by the majority of the studied species (Inostroza-Blancheteau et al., 2008; Kochian et al., 2015). The gramineous species such as rye, wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), sorghum (*Sorghum bicolor*), rice and maize share this ubiquitous mechanism (Ma et al., 2014). The organic acids (OA) commonly secreted are malate, citrate and oxalate which are able to form stable complexes with Al, being Al-citrate complex the strongest and Al-malate the weakest. The Al affinity for the oxygen donor ligands allows the formation of these strong complexes (Barceló and Poschenrieder, 2002; Inostroza-Blancheteau et al., 2008, 2012). The Al-induced secretion of OA anions is localized to the root apex, which is in agreement with the targeting site for Al toxicity, and their secretion is highly specific to Al (Yang et al., 2013a). Several studies describe an Al-triggered increase of organic acid anion release from root tips in Al tolerant plants (Barceló and Poschenrieder, 2002).

The primary differences between plant species that employ this Al resistance mechanism are the OA released (malate, citrate or oxalate), the magnitude of the OA exudation and the time course for Al-dependent OA exudation (Kochian et al., 2015). Many plant species release only one type of OA, however, it is not unusual to find plant species that release more than one OA in response to Al stress, suggesting that multiple release mechanisms or transporters may be operating in these species (Yang et al., 2013; Kochian et al., 2015). For example, citrate exudation is the major exclusion mechanism for barley (Zhao et al., 2003), rice (Ma et al., 2002), maize (Pellet et al., 1995) and

sorghum (Magalhaes et al., 2007) whereas oxalate secretion was reported in buckwheat (Zheng et al., 1998b). On the other hand, both citrate and malate are released from rye (Li et al., 2000), wheat (Delhaize et al., 1993; Ryan et al., 2009), oat *(Avena sativa*, Zheng et al., 1998a) and Yorkshire fog (*Holcus lanatus*, Chen et al., 2013). The amount of OA anion exudation vary among species, cultivars and even between tissues of the same plant, furthermore, there is a lack of correlation among Al tolerance degree and the amount of OA released by some plants (Ishikawa et al., 2000; Inostroza-Blancheteau et al., 2012; Yang et al., 2013; Ma et al., 2014). Two different patterns of Al-induced OA exudation have been described based on the timing of response/secretion (Ma, 2000). In Pattern I plants, the onset of OA anion secretion occurs immediately after the addition of Al such as in buckwheat, barley and wheat. However, in Pattern II plants, OA anion secretion is delayed for several hours after Al exposure such as in rye, sorghum, rice and maize. Furthermore, Pattern I behavior can be triggered by the Al-induced activation of anion efflux channels or the constitutively high release of OA, while in Pattern II plants gene activation may be implied (Poschenrieder et al., 2008).

The Al^{3+}-dependent efflux of malate and citrate from plant root cells into the rhizosphere are controlled by members of two gene families, both encoding transport proteins and localized in the plasma membrane. Malate efflux is encoded by members of the Al-activated malate transporter (*ALMT*) gene family and citrate efflux is encoded by members of the multidrug and toxic compound extrusion (*MATE*) gene family, also named Al-activated citrate transporter (*AACT*) (Ryan and Delhaize, 2012; Kochian et al., 2015). Most of the *ALMT* and *MATE/AACT* genes are expressed in the root tips which are the target site for Al toxicity and the high expression in this site can efficiently detoxify Al externally (Ma et al., 2014). As *ALMTs* and *MATEs* both confer Al resistance through root OA release, this appears to be a striking example of functional coevolution of Al resistance by two transporters that are structurally and functionally quite different (Kochian et al., 2015).

3.2.1.1. *ALMT* genes

The *TaALMT1* gene, which underlies a major wheat Al resistance locus (Alt_{BH}), was the first Al^{3+} resistance gene isolated from plant species (Sasaki et al., 2004). The expression of this gene results in an enhanced Al-activated malate efflux and an increased Al resistance. Subsequently, homologous genes of *TaALMT1* have been identified in

Arabidopsis (*AtALMT1*, Hoekenga et al., 2006), rapeseed (*Brassica napus*; *BnALMT1* and *BnALMT2*, Ligaba et al., 2006), rye (*ScALMT1*, Fontecha et al., 2007; Collins et al., 2008), maize (*ZmALMT1* and *ZmALMT2*, Piñeros et al., 2008; Ligaba et al., 2012), barley (*HvALMT1*, Gruber et al., 2010), Yorkshire fog (*HlALMT1*, Chen et al., 2013) and *Brachypodium distachyon* (*BdALMT1*, Contreras et al., 2014). Most of these genes performs similar functions as *TaALMT1* excepting the homologous genes from maize and barley whose are not directly involved in the secretion of malate and are not associated with Al tolerance in the respective species. For a recent review of the *ALMT* family see Sharma et al. (2016).

3.2.1.2. *MATE* genes

MATEs were first identified as Al resistance genes of the major Al resistance loci in sorghum (*SbMATE*, Magalhaes et al., 2007) and barley (*HvAACT1*, Furukawa et al., 2007). These genes are responsible for Al detoxification based on root citrate efflux in response to Al stress. Functional MATE homologous associated with Al tolerance were also identified in *Arabidopsis* (*AtMATE1*, Liu et al., 2009), maize (*ZmMATE1*, Maron et al., 2010), rice bean (*Vigna umbellata*; *VuMATE*, Yang et al., 2011), wheat (*TaMATE1*, Tovkach et al., 2013; Garcia-Oliveira et al., 2014), rye (*ScMATE1* and *ScMATE2*, Yokosho et al., 2010; Silva-Navas et al., 2012; Santos et al., 2018) and rice (*OsFRDL4*, *Ferric reductase defective3-like4*; Yokosho et al., 2011). Other homologous of these genes were reported in maize (*ZmMATE2*, Maron et al., 2010) and *Brachypodium* (*BdMATE1* and *BdMATE2*, Contreras et al., 2014) but a possible role in Al tolerance was not clear. Plant MATEs can transport substrates other than citrate, which may also play a role in Al tolerance (Yang et al., 2013a). For reviews of the *MATE* family see Magalhaes (2010) and Takanashi et al. (2014).

3.2.2. Al exclusion via release of phenolic compounds

Other ligands, such as phenolic compounds, with potential for Al detoxification in the rhizosphere have received less attention (Poschenrieder et al., 2008). Phenolic compounds are less potent chelators of Al^{3+} than OAs are, but the electrophilic nature of oxygen atoms in –OH groups of phenolic rings imparts a reasonable capacity for Al chelation (Kochian et al., 2015). Varietal differences in the exudation rate of flavonoid-type phenolics by root tips of maize have been related to differences in Al resistance,

meaning that root phenolic exudation may play a role in maize root Al exclusion (Kidd et al., 2001). In light of this work, the role of phenolic compounds in Al exclusion mechanisms must deserves additional attention being able to be involved in other grass species.

4. Transcriptional regulation of Al-tolerance genes

As we saw before, an assortment of Al-tolerance genes was identified and characterized in several crop species. Al-tolerant genes expression have been featured according the following: 1) greater gene expression level of tolerant genotypes comparing to sensitive ones, 2) gene expression localization on root apices, 3) Al-induced gene expression increment (e.g., *AtALMT1*, *BnALMT1*, *ScALMT1*, *SbMATE*, *VuMATE1*, *ZmMATE1*, *ScMATE2* and *OsFRDL4*) as well as, 4) constitutively high gene expression (e.g., *TaALMT1*, *TaMATE1* and *HvAACT1*) (Kochian et al., 2015). Some mechanisms regulating the expression of Al-tolerant genes include increased tandem repeated element, insertion of transposon, increase of copy number and alteration of cis-acting elements (Delhaize et al., 2012). The constitutively high expression of *TaALMT1* in root tips of Al-tolerant wheat lines is associated with duplicated and triplicated tandem repeat elements in the promoter region, which could function as enhancers of gene expression (Sasaki et al., 2006; Ryan et al., 2010a). In sorghum, tourist-like miniature inverted repeat transposable elements (MITEs) occur upstream of the *SbMATE* gene and the number of these repeats is broadly correlated with the level of *SbMATE* expression (Magalhaes et al., 2007). The insertion of other transposable elements in the promoter are found to regulate the expression level in barley (*HvAACT1*, Fujii et al., 2012) and wheat (*TaMATE1B*, Tovkach et al., 2013). On the other hand, copy number variation plays a role in the increment of both Al tolerance and gene expression for *ScALMT1* in rye (Collins et al., 2008) and *ZmMATE1* in maize (Maron et al., 2013) in the Al-tolerant genotypes of both species. *ScALMT1* copies range from one to, at least, five copies whereas three copies were identified in *ZmMATE1*. Finally, the high expression of *HlALMT1* in Yorkshire fog is achieved by increasing the number of cis-acting elements of the transcription factor for Al tolerance HlART1 (described below) in the promoter region (Chen et al., 2013).

With regard to transcription factors involved in Al induction of Al tolerance gene expression, AtSTOP1 (Iuchi et al., 2007) and OsART1 (Yamaji et al., 2009) are two

related members of the C_2H_2-type zinc-finger transcription factor family that positively regulate Al-induced expression of Al tolerance genes in *Arabidopsis* and rice, respectively. AtSTOP1 is involved in the expression of proton-tolerance genes (Delhaize et al., 2012) and in the Al-induced expression of several *Arabidopsis* Al resistance genes, including *AtALMT1*, *AtMATE1*, and *AtALS3* (Liu et al., 2009; Sawaki et al., 2009). This transcription factor is constitutively expressed, indicating its involvement in the Al induction of gene expression must involve a posttranslational processes (Delhaize et al., 2012; Kochian et al., 2015). Like *AtSTOP1*, *OsART1* is constitutively expressed in rice roots, but unlike *AtSTOP1*, it is involved only in Al resistance. OsART1 regulates the expression of at least 31 genes, some of which have been implicated in both internal and external detoxification mechanisms (Yamaji et al., 2009). The transcription factor ART1 plays also a central role in Al tolerance in Yorkshire fog (HlART1, Chen et al., 2013), suggesting that this species share a similar regulatory pathway with rice. The expression of the *ART1* homolog in wheat, *TaSTOP1*, is not induced by Al like in rice and Yorkshire fog but the role in Al tolerance remains unclear (Garcia-Oliveira et al., 2013). Recently, it was identified a paralogous of AtSTOP1, AtSTOP2, that may partner with AtSTOP1 in regulating the expression of some of the Al and low-pH resistance genes in *Arabidopsis* (Kobayashi et al., 2014). For a review of this thematic see Daspute et al. (2017).

5. Rye: a model species in Al tolerance studies

There is a wide variability in Al tolerance between and within plant species and this has been useful to breeders in developing Al-tolerant cultivars of several crops, as well as to researchers for studying the genetic, physiology and biochemistry of Al tolerance, helping in the identification of Al-tolerance genes (Delhaize and Ryan, 1995; Ma et al., 2014). Genetic improvement of crops for acid soil tolerance has been accelerated by the availability of screening criteria for detecting Al tolerance. Different screening methods have been used to evaluate Al tolerance: hydroponic culture (Gallego and Benito, 1997; Ma et al., 1997b; Pinto-Carnide and Guedes-Pinto, 1999), soil bioassays (Ring et al., 1993) and field evaluations (Carvalho et al., 2016).

Laboratory- and greenhouse-based techniques for Al tolerance screening are widely used because they are quick, highly accurate, non-destructive, and can be applied at early development plant stages. Hydroponic culture is the method currently used, which provides easy access to root systems and tight control over nutrient availability and pH,

and allows the Al tolerance evaluation by root length measurement and root hematoxylin and/or eriochrome cyanine R staining. The use of soil media has received less attention because of the difficulties in creating a soil environment with a specific type and amount of phytotoxicity. On the other hand, field-based techniques have as a major obstacle the inherent spatial variability for pH or plant nutrients in soil, which influences Al stress severity making this method more laborious, time consuming and expensive (Carver and Ownby, 1995; Wang et al., 2006).

The degree of Al tolerance among cereal crops usually follows the order rye (*S. cereale*) ≥ rice (*O. sativa*) > oat (*A. sativa*) > bread wheat (*T. aestivum*) > *Brachypodium hybridum* > *B. distachyon* > barley (*H. vulgare*) > durum wheat (*T. turgidum*), although genotypic differences also exists in each species (Aniol and Gustafson, 1984; Bona et al., 1993; Pinto-Carnide and Guedes-Pinto, 1999; Kim et al., 2001; Ma et al., 2002; Contreras et al., 2014).

Rye (*Secale cereale* L.) is a diploid species, with a chromosome number of $2n = 2x = 14$, which belongs to the Triticeae tribe, along with other economically important cereals, such as wheat and barley. In contrast to most grain crops that are self-pollinating, rye is a cross-pollinating (allogamous) cereal, and such outbreeding nature results in a high intraspecific diversity which makes it suitable for breeding purposes (Chikmawati et al., 2006; Schlegel, 2014). This gramineous plant is commonly grown in Eastern and Northern Europe, mainly for the production of bread, alcohol, and animal feed (Evans, 1995).

Rye is currently a recognized dietary value and owns advantageous features such as nutrient efficiency, tolerance of diseases, allows a reduced usage of pesticides and fertilizers during production making rye into an important genetic resource (Bolibok-Bragoszewska et al., 2009; Ribeiro et al., 2012; Schlegel, 2014). Furthermore, with the high abiotic stress tolerance to frost, drought, acid soils and marginal soil fertility rye becomes a source of favorable agronomic traits being a perfect model for genetic and functional analyses and consequently for improvement by gene introgression in cereal crops like wheat and barley, which are less tolerant to adverse environmental conditions (Martis et al., 2013; Schlegel, 2014). Rye has one of the most efficient groups of genes for Al tolerance (*Alt*) among cultivated species belonging to Triticeae tribe (Matos et al., 2005). Genetic studies showed that Al tolerance in rye is controlled by four dominant and independent loci (*Alt1*, *Alt2*, *Alt3* and *Alt4*) located on chromosome arm *6RS*, *3RS*, *4RL*

and *7RS*, respectively (Aniol and Gustafson, 1984; Gallego and Benito, 1997; Ma et al., 2000; Miftahudin et al., 2002; Matos et al., 2005; Benito et al., 2010).

Wild relatives have an important, increasing role in research on genetic resources. In general, wild plants show high tolerance against various abiotic stresses, such as salinity, metal toxicities, drought, extreme temperature and oxidative stress (Ezaki et al., 2008, 2013). It is therefore strongly expected that genes conferring high tolerance to various stresses can be isolated from high-tolerant wild plants. Thus, wild crops can be an additional germplasm source to maintain high genetic diversity and to provide more opportunities to breed for crops that can be cultivated under unfavorable conditions.

The *Secale* genus includes perennial or annual, self-incompatible or self-compatible, and cultivated, weedy or wild species (Vences et al., 1987). Nowadays, four species are recognized in this genus: the annual outbreeder *S. cereale* L., the perennial outbreeder *S. strictum* (Presl.) Presl. (syn. *S. montanum* Guss.) and both the annual autogamous *S. sylvestre* Host and *S. vavilovii* Grossh. Moreover, *S. cereale* contain eight subspecies including *ancestrale* Zhuk., *cereale* (the only cultivated) and *segetale* Zhuk. and *S. strictum* comprises five subspecies (GRIN, 2017).

The taxonomic relationships among these species have been, and still are, controversy. The first attempts at a systematic classification were carried based in morphological characteristics, life cycle (perennial vs. annual) and geographical distribution while the second set of studies concerns cytogenetics features, which were followed by works based on chemotaxonomic approaches, isozymes and molecular data (For references see Chikmawati et al., 2006). More recently, advances in molecular plant biology and in plant genomics lead to the development of molecular markers which are expected to elucidate many patterns of rye evolution. A variety of molecular techniques have been developed providing genetic diversity and genetic relationship information that can be used as DNA fingerprinting (Chikmawati et al., 2006). It is crucial to assess the genetic diversity of rye and wild rye in order to preserve its biodiversity for breeding and improvement purposes. Over the years, both genetic relationships and genetic diversity have been achieved within the genus *Secale* based on ISSRs – Inter Simple Sequence Repeat (Matos et al., 2001; Bolibok et al., 2005; Myśków et al., 2010; Santos et al., 2016), RAPDs – Random Amplification of Polymorphic DNA (Del Pozo et al., 1995; Matos et al., 2001; Persson et al., 2001; Ma et al., 2004b; Santos et al., 2016), RFLPs – Restriction Fragment Length Polymorphism (Petersen and Doebley, 1993; Loarce et al., 1996), SSRs – Simple Sequence Repeats (Shang et al., 2006; Akhavan et al., 2010; Fu et al., 2010;

Ren et al., 2011; Targońska et al., 2016), AFLPs – Amplified Fragment Length Polymorphism (Chikmawati et al., 2005, 2012), DArT – Diversity Array Technology (Bolibok-Bragoszewska et al., 2009) and DArTseq markers (Al-Beyroutiová et al., 2016).

The widespread use of DNA polymorphisms along with the growing technology of molecular markers has a significant impact on plant improvement with regards to genetic diversity and genotyping studies, linkage map construction, trait tagging, gene cloning, and Marker-Assisted Selection (MAS) (Inostroza-Blancheteau et al., 2010).

So far, several Al-tolerance genes were isolated and characterized in rye, as we could see earlier, which summarizing are: *ScALMT1* (Fontecha et al., 2007; Collins et al., 2008), *ScMATE1* (Yokosho et al., 2010; Silva-Navas et al., 2012; Santos et al., 2018), *ScMATE2* (Yokosho et al., 2010), *ScMDH* (El-Moneim et al., 2014a), *ScCS* (El-Moneim, 2012), *ScFH* (El-Moneim, 2012), *ScSTOP1* (Silva-Navas, 2016), *ScSOD* (Sánchez-Parra et al., 2014) and *ScPME* (El-Moneim et al., 2014b). Taken all together, we can confirm rye as a useful model species for Al-tolerance approaches.

I-2. Aims

Considering the above mentioned, the importance of rye and its wild relatives is clear. It is known that rye is one of the most tolerant cereals to biotic and abiotic stresses, including Al tolerance, making it a valuable germplasm source. Wild crops are usually very resistant under harsh conditions, however, there are no data about the behavior of wild *Secale* species as regards Al tolerance. Most of the studies on Al tolerance using cereals focus rice, barley, maize and wheat species, so it is necessary to broaden the knowledge about the genetics and molecular aspects of Al tolerance in rye and wild rye. Thus, the main goals of this thesis are based on cultivated and wild ryes as plant material and consists of:

1- Assessment of the molecular diversity and genetic relationships through molecular markers;
2- Al tolerance evaluation: intra-cultivar, inter-cultivar, intraspecific and interspecific variability analysis;
3- Identification of potential Al tolerance linked markers;
4- Observation of cell disorders related to Al toxicity;
5- Estimation of the organic acids exudation;
6- Isolation and characterization of putative Al tolerance genes (sequencing);

7- Candidate gene expression studies.

References

Aggarwal, A., Ezaki, B., Munjal, A., and Tripathi, B. N. (2015). "Physiology and Biochemistry of Aluminum Toxicity and Tolerance in Crops," in *Stress Responses in Plants - Mechanisms of Toxicity and Tolerance*, eds. B. N. Tripathi and M. Muller (Springer International Publishing Switzerland), 35–58. doi:10.1007/978-3-319-13368-3.

Akhavan, A., Saeidi, H., and Rahiminejad, M. R. (2010). Genetic diversity of *Secale cereale* L. in Iran as measured using microsatellites. *Genet. Resour. Crop Evol.* 57, 415–422. doi:10.1007/s10722-009-9480-9.

Al-Beyroutiová, M., Sabo, M., Sleziak, P., Dušinský, R., Birčák, E., Hauptvogel, P., et al. (2016). Evolutionary relationships in the genus *Secale* revealed by DArTseq DNA polymorphism. *Plant Syst. Evol.* 302, 1083–1091. doi:10.1007/s00606-016-1318-2.

Aniol, A., and Gustafson, J. P. (1984). Chromosome location of genes controlling aluminium tolerance in wheat, rye and triticale. *Can. J. Genet. Cytol.* 26, 701–705.

Barceló, J., and Poschenrieder, C. (2002). Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: A review. *Environ. Exp. Bot.* 48, 75–92. doi:10.1016/S0098-8472(02)00013-8.

Basu, U., Good, A. G., and Taylor, G. J. (2001). Transgenic *Brassica napus* plants overexpressing aluminum-induced mitochondrial manganese superoxide dismutase cDNA are resistant to aluminum. *Plant Cell Environ.* 24, 1269–1278. doi:10.1046/j.0016-8025.2001.00783.x.

Benito, C., Silva-Navas, J., Fontecha, G., Hernández-Riquer, M. V., Eguren, M., Salvador, N., et al. (2010). From the rye *Alt3* and *Alt4* aluminum tolerance loci to orthologous genes in other cereals. *Plant Soil* 327, 107–120. doi:10.1007/s11104-009-0035-9.

Bian, M., Zhou, M., Sun, D., and Li, C. (2013). Molecular approaches unravel the mechanism of acid soil tolerance in plants. *Crop J.* 1, 91–104. doi:10.1016/j.cj.2013.08.002.

Bolan, N., Hedley, M., and White, R. (1991). Processes of soil acidification during nitrogen cycling with emphasis on legume based pastures. *Plant Soil* 134, 53–63.

Bolibok-Bragoszewska, H., Heller-Uszyńska, K., Wenzl, P., Uszyński, G., Kilian, A., and Rakoczy-Trojanowska, M. (2009). DArT markers for the rye genome - genetic diversity and mapping. *BMC Genomics* 10, 578. doi:10.1186/1471-2164-10-578.

Bolibok, H., Rakoczy-Trojanowska, M., Hromada, A., and Pietrzykowski, R. (2005). Efficiency of different PCR-based marker systems in assessing genetic diversity among winter rye (*Secale cereale* L.) inbred lines. *Euphytica* 146, 109–116. doi:10.1007/s10681-005-0548-0.

Bona, L., Wright, R. J., Baligar, V. C., and Matuz, J. (1993). Screening wheat and other small grains for acid soil tolerance. *Landsc. Urban Plan.* 27, 175–178. doi:10.1016/0169-2046(93)90046-G.

Carvalho, G., Schaffert, R. E., Malosetti, M., Viana, J. H. M., Menezes, C. B., Silva, L. A., et al. (2016). Back to Acid Soil Fields: The Citrate Transporter SbMATE Is a Major Asset for Sustainable Grain Yield for Sorghum Cultivated on Acid Soils. *G3*

Genes, Genomes, Genetics 6, 475–484. doi:10.1534/g3.115.025791.

Carver, B. F., and Ownby, J. D. (1995). Acid soil tolerance in wheat. *Adv. Agron.* 54, 117–173.

Chen, Z. C., Yokosho, K., Kashino, M., Zhao, F. J., Yamaji, N., and Ma, J. F. (2013). Adaptation to acidic soil is achieved by increased numbers of cis-acting elements regulating *ALMT1* expression in *Holcus lanatus*. *Plant J.* 76, 10–23. doi:10.1111/tpj.12266.

Chikmawati, T., Ma, X., Ross, K., and Gustafson, J. P. (2006). "*Secale*," in *Genome Mapping and Molecular Breeding in Plants - Cereals and Millets*, ed. C. Kole (Springer-Verlag Berlin Heidelberg), 243–255.

Chikmawati, T., Miftahudin, M., Skovmand, B., and Gustafson, J. P. (2012). Amplified fragment length polymorphism-based genetic diversity among cultivated and weedy rye (*Secale cereale* L.) accessions. *Genet. Resour. Crop Evol.* 59, 1743–1752. doi:10.1007/s10722-012-9796-8.

Chikmawati, T., Skovmand, B., and Gustafson, J. P. (2005). Phylogenetic relationships among *Secale* species revealed by amplified fragment length polymorphisms. *Genome* 48, 792–801. doi:10.1139/g05-043.

Collins, N. C., Shirley, N. J., Saeed, M., Pallotta, M., and Gustafson, J. P. (2008). An *ALMT1* gene cluster controlling aluminum tolerance at the *Alt4* locus of rye (*Secale cereale* L.). *Genetics* 179, 669–682. doi:10.1534/genetics.107.083451.

Contreras, R., Figueiras, A. M., Gallego, F. J., and Benito, C. (2014). *Brachypodium distachyon*: A model species for aluminium tolerance in Poaceae. *Funct. Plant Biol.* 41, 1270–1283. doi:10.1071/FP13362.

Daspute, A. A., Sadhukhan, A., Tokizawa, M., Kobayashi, Y., Panda, S. K., and Koyama, H. (2017). Transcriptional Regulation of Aluminum-Tolerance Genes in Higher Plants: Clarifying the Underlying Molecular Mechanisms. *Front. Plant Sci.* 8, 1–12. doi:10.3389/fpls.2017.01358.

Del Pozo, J. C., Figueiras, A. M., Benito, C., and De La Peña, A. (1995). PCR derived molecular markers and phylogenetic relationships in the *Secale* genus. *Biol. Plant.* 37, 481–489. doi:10.1007/BF02908823.

Delhaize, E., Ma, J. F., and Ryan, P. R. (2012). Transcriptional regulation of aluminium tolerance genes. *Trends Plant Sci.* 17, 341–348. doi:10.1016/j.tplants.2012.02.008.

Delhaize, E., and Ryan, P. R. (1995). Aluminum Toxicity and Tolerance in Plants. *Plant Physiol* 107, 315–321.

Delhaize, E., Ryan, P. R., and Randall, P. J. (1993). Aluminum Tolerance in Wheat (*Triticum aestivum* L.) (II. Aluminum-Stimulated Excretion of Malic Acid from Root Apices). *Plant Physiol* 103, 695–702. doi:103/3/695 [pii].

El-Moneim, D. A., Contreras, R., Silva-Navas, J., Gallego, F. J., Figueiras, A. M., and Benito, C. (2014a). On the consequences of aluminium stress in rye: Repression of two mitochondrial malate dehydrogenase mRNAs. *Plant Biol.* 17, 123–133. doi:10.1111/plb.12219.

El-Moneim, D. A., Contreras, R., Silva-Navas, J., Gallego, F. J., Figueiras, A. M., and Benito, C. (2014b). Pectin methylesterase gene and aluminum tolerance in *Secale cereale*. *Environ. Exp. Bot.* 107, 125–133. doi:10.1016/j.envexpbot.2014.06.006.

El-Moneim, D. A. (2012). Aislamiento, caracterización estructural y regulación de la expresión de genes implicados en la tolerancia al aluminio en centeno. Tesis Doctoral en Ciencias Biologicas. Madrid: Universidad Complutense de Madrid, 277p.

Evans, G. M. (1995). "Rye," in *Evolution of Crop Plants*, eds. J. Smartt and N. Simmonds (Harlow: Longman Scientific&Technical), 166–170.

Ezaki, B., Gardner, R. C., Ezaki, Y., and Matsumoto, H. (2000). Expression of aluminum-induced genes in transgenic *Arabidopsis* plants can ameliorate aluminum stress and/or oxidative stress. *Plant Physiol.* 122, 657–665. doi:10.1104/pp.122.3.657.

Ezaki, B., Jayaram, K., Higashi, A., and Takahashi, K. (2013). A combination of five mechanisms confers a high tolerance for aluminum to a wild species of Poaceae, *Andropogon virginicus* L. *Environ. Exp. Bot.* 93, 35–44. doi:10.1016/j.envexpbot.2013.05.002.

Ezaki, B., Nagao, E., Yamamoto, Y., Nakashima, S., and Enomoto, T. (2008). Wild plants, *Andropogon virginicus* L. and *Miscanthus sinensis* Anders, are tolerant to multiple stresses including aluminum, heavy metals and oxidative stresses. *Plant Cell Rep.* 27, 951–961. doi:10.1007/s00299-007-0503-8.

FAO: Food and Agriculture Organization (2018). FAO SOILS PORTAL - Acid soils. Visit. http://www.fao.org/soils-portal/soil-management/management-of-some-problem-soils/acid-soils/en/

Fontecha, G., Silva-Navas, J., Benito, C., Mestres, M. A., Espino, F. J., Hernández-Riquer, M. V., et al. (2007). Candidate gene identification of an aluminum-activated organic acid transporter gene at the *Alt4* locus for aluminum tolerance in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 114, 249–260. doi:10.1007/s00122-006-0427-7.

Foy, C. D. (1988). Plant adaptation to acid aluminum-toxic soils. *Commun. Soil Sci Plant Anal.* 19, 959–988.

Fujii, M., Yokosho, K., Yamaji, N., Saisho, D., Yamane, M., Takahashi, H., et al. (2012). Acquisition of aluminium tolerance by modification of a single gene in barley. *Nat. Commun.* 3, 713. doi:10.1038/ncomms1726.

Furukawa, J., Yamaji, N., Wang, H., Mitani, N., Murata, Y., Sato, K., et al. (2007). An aluminum-activated citrate transporter in barley. *Plant Cell Physiol.* 48, 1081–1091.

Gallego, F. J., and Benito, C. (1997). Genetic control of aluminium tolerance in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 95, 393–399.

Garcia-Oliveira, A. L., Benito, C., Prieto, P., de Andrade Menezes, R., Rodrigues-Pousada, C., Guedes-Pinto, H., et al. (2013). Molecular characterization of *TaSTOP1* homoeologues and their response to aluminium and proton (H^+) toxicity in bread wheat (*Triticum aestivum* L.). *BMC Plant Biol.* 13, 134. doi:10.1186/1471-2229-13-134.

Garcia-Oliveira, A. L., Martins-Lopes, P., Tolrá, R., Poschenrieder, C., Tarquis, M., Guedes-Pinto, H., et al. (2014). Molecular characterization of the citrate transporter gene *TaMATE1* and expression analysis of upstream genes involved in organic acid transport under Al stress in bread wheat (*Triticum aestivum*). *Physiol. Plant.* 152, 441–452. doi:10.1111/ppl.12179.

Grevenstuk, T., and Romano, A. (2013). Aluminium speciation and internal detoxification mechanisms in plants: where do we stand? *Metallomics* 5, 1584–94. doi:10.1039/c3mt00232b.

Gruber, B., Ryan, P., Richardson, A., Tyerman, S., Ramesh, S., Hebb, D., et al. (2010). *HvALMT1* from barley is involved in the transport of organic anions. *J Exp Bot* 61, 1455–1467.

Guo, J. H., Liu, X. J., Zhang, Y., Shen, J. L., Han, W. X., Zhang, W. F., et al. (2010). Significant acidification in major Chinese croplands. *Science (80-.).* 327, 1008–1010.

Hede, A. R., Skovmand, B., and López-Cesati, J. (2001). "Acid Soils and Aluminum Toxicity," in *Application of physiology in wheat breeding*, eds. R. MP and M. A (Mexico: CIMMYT), 172–182.

Hoekenga, O. A., Maron, L. G., Pineros, M. A., Cancado, G. M. A., Shaff, J., Kobayashi, Y., et al. (2006). *AtALMT1*, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in *Arabidopsis*. *Proc. Natl. Acad. Sci.* 103, 9738–9743. doi:10.1073/pnas.0602868103.

Horst, W. J., Puschel, A. K., and Schmohl, N. (1997). Induction of callose formation is a sensitive marker for genotypic aluminum sensitivity in maize. *Plant Soil* 192, 23–30.

Horst, W. J., Wang, Y., and Eticha, D. (2010). The role of the root apoplast in aluminium-induced inhibition of root elongation and in aluminium resistance of plants: A review. *Ann. Bot.* 106, 185–197. doi:10.1093/aob/mcq053.

Huang, C. F., Yamaji, N., Chen, Z., and Ma, J. F. (2012). A tonoplast-localized half-size ABC transporter is required for internal detoxification of aluminum in rice. *Plant J.* 69, 857–867. doi:10.1111/j.1365-313X.2011.04837.x.

Huang, C., Yamaji, N., and Ma, J. (2010). Knockout of a bacterial-type ATP-binding cassette transporter gene, *AtSTAR1*, results in increased aluminum sensitivity in *Arabidopsis*. *Plant Physiol.* 153, 1669–77.

Huang, C., Yamaji, N., Mitani, N., Yano, M., Nagamura, Y., and Ma, J. (2009). A bacterial-type ABC transporter is involved in aluminum tolerance in rice. *Plant Cell* 21, 655–67.

Inostroza-Blancheteau, C., Renge, Z., Alberdi, M., De La Luz Mora, M., Aquea, F., Arce-Johnson, P., et al. (2012). Molecular and physiological strategies to increase aluminum resistance in plants. *Mol. Biol. Rep.* 39, 2069–2079. doi:10.1007/s11033-011-0954-4.

Inostroza-Blancheteau, C., Soto, B., Ibáñez, C., Ulloa, P., Aquea, F., Arce-Johnson, P., et al. (2010). Mapping aluminum tolerance loci in cereals: A tool available for crop breeding. *Electron. J. Biotechnol.* 13, 1–12. doi:10.2225/vol13-issue4-fulltext-4.

Inostroza-Blancheteau, C., Soto, B., Ulloa, P., Aquea, F., and Reyes-Díaz, M. (2008). Resistance mechanisms of aluminum (Al^{3+}) phytotoxicity in cereals: physiological, genetic and molecular bases. *J. Soil Sc. Plant Nutr* 8, 57–71.

Ishikawa, S., Wagatsuma, T., Sasaki, R., and Ofei-Manu, P. (2000). Comparison of the amount of citric and malic acids in Al media of seven plant species and two cultivars each in five plant species. *Soil Sci. Plant Nutr.* 46, 751–758.

Iuchi, S., Koyama, H., Iuchi, A., Kobayashi, Y., Kitabayashi, S., Kobayashi, Y., et al. (2007). Zinc finger protein STOP1 is critical for proton tolerance in *Arabidopsis* and coregulates a key gene in aluminum tolerance. *Proc. Natl. Acad. Sci.* 104, 9900–9905. doi:10.1073/pnas.0700117104.

Kidd, P., Llugany, M., Poschenrieder, C., Gunse, B., and Barcelo, J. (2001). The role of root exudates in aluminium resistance and silicon-induced amelioration of aluminium toxicity in three varieties of maize (*Zea mays* L.). *J. Exp. Bot.* 52, 1339–52.

Kim, B. Y., Baier, A. C., Somers, D. J., and Gustafson, J. P. (2001). Aluminium tolerance in triticale, wheat and rye. *Euphytica* 120, 329–337.

Kinraide, T. B., Yermiyahu, U., and Rytwo, G. (1998). Computation of surface electrical potentials of plant cell membranes. Correspondence to published zeta potentials from diverse plant sources. *Plant Physiol.* 118, 505–512.

Klug, B., and Horst, W. J. (2010). Spatial characteristics of aluminum uptake and

translocation in roots of buckwheat (*Fagopyrum esculentum*). *Physiol. Plant.* 139, 181–191. doi:10.1111/J.1399-3054.2010.01355.X.

Kobayashi, Y., Ohyama, Y., Kobayashi, Y., Ito, H., Iuchi, S., Fujita, M., et al. (2014). STOP2 activates transcription of several genes for Al- and low pH-tolerance that are regulated by STOP1 in *Arabidopsis*. *Mol. Plant* 7, 311–322. doi:10.1093/mp/sst116.

Kochian, L. V., Hoekenga, O. A., and Piñeros, M. A. (2004). How Do Crop Plants Tolerate Acid Soils? Mechanisms of Aluminum Tolerance and Phosphorous Efficiency. *Annu. Rev. Plant Biol.* 55, 459–493. doi:10.1146/annurev.arplant.55.031903.141655.

Kochian, L. V., Pence, N. S., Letham, D. L. D., Pineros, M. A., Magalhaes, J. V., Hoekenga, O. A., et al. (2002). Mechanisms of metal resistance in plants: Aluminum and heavy metals. *Plant Soil* 247, 109–119. doi:10.1023/A:1021141212073.

Kochian, L. V., Piñeros, M. A., and Hoekenga, O. A. (2005). The physiology, genetics and molecular biology of plant aluminum resistance and toxicity. *Plant Soil* 274, 175–195. doi:10.1007/s11104-004-1158-7.

Kochian, L. V., Piñeros, M. A., Liu, J., and Magalhaes, J. V. (2015). Plant Adaptation to Acid Soils: The Molecular Basis for Crop Aluminum Resistance. *Annu. Rev. Plant Biol.* 66, 571–598. doi:10.1146/annurev-arplant-043014-114822.

Larsen, P., Geisler, M., Jones, C., Williams, K., and Cancel, J. (2005). *ALS3* encodes a phloem-localized ABC transporter-like protein that is required for aluminum tolerance in *Arabidopsis*. *Plant J.* 41, 353–63.

Li, J., Liu, J., Dong, D., Jia, X., McCouch, S., and Kochian, L. (2014). Natural variation underlies alterations in Nramp aluminum transporter (*NRAT1*) expression and function that play a key role in rice aluminum tolerance. *Proc Natl Acad Sci U S A* 111, 6503–6508.

Li, X. F., Ma, J. F., and Matsumoto, H. (2000). Pattern of aluminum-induced secretion of organic acids differs between rye and wheat. *Plant Physiol.* 123, 1537–1544. doi:10.1104/pp.123.4.1537.

Ligaba, A., Katsuhara, M., Ryan, P., Shibasaka, M., and Matsumoto, H. (2006). The *BnALMT1* and *BnALMT2* genes from rape encode aluminum-activated malate transporters that enhance the aluminum resistance of plant cells. *Plant Physiol.* 142, 1294–1303.

Ligaba, A., Maron, L., Shaff, J., Kochian, L., and Piñeros, M. (2012). Maize ZmALMT2 is a root anion transporter that mediates constitutive root malate efflux. *Plant Cell Env.* 35, 1185–1200.

Liu, J., Magalhaes, J. V., Shaff, J., and Kochian, L. V. (2009). Aluminum-activated citrate and malate transporters from the *MATE* and *ALMT* families function independently to confer *Arabidopsis* aluminum tolerance. *Plant J.* 57, 389–399. doi:10.1111/j.1365-313X.2008.03696.x.

Loarce, Y., Gallego, R., and Ferrer, E. (1996). A comparative analysis of the genetic relationships between rye cultivars using RFLP and RAPD markers. *Euphytica* 88, 107–115.

Ma, J. . F., Taketa, S., and Yang, Z. M. (2000). Aluminum tolerance genes on the short arm of chromosome *3R* are linked to organic acid release in triticale. *Plant Physiol* 122, 687–694. doi:10.1104/pp.122.3.687.

Ma, J. F. (2000). Role of organic acids in detoxification of aluminum in higher plants. *Plant Cell Physiol.* 41, 383–390. doi:10.1093/pcp/41.4.383.

Ma, J. F., Chen, Z. C., and Shen, R. F. (2014). Molecular mechanisms of Al tolerance in gramineous plants. *Plant Soil* 381, 1–12. doi:10.1007/s11104-014-2073-1.

Ma, J. F., and Furukawa, J. (2003). Recent progress in the research of external Al detoxification in higher plants: A minireview. *J. Inorg. Biochem.* 97, 46–51. doi:10.1016/S0162-0134(03)00245-9.

Ma, J. F., and Hiradate, S. (2000). Form of aluminium for uptake and translocation in buckwheat (*Fagopyrum esculentum* Moench). *Planta* 211, 355–360.

Ma, J. F., Hiradate, S., Nomoto, K., Iwashita, T., and Matsumoto, H. (1997a). Internal detoxification mechanism of Al in hydrangea: identification of Al form in the leaves. *Plant Physiol.* 113, 1033–1039.

Ma, J. F., and Ryan, P. R. (2010). Foreword: Understanding how plants cope with acid soils. *Funct. Plant Biol.* 37, 2008–2011. doi:10.1071/FPv37n4-FO.

Ma, J. F., Ryan, P. R., and Delhaize, E. (2001). Aluminium tolerance in plants and the complexing role of organic acids. *Trends Plant Sci.* 6, 273–278. doi:10.1016/S1360-1385(01)01961-6.

Ma, J. F., Shen, R. F., Nagao, S., and Tanimoto, E. (2004a). Aluminum targets elongating cells by reducing cell wall extensibility in wheat roots. *Plant Cell Physiol.* 45, 583–589.

Ma, J. F., Shen, R., Zhao, Z., Wissuwa, M., Takeuchi, Y., T, E., et al. (2002). Response of rice to Al stress and identification of quantitative trait loci for Al tolerance. *Plant Cell Physiol* 43, 652–659.

Ma, J. F., Zheng, S. J., Li, X. F., Takeda, K., and Matsumoto, H. (1997b). A rapid hydroponic screening for aluminium tolerance in barley. *Plant Soil* 191, 133–137. doi:10.1023/A:1004257711952.

Ma, R., Yli-Mattila, T., and Pulli, S. (2004b). Phylogenetic relationships among genotypes of worldwide collection of spring and winter ryes (*Secale cereale* L.) determined by RAPD-PCR markers. *Hereditas* 140, 210–221. doi:10.1111/j.1601-5223.2004.01844.x.

Magalhaes, J. V. (2010). How a microbial drug transporter became essential for crop cultivation on acid soils: Aluminium tolerance conferred by the multidrug and toxic compound extrusion (*MATE*) family. *Ann. Bot.* 106, 199–203. doi:10.1093/aob/mcq115.

Magalhaes, J. V, Liu, J., Guimarães, C. T., Lana, U. G. P., Alves, V. M. C., Wang, Y.-H., et al. (2007). A gene in the multidrug and toxic compound extrusion (*MATE*) family confers aluminum tolerance in sorghum. *Nat. Genet.* 39, 1156–1161. doi:10.1038/ng2074.

Maron, L. G., Guimaraes, C. T., Kirst, M., Albert, P. S., Birchler, J. A., Bradbury, P. J., et al. (2013). Aluminum tolerance in maize is associated with higher *MATE1* gene copy number. *Proc. Natl. Acad. Sci.* 110, 5241–5246. doi:10.1073/pnas.1220766110.

Maron, L. G., Kirst, M., Mao, C., Milner, M. J., Menossi, M., and Kochian, L. V. (2008). Transcriptional profiling of aluminum toxicity and tolerance responses in maize roots. *New Phytol.* 179, 116–128. doi:10.1111/j.1469-8137.2008.02440.x.

Maron, L. G., Piñeros, M. A., Guimarães, C. T., Magalhaes, J. V., Pleiman, J. K., Mao, C., et al. (2010). Two functionally distinct members of the *MATE* (multi-drug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. *Plant J.* 61, 728–740. doi:10.1111/j.1365-313X.2009.04103.x.

Martis, M. M., Zhou, R., Haseneyer, G., Schmutzer, T., Vrana, J., Kubalakova, M., et al. (2013). Reticulate Evolution of the Rye Genome. *Plant Cell* 25, 3685–3698. doi:10.1105/tpc.113.114553.

Matos, M., Camacho, M. V., Pérez-Flores, V., Pernaute, B., Pinto-Carnide, O., and Benito, C. (2005). A new aluminum tolerance gene located on rye chromosome arm *7RS*. *Theor. Appl. Genet.* 111, 360–369. doi:10.1007/s00122-005-2029-1.

Matos, M., Pinto-Carnide, O., and Benito, C. (2001). Phylogenetic relationships among Portuguese rye based on isozyme, RAPD and ISSR markers. *Hereditas* 134, 229–236. doi:10.1111/j.1601-5223.2001.00229.x.

Matsumoto, H., Hirasawa, E., Morimura, S., and Takahashi, E. (1976). Localization of aluminium in tea leaves. *Plant Cell. Physiol.* 17, 627–631.

Matsumoto, H., and Motoda, H. (2012). Aluminum toxicity recovery processes in root apices. Possible association with oxidative stress. *Plant Sci.* 185–186, 1–8. doi:10.1016/j.plantsci.2011.07.019.

Merino-Gergichevich, C., Alberdi, M., Ivanov, A. G., and Reyes-Diaz, M. (2010). Al^{3+}–Ca^{2+} interaction in plants growing in acid soils: Al-phytotoxicity response to calcareous amendments,. *J. Soil Sci. Plant Nutr.* 10, 217–243.

Miftahudin, T., Scoles, G., and Gustafson, J. (2002). AFLP markers tightly linked to the aluminum tolerance gene *Alt3* in rye. *Theor. Appl. Genet.* 104, 626–631. doi:10.1007/s00122-001-0782-3.

Myśków, B., Milczarski, P., and Masojć, P. (2010). Comparison of RAPD, ISSR and SSR markers in assessing genetic diversity among rye (*Secale cereale* L.) inbred lines. *Plant Breed. Seed Sci.* 62, 107–115.

Negishi, T., Oshima, K., Hattori, M., Kanai, M., Mano, S., Nishimura, M., et al. (2012). Tonoplast- and Plasma Membrane-Localized Aquaporin-Family Transporters in Blue Hydrangea Sepals of Aluminum Hyperaccumulating Plant. *PLoS One* 7. doi:10.1371/journal.pone.0043189.

Panda, S. K., and Matsumoto, H. (2007). Molecular physiology of aluminum toxicity and tolerance in plants. *Bot. Rev.* 73, 326–347.

Pellet, D., Grunes, D., and Kochian, L. (1995). Organic acid exudation as an aluminum-tolerance mechanism in maize (*Zea mays* L.). *Planta* 196, 788–795.

Persson, K., Díaz, O., and von Bothmer, R. (2001). Extent and patterns of RAPD variation in landraces and cultivars of rye (*Secale cereale* L.) from northern Europe. *Hereditas* 134, 237–43. doi:10.1111/j.1601-5223.2001.00237.x.

Petersen, G., and Doebley, J. F. (1993). Chloroplast DNA variation in the genus *Secale* (Poaceae). *Plant Syst. Evol.* 187, 115–125. doi:10.1007/BF00994094.

Piñeros, M., Cançado, G., Maron, L., Lyi, S., Menossi, M., and Kochian, L. (2008). Not all ALMT1-type transporters mediate aluminum-activated organic acid responses: the case of ZmALMT1 – an anion-selective transporter. *Plant J* 53, 352–367.

Pinto-Carnide, O., and Guedes-Pinto, H. (1999). Aluminium tolerance variability in rye and wheat Portuguese germplasm. *Genet. Resour. Crop Ev.* 46, 81–85.

Pinto-Carnide, O., Guedes-Pinto, H., and Vaz, E. (1990). Tolerância ao alumínio de híbridos F2 de triticales hexaploides. *Melhoramento* 32, 285–380.

Poschenrieder, C., Gunsé, B., Corrales, I., and Barceló, J. (2008). A glance into aluminum toxicity and resistance in plants. *Sci. Total Environ.* 400, 356–368. doi:10.1016/j.scitotenv.2008.06.003.

Rao, I. M., Zeigler, R. S., Vera, R., and Sarkarung, S. (1993). Selection and breeding for

acid-soil tolerance in crops,. *Bioscience* 43, 454–465.

Rengel, Z., and Zhang, W. (2003). Role of dynamics of intracellular calcium in aluminium-toxicity syndrome. *New Phytol.* 159, 295–314.

Ribeiro, M., Seabra, L., Ramos, A., Santos, S., Pinto-Carnide, O., Carvalho, C., et al. (2012). Polymorphism of the storage proteins in Portuguese rye (*Secale cereale* L.) populations. *Hereditas* 149, 72–84. doi:10.1111/j.1601-5223.2012.02239.x.

Ring, S. M., Fisher, R. P., Poile, G. J., Helyar, K. R., Conyers, M. K., and Morris, S. G. (1993). Screening species and cultivars for their tolerance to acidic soil conditions. *Plant Soil* 155., 521–524. doi:https://doi.org/10.1007/BF00025098.

Ryan, P. R., and Delhaize, E. (2010). The convergent evolution of aluminium resistance in plants exploits a convenient currency. *Funct. Plant Biol.* 37, 275–284. doi:10.1071/FP09261.

Ryan, P. R., and Delhaize, E. (2012). "Adaptations to Aluminum Toxicity," in *Plant Stress Physiology*, ed. S. Shabala (Boston, MA, USA: CAB International), 171–193.

Ryan, P. R., Ditomaso, J. M., and Kochian, L. V. (1993). Aluminum toxicity in roots: an investigation of spatial sensitivity and the role of the root cap. *J. Exp. Bot.* 44, 437–446.

Ryan, P. R., Raman, H., Gupta, S., Horst, W. J., and Delhaize, E. (2009). A Second Mechanism for Aluminum Resistance in Wheat Relies on the Constitutive Efflux of Citrate from Roots. *Plant Physiol.* 149, 340–351. doi:10.1104/pp.108.129155.

Ryan, P. R., Raman, H., Gupta, S., Sasaki, T., Yamamoto, Y., and Delhaize, E. (2010a). The multiple origins of aluminium resistance in hexaploid wheat include *Aegilops tauschii* and more recent cis mutations to *TaALMT1*. *Plant J.* 64, 446–455. doi:10.1111/j.1365-313X.2010.04338.x.

Ryan, P. R., Tyerman, S. D., Sasaki, T., Furuichi, T., Yamamoto, Y., Zhang, W. H., et al. (2010b). The identification of aluminium-resistance genes provides opportunities for enhancing crop production on acid soils. *J. Exp. Bot.* 62, 9–20. doi:10.1093/jxb/erq272.

Samac, D. A., and Tesfaye, M. (2003). Plant improvement for tolerance to aluminum in acid soils – a review. *Plant Cell. Tissue Organ Cult.* 75, 189–207.

Sánchez-Parra, B., Figueiras, A. M., El-Moneim, D. A., Contreras, R., Rouco, R., Gallego, F. J., et al. (2014). The role of two superoxide dismutase mRNAs in rye aluminium tolerance. *Plant Biol.* 17, 694–702. doi:10.1111/plb.12281.

Santos, E., Benito, C., Silva-Navas, J., Gallego, F. J., Figueiras, A. M., Pinto-Carnide, O., et al. (2018). Characterization, genetic diversity, phylogenetic relationships, and expression of the aluminum tolerance *MATE1* gene in *Secale* species. *Biol. Plant.* 62, 109–120. doi:10.1007/s10535-017-0749-0.

Santos, E., Matos, M., Silva, P., Figueiras, A. M., Benito, C., and Pinto-Carnide, O. (2016). Molecular diversity and genetic relationships in *Secale*. *J. Genet.* 95, 273–281. doi:10.1007/s12041-016-0632-3.

Sasaki, T., Ryan, P., Delhaize, E., Hebb, D., Ogihara, Y., Kawaura, K., et al. (2006). Sequence upstream of the wheat (*Triticum aestivum* L.) *ALMT1* gene and its relationship to aluminum resistance. *Plant Cell Physiol* 47, 1343–1354.

Sasaki, T., Yamamoto, Y., Ezaki, B., Katsuhara, M., Ahn, S., Ryan, P., et al. (2004). A wheat gene encoding an aluminum-activated malate transporter. *Plant J* 37, 645–653.

Sawaki, Y., Iuchi, S., Kobayashi, Y., Kobayashi, Y., Ikka, T., Sakurai, N., et al. (2009). STOP1 Regulates Multiple Genes That Protect *Arabidopsis* from Proton and

Aluminum Toxicities. *Plant Physiol.* 150, 281–294. doi:10.1104/pp.108.134700.

Schlegel, R. H. J. ed. (2014). *Rye: Genetics, Breeding, and Cultivation*. Boca Raton, Florida: CRC Press; Taylor&Francis Group.

Shang, H. Y., Wei, Y. M., Wang, X. R., and Zheng, Y. L. (2006). Genetic diversity and phylogenetic relationships in the rye genus *Secale* L. (rye) based on *Secale cereale* microsatellite markers. *Genet. Mol. Biol.* 29, 685–691. doi:10.1590/S1415-47572006000400018.

Sharma, T., Dreyer, I., Kochian, L., and Piñeros, M. A. (2016). The ALMT Family of Organic Acid Transporters in Plants and Their Involvement in Detoxification and Nutrient Security. *Front. Plant Sci.* 7, 1–12. doi:10.3389/fpls.2016.01488.

Shen, R., Iwashita, T., and Ma, J. (2004). Form of Al changes with Al concentration in leaves of buckwheat. *J Exp Bot* 55, 131–136.

Silva-Navas, J. (2016). Modulación del sistema radicular en condiciones de estrés. Tesis Doctoral en Ciencias Biologicas. Madrid: Universidad Complutense de Madrid, 209p.

Silva-Navas, J., Benito, C., Téllez-Robledo, B., Abd El-Moneim, D., and Gallego, F. J. (2012). The *ScAACT1* gene at the *Qalt5* locus as a candidate for increased aluminum tolerance in rye (*Secale cereale* L.). *Mol. Breed.* 30, 845–856. doi:10.1007/s11032-011-9668-5.

Silva, I., Smyth, T., Moxley, D., Carter, T., Allen, N., and Rufty, T. (2000). Aluminum accumulation at nuclei of cells in the root tip. Fluorescence detection using lumogallion and confocal laser scanning microscopy. *Plant Physiol.* 123, 543–552.

Silva, S., Santos, C., Matos, M., and Pinto-Carnide, O. (2012). Al toxicity mechanism in tolerant and sensitive rye genotypes. *Environ. Exp. Bot.* 75, 89–97. doi:10.1016/j.envexpbot.2011.08.017.

Simões, C. C., Melo, J. O., Magalhaes, J. V, and Guimarães, C. T. (2012). Genetic and molecular mechanisms of aluminum tolerance in plants. *Genet. Mol. Res.* 11, 1949–1957. doi:10.4238/.

Sivaguru, M., Pike, S., Gassmann, W., and Baskin, T. (2003). Aluminum rapidly depolymerizes cortical rnicrotubules and depolarizes the plasma membrane: evidence that these responses are mediated by a glutamate receptor. *Plant Cell Physiol.* 44, 667–675.

Takanashi, K., Shitan, N., and Yazaki, K. (2014). The multidrug and toxic compound extrusion (*MATE*) family in plants. *Plant Biotechnol.* 31, 417–430. doi:10.5511/plantbiotechnology.14.0904a.

Tolrà, R., Barceló, J., and Poschenrieder, C. (2009). Constitutive and aluminium-induced patterns of phenolic compounds in two maize varieties differing in aluminium tolerance. *J. Inorg. Biochem.* 103, 1486–1490. doi:10.1016/j.jinorgbio.2009.06.013.

Tolrà, R., Vogel-Mikus, K., Hajiboland, R., Kump, P., Pongrac, P., Kaulich, B., et al. (2011). Localization of aluminium in tea (*Camellia sinensis*) leaves using low energy X-ray fluorescence spectro-microscopy. *J. Plant Res.* 124, 165–172. doi:10.1007/S10265-010-0344-3.

Tovkach, A., Ryan, P. R., Richardson, A. E., Lewis, D. C., Rathjen, T. M., Ramesh, S., et al. (2013). Transposon-mediated alteration of *TaMATE1B* expression in wheat confers constitutive citrate efflux from root apices. *Plant Physiol.* 161, 880–92. doi:10.1104/pp.112.207142.

Ulrich, B., Mayer, R., and Khanna, P. K. (1980). Chemical changes due to acid precipitation in a less-derived soil in Central Europe. *Soil Sci.* 130, 1993–1999.

Vences, F. J., Vaquero, F., and Pérez de la Vega, M. (1987). Phylogenetic relationships in *Secale* (Poaceae): An isozymatic study. *Plant Syst. Evol.* 157, 33–47. doi:10.1007/BF00939179.

Von Uexkull, H. R., and Mutert, E. (1995). Global extent , development and economic impact of acid soils. *Plant Soil* 171, 1–15.

Wang, J., Raman, H., Zhang, G., Mendham, N., and Zhou, M. (2006). Aluminium tolerance in barley (*Hordeum vulgare* L.): Physiological mechanisms, genetics and screening methods. *J. Zhejiang Univ. Sci. B* 7, 769–787. doi:10.1631/jzus.2006.B0769.

Wang, Y., Li, R., Li, D., Jia, X., Zhou, D., Li, J., et al. (2017). NIP1;2 is a plasma membrane-localized transporter mediating aluminum uptake, translocation, and tolerance in *Arabidopsis*. *Proc. Natl. Acad. Sci.* 114, 5047–5052. doi:10.1073/pnas.1618557114.

Xia, J., Yamaji, N., and Ma, J. (2013). A plasma membrane-localized small peptide is involved in rice aluminum tolerance. *Plant J.* 76, 345–55.

Yamaji, N., Huang, C. F., Nagao, S., Yano, M., Sato, Y., Nagamura, Y., et al. (2009). A Zinc Finger Transcription Factor ART1 Regulates Multiple Genes Implicated in Aluminum Tolerance in Rice. *Plant Cell* 21, 3339–3349. doi:10.1105/tpc.109.070771.

Yamamoto, Y., Kobayashi, Y., and Matsumoto, H. (2001). Lipid Peroxidation Is an Early Symptom Triggered by Aluminum, But Not the Primary Cause of Elongation Inhibition in Pea Roots. *Plant Physiol.* 125, 199–208. doi:10.1104/pp.125.1.199.

Yang, L. T., Qi, Y. P., Jiang, H. X., and Chen, L. S. (2013a). Roles of organic acid anion secretion in aluminium tolerance of higher plants. *Biomed Res. Int.* 2013, 9–15. doi:10.1155/2013/173682.

Yang, X. Y., Yang, J. L., Zhou, Y., Pineros, M. A., Kochian, L. V., Li, G. X., et al. (2011). A de novo synthesis citrate transporter, *Vigna umbellata* multidrug and toxic compound extrusion, implicates in Al-activated citrate efflux in rice bean (*Vigna umbellata*) root apex. *Plant Cell Environ.* 34, 2138–2148.

Yang, X. Y., Zeng, Z. H., Yan, J. Y., Fan, W., Bian, H. W., Zhu, M. Y., et al. (2013b). Association of specific pectin methylesterases with Al-induced root elongation inhibition in rice. *Physiol. Plant.* 148, 502–511. doi:10.1111/ppl.12005.

Yokosho, K., Yamaji, N., and Ma, J. F. (2010). Isolation and characterisation of two *MATE* genes in rye. *Funct. Plant Biol.* 37, 296–303. doi:10.1071/FP09265.

Yokosho, K., Yamaji, N., and Ma, J. F. (2011). An Al-inducible *MATE* gene is involved in external detoxification of Al in rice. *Plant J.* 68, 1061–1069. doi:10.1111/j.1365-313X.2011.04757.x.

Zeng, Q. L., Chen, R. F., Zhao, X. Q., Shen, R. F., Noguchi, A., Shinmachi, F., et al. (2013). Aluminum could be transported via phloem in *Camellia oleifera* Abel. *Tree Physiol.* 33, 96–105. doi:10.1093/treephys/tps117.

Zhao, Z., Ma, J. F., Sato, K., and Takeda, K. (2003). Differential Al resistance and citrate secretion in barley (*Hordeum vulgare* L.). *Planta* 217, 794–800. doi:10.1007/s00425-003-1043-2.

Zheng, S. J., Ma, J. F., and Matsumoto, H. (1998a). Continuous secretion of organic acids is related to aluminium resistance during relatively long-term exposure to aluminium stress. *Physiol. Plant.* 103, 209–214.

Zheng, S. J., Ma, J. F., and Matsumoto, H. (1998b). High aluminum resistance in

buckwheat: I. Al-induced specific secretion of oxalic acid from root tips. *Plant Physiol.* 117, 745–751.

Zhou, L.-L., Bai, G.-H., Carver, B., and Zhang, D.-D. (2007). Identification of new sources of aluminum resistance in wheat. *Plant Soil* 297, 105–118. doi:10.1007/s11104-007-9324-3.

Zhu, X. F., Shi, Y. Z., Lei, G. J., Fry, S. C., Zhang, B. C., Zhou, Y. H., et al. (2012). *XTH31*, Encoding an in Vitro XEH/XET-Active Enzyme, Regulates Aluminum Sensitivity by Modulating in Vivo XET Action, Cell Wall Xyloglucan Content, and Aluminum Binding Capacity in *Arabidopsis*. *Plant Cell* 24, 4731–4747. doi:10.1105/tpc.112.106039.

CHAPTER II

Molecular Markers as tools for genetic diversity, phylogeny and association studies

SUBCHAPTER II-1

Molecular diversity and genetic relationships in *Secale*

E. Santos[1,2], M. Matos[1,2], P. Silva[1], A. M. Figueiras[3], C. Benito[3] and O. Pinto-Carnide[1,4]

[1]*Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, Quinta de Prados, 5001-801 Vila Real, Portugal*
[2]*BioISI- Biosystems & Integrative Sciences Institute, University of Lisboa, Faculty of Sciences, Campo Grande, Lisboa, Portugal*
[3]*Departamento de Genética, Facultad de Biologia, Universidad Complutense, C/ José Antonio Novais, 12, 28040 Madrid, Spain*
[4]*CITAB -Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*

Keywords: random amplified polymorphic DNAs; inter simple sequence repeats; genetic diversity; *ScMATE1* gene; exons; *Secale*.

*Published in *Journal of Genetics* (2016) DOI: 10.1007/s12041-016-0632-3

Abstract

The objective of this study was to quantify the molecular diversity and to determine the genetic relationships among *Secale* spp. and among cultivars of *Secale cereale* using RAPDs, ISSRs and sequence analysis of six exons of *ScMATE1* gene. Thirteen ryes (cultivated and wild) were genotyped using 21 RAPD and 16 ISSR primers. A total of 435 markers (242 RAPDs and 193 ISSRs) were obtained, with 293 being polymorphic (146 RAPDs and 147 ISSRs). Two RAPD and nine ISSR primers generated more than 80% of polymorphism. The ISSR markers were more polymorphic and informative than RAPDs. Further, 69% of the ISSR primers selected achieved at least 70% of DNA polymorphism. The study of six exons of the *ScMATE1* gene also demonstrated a high genetic variability that subsists in *Secale* genus. One difference observed in exon 1 sequences from *S. vavilovii* seems to be correlated with Al sensitivity in this species. The genetic relationships obtained using RAPDs, ISSRs and exons of *ScMATE1* gene were similar. *S. ancestrale*, *S. kuprijanovii* and *S. cereale* were grouped in the same cluster and *S. segetale* was in another cluster. *S. vavilovii* showed evidences of not being clearly an isolate species and having great intraspecific differences.

1. Introduction

Rye (*Secale cereale* L.) is an important crop species with the ability to tolerate biotic and abiotic stresses. This species can get a good income in areas where there are no other crops grown, i.e. in areas that are not suitable for cultivation of other cereals (Bushuk, 2001). Rye belongs to genus *Secale* which is small but a significant taxon in tribe Triticeae. *Secale* is a typical representative of the Mediterranean flora (Sencer and Hawkes, 1980). It includes one cultivated and three wild species; perennial or annual, self-incompatible or self-compatible (Vences et al., 1987). *Secale* are mostly allogamous promoting a great diversity of genotypes. All species in this genus have $2n = 14$ chromosomes (Frederiksen and Petersen, 1998); however, there are also some tetraploid derivatives artificially obtained.

Over the years, there has been much controversy about the taxonomy of the *Secale* genus. It remains uncertain despite the large number of studies performed. At present, there are four recognized species of the genus *Secale*: the annual out breeder *S. cereale* L., the annual autogamous *S. sylvestre* Host and *S. vavilovii* Grossh, and the perennial out breeder *S. strictum* (Presl.) Presl. (syn. *S. montanum* Guss.) (De Bustos and Jouve, 2002; Germplasm Resources Informative Network (GRIN) 2015 (http://www.ars-grin.gov)). There are eight subspecies included in *S. cereale*: *afghanicum* (Vavilov) Hammer, *ancestrale* Zhuk., *cereale* (the only cultivated), *dighoricum* Vavilov, *rigidum* Vavilov and Antropov (syn. *S. turkestanicum* Bensin), *segetale* Zhuk., *tetraploidum* Kobyl. and *tsitsinii* Kobyl. Within *S. strictum*, there are five subspecies: *africanum* (Stapf) K. Hammer (unlike the others, it is autogamous), *anatolicum* (Boiss.) K. Hammer, *ciliatoglume* (Boiss.) K. Hammer, *kuprijanovii* (Grossh.) K. Hammer and *strictum* (syn. *S. montanum* Guss.) (USDA, ARS, National Genetic Resources Program. Germplasm Resources Informative Network (GRIN) 2015 (http://www.ars-grin.gov/cgi-bin/npgs/html/splist.pl?11022 (10 April 2015)).

Phylogenetic relationships between and among *Secale* species have been studied using different approaches, including morphological and ecological (e.g. Vavilov, 1926; Stutz, 1972), cytogenetic (Khush, 1962; Cuadrado and Jouve, 2002; Zhou et al., 2010), chemotaxonomic (Dedio et al., 1969), biochemical, like isozymes (Vences et al., 1987; Matos et al., 2001); and molecular methods such as amplified fragment length polymorphism (AFLP) (Chikmawati et al., 2005, 2012), ISSR (Matos et al., 2001; Ren et al., 2011), random amplified polymorphic DNA (RAPD) (Matos et al., 2001; Ma et al.,

2004), restriction fragment length polymorphism (RFLP) (Loarce et al., 1996), simple sequence repeat (SSR) (Shang et al., 2006; Jenabi et al., 2011) and ribosomal DNA studies (De Bustos and Jouve, 2002).

Several classifications were obtained resulting from the diversity of techniques used in phylogenetic analysis. Genetic diversity studies on rye and wild rye could contribute to the maintenance and rational use of germplasm resources in the improvement of rye and its related species (Ma et al., 2004). In the past two decades, use of molecular markers have become routine in plant biotechnology, such as genetic diversity studies. ISSR (Zietkiewicz et al., 1994) and RAPD (Williams et al., 1990) are polymerase chain reaction (PCR)-based markers popular for its advantages, cheap, quick and easy to assay. PCR amplification needs low quantities of DNA and, moreover, these markers have a high genomic abundance and good genome coverage, and does not require sequence information.

Al-induced citrate transporter (*ScMATE1*) gene is of interest in rye since it is involved in aluminum tolerance in Poaceae family, and has been studied and characterized in *S. cereale* (Silva-Navas et al., 2012). In this work, ISSR and RAPD markers and six exons of *ScMATE1* gene were used to study the molecular diversity within the *Secale* genus and to estimate the genetic relationships among *Secale* spp. and between cultivars of *S. cereale* ssp. *cereale*. In this study, the *ScMATE1* gene was selected due to its importance in Al tolerance behavior in different cultivars of rye. This is the first study of diversity of *ScMATE1* gene in different species of *Secale* genus.

2. Materials and Methods

2.1. Plant materials and DNA extraction

Three species, *S. cereale* L., *S. strictum* (C. Persl) C. Persl and *S. vavilovii* Grossh. of the *Secale* genus were studied. Also, three subspecies of *S. cereale*: *S. cereale* ssp. *ancestrale* Zhuk., the cultivated *S. cereale* ssp. *cereale* and *S. cereale* ssp. *segetale* Zhuk.; and two of *S. strictum*: *S. strictum* ssp. *kuprijanovii* Grossh. and *S. strictum* ssp. *strictum* syn. *S. montanum* Guss. were also studied. These materials were kindly provided by Dr E. Larter, University of Winnipeg (Manitoba, Canada) (Dedio et al., 1969) and maintained in the germplasm bank of the Department of Genetics and Biotechnology at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Moreover, four rye varieties: Imperial (USA), JNK (Japan), Dankowskie Zlote (Poland) and Alvão (Portugal); three

regional populations from the northern Portugal (two from Lamego and one from Montalegre), and the consanguineous line Riodeva (Spain) belonging to cultivated *S. cereale* were also studied.

For RAPD and ISSR analyses, each rye and wild rye were reduced to a pool of 15 plants and thereafter to 100 mg of young leaves from each plant. In the analyses of bulk samples, only frequently seen fragments in individual plants were scored (Loarce et al., 1996). Fragments seen at frequencies below 10% (Michelmore et al., 1991) or 14% (Loarce et al., 1996) were not amplified in the bulk DNA samples. The number of plants used to construct the bulk samples was similar to that used in the previous works. The fragments seen at frequencies below 13% were not amplified in our bulks. For *ScMATE1* study, individual plants were used. All the green tissues were frozen in liquid nitrogen and stored at 80 ºC until DNA extraction. For DNA extraction, a small- scale DNA isolation method was used (DNeasy Plant Mini kit, Qiagen Hilden, Germany) according to the procedures described by the manufacturer.

2.2. RAPD and ISSR markers

2.2.1. Polymerase chain reaction

For RAPDs, 21 10-mer oligonucleotides from sets A, B and C (Operon Technologies, Alameda, USA) were selected (Supplementary Table S1). For ISSRs, 16 primers from UBC primer set 100/9 (University of British Columbia, Canada) based on dinucleotides and pentanucleotides repeats were chosen (Supplementary Table S1). Primers were selected according to the degree of reproducibility, amplification and polymorphism.

RAPD and ISSR reactions were performed with minor modifications as described by Matos et al. (2001). These reactions were carried out in 25 and 20 μL volume, respectively using T-Professional Thermocycler (Biometra, Göttingen, Germany). PCR products were analyzed on 1.8% agarose gel stained with ethidium bromide.

2.2.2. Data analysis

RAPD and ISSR amplifications were repeated at least thrice and only repetitive PCR products were scored. RAPD and ISSR markers were scored based on the presence (1) or absence (0) of homologous bands of all rye genotypes. Bands with same mobility were treated as identical fragment. Cluster analysis using the simple matching coefficient (SM) and the unweighted pairwise group method with arithmetic average (UPGMA) was

achieved with the NTSYS-pc statistical package v. 2.02 (Rohlf, 1998). Further, cluster analyses were performed for each marker system based on Dice and Jaccard coefficient to reinforce the study (Nei and Li, 1979).

To check the goodness of fit of ISSR and RAPD cluster analysis to the similarity matrix on which it was used, the cophenetic correlation coefficient (r) was calculated and Mantel test was performed with 1000 permutations (Mantel, 1967) using COPH and MXCOMP tools from NTSYS-pc package. The coefficients values obtained for both markers were compared. Further, the correlation of the combined similarity data matrices (RAPD and ISSR) and the cophenetic matrices of the three coefficients used (SM, Dice and Jaccard) were estimated and compared to verify their reliability.

To determine the confidence limits of UPGMA-based dendrograms, bootstrap tests were performed using 10 000 replications for phylogenetic analysis using the Winboot program (Yap and Nelson, 1996).

2.2.3. Discriminatory power

Primer resolving power (Rp) was calculated according to Prevost and Wilkinson (1999) formula Rp = ΣIb, where Ib (band informativeness) takes the value of 1–[2x(0.5–p)], p being the proportion of the 13 rye genotypes analyzed containing the band. Polymorphic information content (PIC) was calculated as PIC = 2fi(1–fi), as proposed by Roldán-Ruiz et al. (2000), where fi is the frequency of the marker bands present and (1–fi) is the frequency of absent marker bands. PIC was averaged over the bands for each primer. To estimate the effectiveness of each marker system, marker index (MI) was calculated according to Sorkheh et al. (2007) that defined it as the product of the polymorphism percentage and PIC value.

2.3. *ScMATE1* gene

2.3.1. Exon amplifications

ScMATE1 complete gene (genomic sequence) was previously obtained from Silva-Navas et al. (2012). Different pair of primers which were designed from the sequences of *ScMATE1* gene were used to amplify the exons 1, 3, 4, 8, 9 and 10 from rye genomic DNA (gDNA) by PCR (Supplementary Table S2).

2.3.2. Sequence and phylogenetic analyses

Sequences were analyzed with Chromas Lite 1.0 (Technelysium, South Brisbane, Australia). A BLASTN search (http://www.ncbi.nlm.nih.gov/) was performed to confirm the DNA sequences predicted from the analysis. Alignments between different *ScMATE1* sequences were made using the ClustalW algorithm (http://www.ebi.ac.uk/Tools/clustalw). The sequences obtained in this study were compared and several diversity parameters were utilized to study the difference among the DNA sequences of *ScMATE1* using DnaSP v. 5.0 (Librado and Rozas, 2009). Genetic relationships among exons of *ScMATE1* gene in different *Secale* genotypes were studied using MEGA 4.0 (Tamura et al., 2007) with the evolutionary distance (Kimura 2-parameter) and the UPGMA clustering method. Bootstraps with 10 000 replicates were performed to test the robustness of the dendrograms. The sequence of *HvMATE1* gene from *Hordeum vulgare* was used as outgroup.

3. Results

3.1. RAPD analysis

A total of 242 fragments were produced from all the primers ranging from 250 to 2800 bp with 60.33% polymorphic (Supplementary Fig. S1). The average band per primer and the average polymorphic band per primer were 11.52 and 6.95, respectively. Moreover, a total of 22 exclusive bands were observed with 13 RAPDs primers, *OPC13* being the most significant. More number of unique bands were found in the cultivated species, especially in the consanguineous line Riodeva. The Rp of 21 RAPD primer was 6.25. Primer *OPC13* reached the highest Rp value (10.46), being able to distinguish all the 13 rye genotypes as *OPC9* primer. The average PIC and MI values were 0.18 and 11.69, respectively (Table 1).

Table 1. Genetic diversity parameters for 21 RAPDs and 16 ISSRs primers used for analyzing 13 rye genotypes.

Primer	NB	PB	%P	UB	Rp	PIC	MI	DG
RAPDs								
A1	10	5	50.00	1	2.15	0.16	7.81	4
A4	8	5	62.50	0	5.38	0.22	13.50	8
A5	11	8	72.73	3	6.15	0.21	15.02	6
A17	13	9	69.23	3	6.62	0.14	9.92	5
B1	12	7	58.33	1	6.83	0.18	10.73	6
B5	12	7	58.33	1	7.69	0.17	10.00	8
B7	15	10	66.67	1	8.62	0.19	12.42	9
B10	13	5	38.46	0	3.23	0.17	6.45	7
C1	9	7	77.78	0	7.23	0.31	23.73	11
C2	7	2	28.57	1	0.62	0.07	2.03	1
C4	9	4	44.44	0	2.31	0.18	7.94	7
C5	13	8	61.54	1	8.62	0.20	12.32	11
C6	9	1	11.11	0	0.31	0.03	0.32	0
C7	11	10	90.91	2	8.31	0.27	24.25	6
C9	13	10	76.92	1	10.46	0.26	20.30	13
C11	9	4	44.44	0	3.23	0.19	8.41	6
C12	10	5	50.00	2	5.38	0.10	5.21	2
C13	21	20	95.24	4	18.00	0.29	27.79	13
C16	14	5	35.71	0	4.62	0.11	4.10	5
C19	15	10	66.67	1	9.69	0.21	14.31	10
C20	8	4	50.00	0	5.85	0.18	8.88	3
Total	242	146	60.33	22	6.25	0.18	11.69	6.71
ISSRs								
808	16	13	81.25	4	8.31	0.23	18.87	11
810	14	10	71.43	4	8.77	0.15	10.75	7
811	15	13	86.67	3	9.85	0.23	19.97	13
812	13	12	92.31	3	9.85	0.28	25.55	9
815	6	4	66.67	0	4.92	0.21	13.94	2
824	10	8	80.00	1	9.54	0.26	20.64	9
827	8	3	37.50	1	2.62	0.13	4.99	2
835	7	3	42.86	2	1.38	0.11	4.78	1
836	19	16	84.21	4	8.92	0.23	19.72	13
842	20	16	80.00	5	12.31	0.20	16.00	11
844	9	8	88.89	2	7.69	0.20	18.00	2
845	9	6	66.67	2	4.62	0.17	11.05	5
846	12	10	83.33	3	8.00	0.25	21.20	9
881	15	12	80.00	4	10.31	0.21	16.79	7
889	12	9	75.00	3	6.62	0.17	12.87	9
891	8	4	50.00	1	3.54	0.13	6.36	2
Total	193	147	76.17	42	7.33	0.20	15.09	7
RAPDs and ISSRs								
Total	435	293	67.36	64	6.79	0.19	13.39	6.86

NB – Number of bands; PB – polymorphic bands; %P – percentage of polymorphism, UB –unique bands; Rp – Resolving power values; PIC – Polymorphism Information Content values; MI – Marker Index values; DG – distinguished genotypes.

3.2. ISSR analysis

The average degree of polymorphism obtained with ISSR markers was higher than that of RAPD markers, reaching 76.17% (Table 1). The total number of amplified products was 193 ranging from 320 to 3000 bp, with 147 being polymorphic. The average band per primer and the average polymorphic band per primer were 12.06 and 9.19, respectively. The polymorphism obtained with ISSR markers was significant because 69% of selected primers achieved at least 70% of polymorphism. The number of exclusive bands was 42 (twice as much as RAPD markers). Five unique bands were obtained with only *UBC842* primer. As in RAPDs, ISSR markers revealed the highest exclusive band number in the cultivated species, with special relevance to the consanguineous line Riodeva and JNK varieties, with 14 and 15 exclusive bands, respectively, average of Rp was 7.33. Primers with higher Rp were able to distinguish a greater number of genotypes, although it was not so linear. Primers *UBC811* and *UBC836* distinguished all rye genotypes (Supplementary Fig. S1). The average PIC and MI were 0.2 and 15.09, respectively (Table 1).

3.3. Genetic relationships using RAPDs and ISSRs data

Dendrograms using only RAPD markers, only ISSR markers and both the markers combined were obtained. These dendrograms were generated using three coefficients (SM, Dice and Jaccard) with the UPGMA grouping method. The three coefficients used produced identical dendrograms with slight variations in the similarity degree obtained (data not shown). The best results were obtained by the combined data (ISSR and RAPD). Two different kinds of dendrograms were created for this study: one with seven distinct cultivars of *S. cereale* (Fig. 1a) and another with six different species/subspecies of the *Secale* genus (Fig. 2a), where the cultivar Imperial was the representative of the *S. cereale* ssp. *cereale*. The results obtained with the combined data and with ISSRs data were the same, whereas the dendrograms obtained with RAPD data showed minor modifications.

The dendrogram that resulted from the cultivated rye data (Fig. 1a) had three main clusters: one with five cultivars split into two subclusters (cluster I) and two divergent samples, the consanguineous line Riodeva (cluster II) and the JNK variety (cluster III). In cluster I, rye varieties Alvão, Imperial and D. Zlote were included in the same subcluster (A) and the regional Portuguese populations from Montalegre and Lamego were grouped together (subcluster B). The dendrogram obtained with one representative

of each *Secale* species/subspecies, which originated three main groups are shown in figure 2a. Cluster I was divided into two subclusters, one with the subspecies *S. ancestrale* and *S. kuprijanovii* (subcluster A) and another with the variety Imperial (*S. cereale*) and the species *S. vavilovii* (subcluster B). In the clusters II and III, the subspecies *S. segetale* and the species *S. strictum*, respectively, were isolated. Mantel test revealed high correlation values between the dendrograms and the original matrices for both approaches (*S. cereale*: $r = 0.960$ and *Secale* species/subspecies: $r = 0.776$). The correlation values were also significant using Jaccard and Dice coefficients (data not shown).

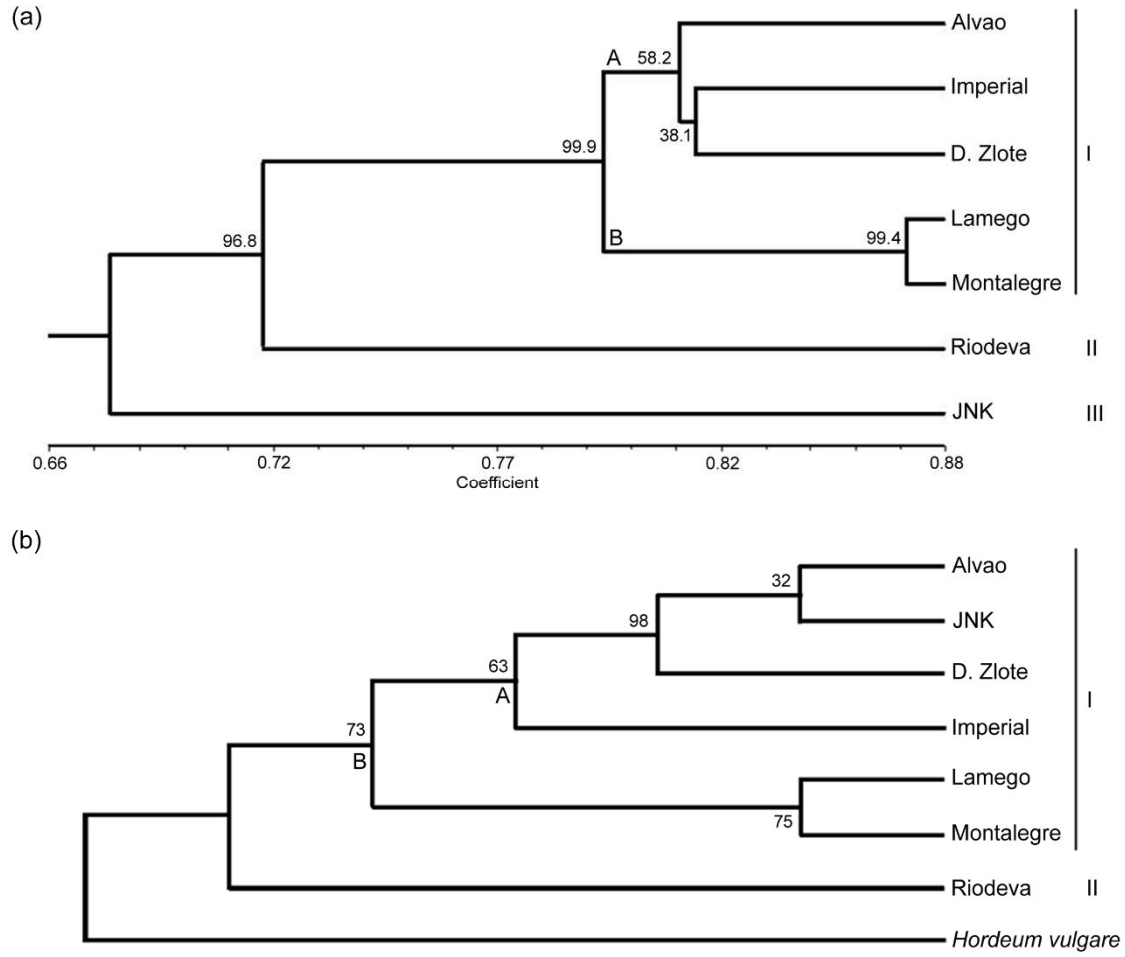


Figure 1. Dendrogram showing the genetic similarity within cultivated ryes belonging to *S. cereale* ssp. *cereale* using (a) RAPD and ISSR markers data and (b) data of six exons of *ScMATE1* gene. I, II, III indicate cluster denomination and (A) and (B) subclusters denomination. Dendrogram (a) was generated using UPGMA method and SM coefficient, whereas dendrogram (b) was generated using the evolutionary distance Kimura 2-parameter and the clustering method UPGMA. The numbers at the nodes are the bootstrap probability values (%).

3.4. *ScMATE1*: sequences and genetic relationships

At least one sequence from each rye genotype per exon was obtained. Exon 1 was the most variable with haplotype diversity (Hd), almost reaching the value 1 (0.918) and with

the highest average number of nucleotide differences (k) and nucleotide diversity (Pi) with 3.9 and 0.02066, respectively (Table 2). Two different sequences of exon 1 were detected in *vavilovii*, one showed two indels with 3 bp each. Exon 3 had the largest size (207 bp), whereas exon 9 had the smallest (72 bp) and both exhibited the highest sequence conservation (SC) with 0.976 and 0.972, respectively. While, exon 3 revealed the lowest Pi value (0.0041), exon 9 showed the smallest Hd (0.453) and k (0.487) values. The sequences comparison revealed intra-cultivar, inter-cultivar, intraspecific and interspecific variabilities.

Table 2. Diversity parameter, for six *ScMATE* exons, obtained comparing different sequences of gDNA from different cultivated and wild ryes using the DnaSP v. 5.0 software.

	Ex1	**Ex3**	**Ex4**	**Ex8**	**Ex9**	**Ex10**
Ex size (bp)	200	207	194	164	72	117
SWAG	6	0	0	0	0	0
IMS	177	202	182	150	70	111
VPS	17	5	12	14	2	6
SVS	1	1	2	0	0	1
PIS	16	4	10	14	2	5
h	19	7	14	12	4	6
Hd	0.918	0.648	0.75	0.623	0.453	0.526
ND (Pi)	0.02066	0.00410	0.01149	0.01776	0.00677	0.00767
k	3.9	0.848	2.228	2.913	0.487	0.897
SC	0.912	0.976	0.938	0.915	0.972	0.949

Ex – exon; SWAG – sites with alignments gaps; IMS – invariable (monomorphic) sites; VPS – variable (polymorphic) sites; SVS – singleton variable sites; PIS – parsimony informative sites; Hd – Haplotype (gene) diversity; ND (Pi) – nucleotide diversity; k – average number of nucleotide differences; SC – sequence conservation.

All exon sequences were joined to construct two dendrograms, the first was made with one sequence of every cultivar of *S. cereale* ssp. *cereale* (Fig. 1b) and the second was made with one sequence of each *Secale* species/subspecies (Fig. 2b) with cultivar Imperial as the representative of the *S. cereale*. This first dendrogram (Fig. 1b) was similar with the dendrogram which was obtained with the dominant markers (RAPDs and ISSRs), with only a change in the variety, JNK. The most divergent were the Riodeva inbred line. The second dendrogram (Fig. 2b) originated two main clusters with the subspecies *ancestrale* and *kuprijanovii*, and the cultivar Imperial in cluster I and the species *S. strictum* and *S. vavilovii*, and the subspecies *segetale* in cluster II. This was also very similar to the dendrogram obtained with RAPDs and ISSRs with an alteration in the species *S. vavilovii* (Fig. 2a).

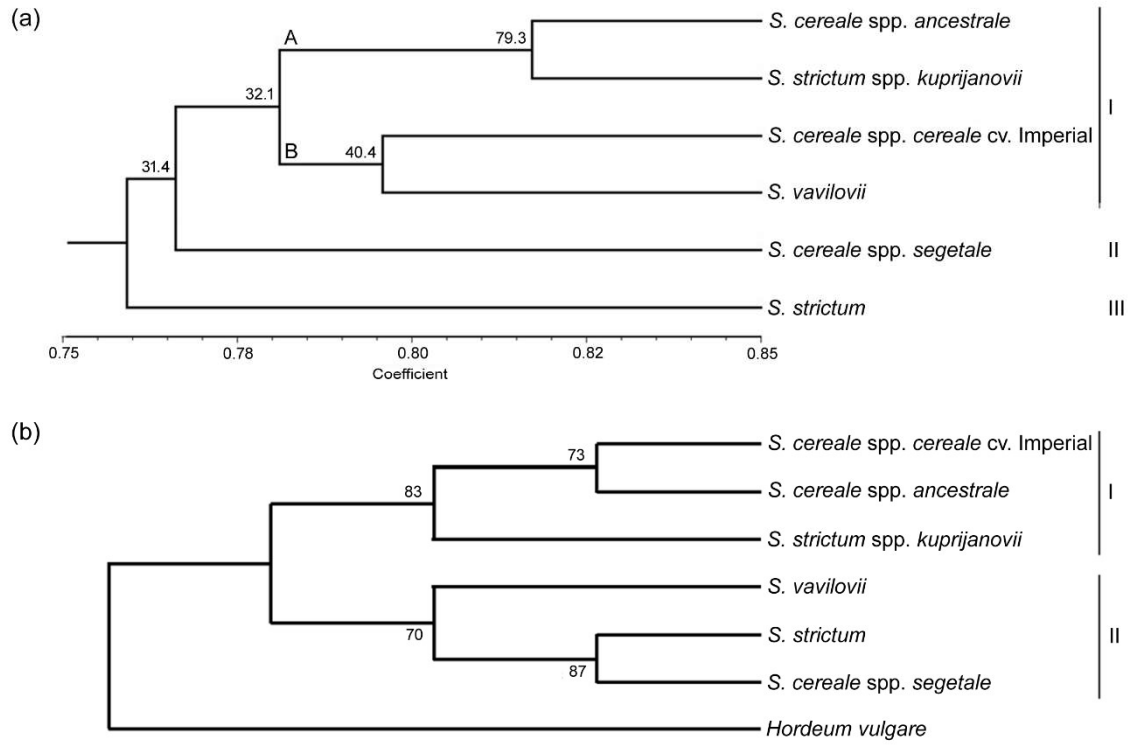


Figure 2. Dendrogram showing the genetic similarity among six different species/subspecies of the *Secale* genus using (a) RAPD and ISSR markers data and (b) data of six exons of *ScMATE1* gene. I, II, III indicate cluster denomination and (A) and (B) subclusters denomination. Dendrogram (a) was generated using UPGMA method and SM coefficient, whereas dendrogram (b) was generated using the evolutionary distance Kimura 2-parameter and the clustering method UPGMA. The numbers at the nodes are the bootstrap probability values (%).

4. Discussion

Molecular markers have various advantages, such as low cost, easy and quick assay, that make them increasingly required as ubiquity among others. Molecular data have shown more effectiveness than the morphological and physiological data. Matos et al. (2001) and Fernández et al. (2002) concluded that for genetic relationships studies, RAPD and ISSR markers were more useful than isozymes. Two RAPD and nine ISSR primers generated more than 80% of polymorphism. The bulk method is very convenient to get information about the interspecific genetic variability. Several authors used bulks in their studies and confirmed their effectiveness and usefulness (Loarce et al., 1996; Matos et al., 2001; Fernández et al., 2002; Tanyolac, 2003).

4.1. Estimation of genetic variability in *Secale* taxa

Polymorphism quantification is crucial in genetic studies because it indicates the variance degree among plants of the same or different species. We found high polymorphism values with both markers. The cultivars of *S. cereale* and wild species of the genus *Secale*

showed a high variability which is important to reduce vulnerability to biotic and abiotic stresses. Raina et al. (2001) reached lower values of polymorphism with the same markers in cultivars and wild species of peanuts. ISSRs were more polymorphic and informative than RAPDs, being the more efficient marker system. Similar results were found with Portuguese rye cultivars (Matos et al., 2001), barley cultivars (Fernández et al., 2002), wild barley populations (Tanyolac, 2003), cultivars and wild species of peanut (Raina et al., 2001) and white mulberry (Srivastava et al., 2004). RAPDs and ISSRs have dominant inheritance. This disadvantage could be partially overcome while analyzing large number of amplification products to enlarge the genome sampling, and to obtain a better assessment of variability and phylogenetic relationships. The 435 fragments (242 RAPDs and 193 ISSRs) analyzed are probably sufficient to obtain accurate genetic relationships. The *ScMATE1* gene has been involved in Al tolerance in some rye cultivars (Silva-Navas et al., 2012). The variability detected in exon 1 is consistent with previous results in *S. cereale* (Silva-Navas et al., 2012) and other *Poaceae* species. Two sequences of exon 1 from *S. vavilovii* suggest that this gene have at least two copies in this species. The same results were obtained by Silva-Navas et al. (2012) in the inbred line Riodeva. These results indicate a potential relation with Al sensitivity/tolerance, since both genotypes were previously classified as sensitive to Al-stress in acidic soil. The diversity parameters analyzed (Table 2) were high indicating the existence of a great genetic variability within the *Secale* genus. This variability has a high selection value that can be exploited through breeding programs to improve the yield/performance of related crops, and even within the *Secale* genus, relative to the presence of aluminum in acidic soil, since this cereal is one of the most tolerant to this stress.

4.2. Genetic relationships among *Secale* taxa

The taxonomy and origin of the *Secale* genus remain highly controversial despite the large number of studies performed over the years. Rye, as barley and wheat, are crops with economic importance that are distributed worldwide. The studies of genetic diversity in the *Secale* genus are important to get a better characterization of its genome for breeding purposes.

Polymorphisms have a great importance in the determination of genetic relationships. The bulk method is a good tool for this approach due to the high degree of polymorphism observed in this study. Use of different markers are essential to obtain more reliable

genetic relationships (Bolibok et al., 2005). The relationships obtained simultaneously using RAPD and ISSR markers are more accurate and the bootstrap values are higher than using one kind of marker. Loarce et al. (1996) and Matos et al. (2001) reached the same conclusion. Both Portuguese landraces (Lamego and Montalegre) grouped together (Fig. 1a) probably due to a possible adaptation to local environmental conditions. Cultivars with the same geographical location have been grouped together previously (Tanyolac, 2003; Ma et al., 2004; Srivastava et al., 2004). Alvão, Imperial and D. Zlote, all rye varieties were together in the subcluster A. Matos et al. (2001) obtained similar results with Lamego, Montalegre and Alvão. Using DArT (diversity arrays technology) markers, Bolibok-Bragoszewska et al. (2009) grouped Imperial and D. Zlote, whereas the inbred lines analyzed were separated in other cluster. In our case, the inbred-line Riodeva was also separated. The consanguineous ryes are characterized by high homozygosity and low genetic variation. The JNK variety was the most divergent (cluster III). This rye exhibited a wide genetic diversity possibly due to the presence of supernumerary chromosomes (Jones et al., 2008).

The subspecies *cereale* and *ancestrale* of *S. cereale* were together (Fig. 2a), whereas the subspecies *segetale* was separated, however, this maintained a close relationship with this group. The subspecies *ancestrale* and *segetale* have been grouped using other molecular markers (De Bustos and Jouve, 2002; Chikmawati et al., 2005; Shang et al., 2006; Fu et al., 2010; Ren et al., 2011). The subspecies *segetale* was generally the farthest in the group (Chikmawati et al., 2005; Shang et al., 2006; Achrem et al., 2014). *S. segetale* was the representative of *S. cereale* species closer to the wild rye *S. strictum*. It appears to be an immediate form among cultivated and wild ryes. This conclusion was also reached by Chikmawati et al. (2012). We had a unique representative of each subspecies, but high genetic similarity among different *S. cereale* subspecies was detected. *S. vavilovii* was closely related to the cultivated rye (Fig. 2a). This species showed strong link to *S. cereale* subspecies which is in agreement with various other works (Cuadrado and Jouve, 2002; De Bustos and Jouve, 2002; Chikmawati et al., 2005; Shang et al., 2006; Fu et al., 2010; Zhou et al., 2010; Ren et al., 2011; Achrem et al., 2014). Moreover, some authors suggested that the species *S. strictum* is the common ancestor of *S. vavilovii* and *S. cereale* species (Cuadrado and Jouve, 2002; De Bustos and Jouve, 2002; Zhou et al., 2010; Achrem et al., 2014). *S. strictum* separated first, being the most divergent (Fig. 2a) and, possibly the most ancient rye in this study. The subspecies *kuprijanovii*, a perennial rye as *S. strictum*, was grouped with annual rye (Fig. 2a). Chikmawati et al. (2005)

separated the perennial from the annual rye using AFLP markers. We only had two perennial ryes and they were separated. However, Shang et al. (2006) and De Bustos and Jouve (2002) also had not grouped *S. strictum* and *S. strictum* ssp. *kuprijanovii* being last, close to the annual rye. Further, the *S. strictum* complex proved to be a heterogeneous group with variations between and within different taxa, mainly in the subspecies *kuprijanovii* (Cuadrado and Jouve, 2002; De Bustos and Jouve, 2002; Shang et al., 2006; Achrem et al., 2014). De Bustos and Jouve (2002) concluded that it was not clear if this perennial rye can be considered a subspecies of *S. strictum*.

The dendrograms obtained using six exons of *ScMATE1* gene (Fig. 1b and 2b) were almost similar to the dendrograms obtained using RAPDs and ISSRs markers (Fig. 1a and 2a). The main differences were in the variety JNK (Fig. 1b) and the species *S. vavilovii* (Fig. 2b). With both dominant markers (RAPD and ISSR), the whole genome was covered, which included the B chromosomes of JNK and as we discussed above, this could be the reason for its remoteness (Fig. 1a). In this case (Fig. 1b), a particular genome location was covered that should not include the supernumerary chromosomes, and JNK grouped with the others rye varieties. *S. vavilovii* was closely related to the cultivated species above (Fig. 2a) and here it was more related to the wild species *S. strictum* (Fig. 2b). This species have great within species differences (Fu et al., 2010). This change may be due to the use of different kinds of molecular markers. Therefore, we obtained good genetic relationships with different molecular markers between different species of the *Secale* genus and within the cultivated species.

5. Conclusion

The bulk method using RAPD and ISSR markers proved to be effective method to detect different levels of polymorphism in rye where ISSRs were more polymorphic and informative than RAPDs. The bulk analysis is an effective strategy for genotype identification in *Secale*. Regarding the genetic relationships, the regional populations of *S. cereale* clustered according to the geographic localization. *S. cereale* cultivars showed high genetic diversity which is favorable for breeding program. Two different species would be expected to have obvious molecular differences but this was not found between *S. cereale* and *S. vavilovii*. The subspecies *segetale* could be an immediate form among cultivated and wild ryes. However, the heterogeneity of the perennial accessions was verified. The *ScMATE1* gene showed a high genetic diversity in the *Secale* genus. Rye

show a high tolerance to Al in acidic soils and the variability detected in this study could be related with the abiotic stress response. Moreover, the availability of plant genetic resources, with different tolerance behaviors and with different sequences, is an important basis for future crop breeding programs. The genetic relationships obtained reinforce the dendrograms with the dominant markers and showed the inconsistency prevailing in *S. vavilovii*. The conclusions were in agreement with the last revision of the genus *Secale* made by Frederiksen and Petersen (1998) who did not recognize *S. vavilovii* as a species and distinguished *S. cereale* and *S. strictum.*

Acknowledgements

This work was supported by Acción Integrada España-Portugal (PT2009-0096 and E-171/10) and PhD grant from Fundação para a Ciência e Tecnologia de Portugal (SFRH/BD/65040/2009).

References

Achrem, M., Kalinka, A., and Rogalska, S. M. (2014). Assessment of genetic relationships among *Secale* taxa by using ISSR and IRAP markers and the chromosomal distribution of the AAC microsatellite sequence. *Turk. J. Botany* 38, 213–225. doi:10.3906/bot-1207-26.

Bolibok-Bragoszewska, H., Heller-Uszyńska, K., Wenzl, P., Uszyński, G., Kilian, A., and Rakoczy-Trojanowska, M. (2009). DArT markers for the rye genome - genetic diversity and mapping. *BMC Genomics* 10, 578. doi:10.1186/1471-2164-10-578.

Bolibok, H., Rakoczy-Trojanowska, M., Hromada, A., and Pietrzykowski, R. (2005). Efficiency of different PCR-based marker systems in assessing genetic diversity among winter rye (*Secale cereale* L.) inbred lines. *Euphytica* 146, 109–116. doi:10.1007/s10681-005-0548-0.

Bushuk, W. (2001). Rye production and uses worldwide. *Cereal Foods World* 46, 70–73.

Chikmawati, T., Miftahudin, M., Skovmand, B., and Gustafson, J. P. (2012). Amplified fragment length polymorphism-based genetic diversity among cultivated and weedy rye (*Secale cereale* L.) accessions. *Genet. Resour. Crop Evol.* 59, 1743–1752. doi:10.1007/s10722-012-9796-8.

Chikmawati, T., Skovmand, B., and Gustafsson, J. P. (2005). Phylogenetic relationships among *Secale* species revealed by amplified fragment length polymorphisms. *Genoma* 48, 792–801. doi:10.1139/g05-043.

Cuadrado, A., and Jouve, N. (2002). Evolutionary trends of different repetitive DNA sequences during speciation in the genus *Secale*. *J. Hered.* 93, 339–345. doi:10.1093/jhered/93.5.339.

De Bustos, A., and Jouve, N. (2002). Phylogenetic relationships of the genus *Secale* based on the characterisation of rDNA ITS sequences. *Plant Syst. Evol.* 235, 147–154. doi:10.1007/s00606-002-0215-z.

Dedio, W., Kaltsikes, P. J., and Larter, E. N. (1969). Numerical chemotaxonomy in the genus *Secale*. *Can. J. Bot.* 47, 1175–1180.

Fernández, M. E., Figueiras, A. M., and Benito, C. (2002). The use of ISSR and RAPD markers for detecting DNA polymorphism, genotype identification and genetic diversity among barley cultivars with known origin. *Theor. Appl. Genet.* 104, 845–851. doi:10.1007/s00122-001-0848-2.

Frederiksen, S., and Petersen, G. (1998). A taxonomic revision of the genus *Secale* (Triticeae; Poaceae). *Nord. J. Bot.* 18, 399–420.

Fu, S., Tang, Z., Ren, Z., Zhang, H., and Yan, B. (2010). Isolation of rye-specific DNA fragment and genetic diversity analysis of rye genus *Secale* L. using wheat SSR markers. *J. Genet.* 89, 489–492. doi:10.1007/s12041-010-0070-6.

Jenabi, T., Saeidi, H., and Rahiminejad, M. R. (2011). Biodiversity of *Secale strictum* in Iran measured using microsatellites. *Genet. Resour. Crop Evol.* 58, 497–505. doi:10.1007/s10722-010-9593-1.

Jones, R. N., González-Sánchez, M., González-García, M., Vega, J. M., and Puertas, M. J. (2008). Chromosomes with a life of their own. *Cytogenet. Genome Res.* 120, 265–280.

Khush, G. S. (1962). Cytogenetic and evolutionary studies in *Secale* II. Interrelationships in the wild species. *Evolution (N. Y).* 16, 484–496.

Librado, P., and Rozas, J. (2009). DnaSP v5.1: a software for comprehensive analysis of DNA polymorphism data. -. *Bioinformatics* 25, 1451–1452.

Loarce, Y., Gallego, R., and Ferrer, E. (1996). A comparative analysis of the genetic relationships between rye cultivars using RFLP and RAPD markers. *Euphytica* 88, 107–115.

Ma, R., Yli-Mattila, T., and Pulli, S. (2004). Phylogenetic relationships among genotypes of worldwide collection of spring and winter ryes (*Secale cereale* L.) determined by RAPD-PCR markers. *Hereditas* 140, 210–221. doi:10.1111/j.1601-5223.2004.01844.x.

Mantel, N. (1967). The detection of disease clustering and a generalized regression approach. *Cancer Res.* 27, 209–220.

Matos, M., Pinto-Carnide, O., and Benito, C. (2001). Phylogenetic relationships among Portuguese rye based on isozyme, RAPD and ISSR markers. *Hereditas* 134, 229–236. doi:10.1111/j.1601-5223.2001.00229.x.

Michelmore, R. W., Paran, I., and Kesseli, R. V. (1991). Identification of markers linked to disease resistance genes by bulk segregant analysis: a rapid method to detect markers in specific genomic regions using segregating populations. *Proc. Natl. Acad. Sci. USA* 88, 9828–9832.

Nei, M. H., and Li, W. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* 76, 5269–5273.

Prevost, A., and Wilkinson, M. J. (1999). A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theor. Appl. Genet.* 98, 107–112. doi:10.1007/s001220051046.

Raina, S. N., Rani, V., Kojima, T., Ogihara, Y., Singh, K. P., and Devarumath, R. M. (2001). RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity, varietal identification, and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species. *Genome* 44, 763–772. doi:10.1139/gen-44-5-763.

Ren, T. H., Chen, F., Zou, Y. T., Jia, Y. H., Zhang, H. Q., Yan, B. J., et al. (2011). Evolutionary trends of microsatellites during the speciation process and phylogenetic relationships within the genus *Secale*. *Genome* 54, 316–326. doi:10.1139/g10-121.

Rohlf, M. (1998). NTSYS-pc. Numerical taxonomy and multivariate analysis system, version 2.02i. Department of Ecology and Evolution. State University of New York, Setauket, USA.

Roldán-Ruiz, I., Dendauw, J., Van Bockstaele, E., Depicker, A., and De Loose, M. (2000). AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.). *Mol. Breed.* 6, 125–134. doi:10.1023/A:1009680614564.

Sencer, H. A., and Hawkes, J. G. (1980). On the origin of cultivated rye. *Biol. J. Linn. Soc.* 13, 299–313.

Shang, H. Y., Wei, Y. M., Wang, X. R., and Zheng, Y. L. (2006). Genetic diversity and phylogenetic relationships in the rye genus *Secale* L. (rye) based on *Secale cereale* microsatellite markers. *Genet. Mol. Biol.* 29, 685–691. doi:10.1590/S1415-47572006000400018.

Silva-Navas, J., Benito, C., Téllez-Robledo, B., El-Moneim, D. A., and Gallego, F. J. (2012). The *ScAACT1* gene at the *Qalt5* locus as a candidate for increased aluminum tolerance in rye (*Secale cereale* L.). *Mol. Breed.* 30, 845–856. doi:10.1007/s11032-011-9668-5.

Sorkheh, K., Shiran, B., Gradziel, T. M., Epperson, B. K., Martínez-Gómez, P., and Asadi, E. (2007). Amplified fragment length polymorphism as a tool for molecular characterization of almond germplasm: genetic diversity among cultivated genotypes and related wild species of almond, and its relationships with agronomic traits. *Euphytica* 156, 327–344. doi:10.1007/s10681-007-9382-x.

Srivastava, P. P., Vijayan, K., Awasthi, A. K., and Saratchandra, B. (2004). Genetic analysis of *Morus alba* through RAPD and ISSR markers. *Indian J. Biotechnol.* 3, 527–532.

Stutz, H. C. (1972). On the origin of cultivated rye. *Am. J. Bot.* 59, 59–70.

Tanyolac, B. (2003). Inter simple sequence repeat (ISSR) and RAPD variation among wild barley (*Hordeum vulgare* ssp. *spontaneum*) populations from west Turkey. *Genet. Resour. Crop Evol.* 50, 611–614. doi:10.1023/A:1024412814757.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. . (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Vavilov, N. (1926). Studies of the origin of cultivated plants. *Bull. Appl. Bot. Genet. Plant Breed.* 16, 1–248.

Vences, F. J., Vaquero, F., and Pérez de la Vega, M. (1987). Phylogenetic relationships in *Secale* (Poaceae): An isozymatic study. *Plant Syst. Evol.* 157, 33–47. doi:10.1007/BF00939179.

Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V (1990). DNA polymorphisms amplified by arbitrary primers are usefil as genetic markers. 18, 6531–6535.

Yap, I. J., and Nelson, R. (1996). Winboot: a program for performing bootstrap analysis of binary data to determine the confidence limits of UPGMA-based dendrograms. IRRI Discussion Paper Series No. 14. International Rice Research Institute, P.O. Box 933, Manila, Philippines.

Zhou, J., Yang, Z., Li, G., Liu, C., Tang, Z., Zhang, Y., et al. (2010). Diversified chromosomal distribution of tandemly repeated sequences revealed evolutionary

trends in *Secale* (Poaceae). *Plant Syst. Evol.* 287, 49–56.

Zietkiewicz, E., Rafalski, A., and Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20, 176–183. doi:10.1006/geno.1994.1151.

Supplementary data

Table S1. RAPD and ISSR primers used in this study.

Primer	5' – 3'	Primer	5' – 3'
RAPDs (Operon)			
A1	CAGGCCCTTC	C5	CATGACCGCC
A4	AATCGGGCTG	C6	GAACGGACTC
A5	AGGGGTCTTG	C7	GTCCCGACGA
A6	GGTCCCTGAC	C8	TGGACCGGTG
A17	GACCGCTTGT	C9	CTCACCGTCC
B1	CTTTCGCTCC	C11	AAAGCTGCGG
B5	TGCGCCCTTC	C12	TGTCATCCCC
B6	TGCTCTGCCC	C13	AAGCCTCGTC
B7	GGTGACGCAG	C15	GACGGATCAG
B10	CTGCTGGGAC	C16	CACACTCCAG
C1	TTCGAGCCAG	C18	TGAGTGGGTG
C2	GTGAGGCGTC	C19	CTTGCCAGCC
C4	CCGCATCTAC	C20	ACTTCGCCAC
ISSRs (UBC)			
807	$(AG)_8T$	836	$(AG)_8YA$
808	$(AG)_8C$	842	$(GA)_8YG$
810	$(GA)_8T$	844	$(CT)_8RC$
811	$(GA)_8C$	845	$(CT)_8RG$
812	$(GA)_8A$	846	$(CA)_8RT$
815	$(CT)_8G$	881	$(GGGTG)_3$
824	$(TC)_8G$	887	$DVD(TC)_7$
827	$(AC)_8G$	889	$DBD(AC)_7$
835	$(AG)_8YC$	891	$HVH(TG)_7$

B = (C, G, T); D = (A, G, T); R = (A, G); V = (A, C, G); Y = (C, T).

Table S2. Primer sequences used to amplify six exons of the *ScMATE1* gene. Primers based on rye *ScMATE1* (*ScAACT1*) gene reported by Silva-Navas et al. (2012).

Primer	Sequence (5' → 3')	T_a (ºC)	Amplified exons
AACT1-1F	TACCTCTTTGCTATGAACATCAGG	56 °C	1
AACT1-1R	GGGTAGATGCACACTTTCGAG		
AACT1-2F	TTTCTATTGCCATATTTAACCAAG	58 °C	3 and 4
AACT1-2R	GAGCCGACATATCAAGATCATA		
AACT1-3F	CTTGATATGTCGGCTCGTC	58 °C	8, 9 and 10
AACT1-3R	GGTGTAGTCTTGTGCTCCGAAGTT		

T_a, annealing temperature

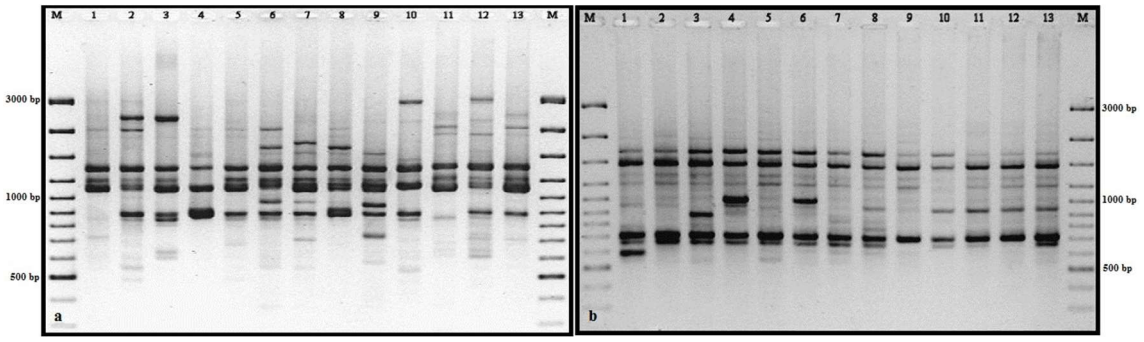


Figure S1. Gel electrophoresis of amplification products obtained using ISSR UBC836 (a) and RAPD OPA17 (b) primers. M – DNA molecular weight marker (GeneRulerTM100-bp plus DNA ladder, Fermentas); 1 – Alvão; 2 – *S. cereale* ssp. *ancestrale*; 3 – Imperial; 4 – JNK; 5 – *S. strictum* ssp. *kuprijanovii*; 6 – Lamego1; 7 – Lamego2; 8 – Montalegre; 9 – *S. strictum*; 10 – Riodeva; 11 – *S. cereale* ssp. *segetale*; 12 – *S. vavilovii*; 13 – D. Zlote.

SUBCHAPTER II-2

Molecular markers associated with Al tolerance in *Secale* genus: a first approach to design specific markers

E. Santos[1,2], P. Silva[1], O. Pinto-Carnide[3], A. M. Figueiras[4], C. Benito[4] and M. Matos[1,2]

[1]*Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, Quinta de Prados, 5001-801 Vila Real, Portugal*
[2]*BioISI- Biosystems & Integrative Sciences Institute, University of Lisboa, Faculty of Sciences, Campo Grande, Lisboa, Portugal*
[3] *CITAB -Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*
[4] *Departamento de Genética, Facultad de Biologia, Universidad Complutense, C/ José Antonio Novais, 12, 28040 Madrid, Spain*

Keywords: Aluminum characterization; rye; ISSR; RAPD; linked markers.

*Submitted in *Euphytica* (EUPH-D-18-00717)

Abstract

Aluminum (Al) toxicity is a major limitation for plant production on the widespread acid soils. The better solution to circumvent this constraint seems to be the development of new Al-tolerant crops. Rye (*Secale cereale* L.) is one of the most Al-tolerant cereal, with a valuable genetic background for breeding purposes. Hydroponic methods have been successfully used for Al tolerance screening in several crop species. In this work, we confirmed his effectiveness with this abiotic trait characterization on all the twelve ryes at study (five wild species/subspecies and seven cultivars). Most of these ryes showed great ability to resist and recover from Al toxicity at huge Al concentrations. This is the first report regarding Al tolerance of wild ryes and results suggest a new and fruitful source of germplasm for crop improvement. RAPD and ISSR markers are highly polymorphic and can be used to identify DNA fragments associated with Al tolerance. Both techniques enabled the identification of 34 possible Al tolerance/sensitivity associated fragments, being ISSRs the most successful (24). Both markers proved to have a great potential to distinguish tolerant and sensitive genotypes based on DNA polymorphisms and provides important resources for molecular marker-assisted selection.

1. Introduction

Aluminum (Al) toxicity comprises about 40% of the world's ice free land area and up to 75% of potentially arable soils (Von Uexkull and Mutert, 1995). As a result of natural processes or human activities, soils pH decrease to 5 or below and become acidic, with the prevalence of the toxic trivalent cation Al^{3+}, which causes severe damage to plants inhibiting root growth and function, and consequently reducing crops yield (Ma et al., 2001; Kochian et al., 2002; Ma and Furukawa, 2003). The increased area of arable land affected by acidity is a threat in world food production. The most advantageous solution, at the economic and practical level, to overcome this limitation seems to be the development of new Al tolerant crops (Simões et al., 2012).

A rapid and reliable screening system is need to discriminate sensitive and resistant genotypes. The nutrient solution culture has been successfully employed for screening Al tolerance in many crops (Ma et al., 1997, 2002; Pinto-Carnide and Guedes-Pinto, 1999; Akhter et al., 2009; Singh et al., 2009). There is a wide genetic variation in Al tolerance both within and between plant species. Among cereals, rye (*Secale cereale* L.) is the most tolerant species along with rice (*Oryza sativa*), followed by oat (*Avena sativa*), triticale (× *Triticosecale*), bread wheat (*Triticum aestivum*), *Brachypodium distachyon* and barley (*Hordeum vulgare*) (Bona et al., 1993; Kim et al., 2001; Contreras et al., 2014). Rye is a very important crop due to its nutritional value and its ability to adapt in adverse environmental conditions, being able to be cultivated in areas that are generally not suitable for other related crops economically more important such as wheat and barley (Bushuk, 2001).

The knowledge of the mechanisms and the genes that control Al cereal tolerance is an important goal that will provide fundamental information that can be used to increase Al tolerance in cereal crops (Benito et al., 2010). Genetic markers are useful tools to reveal Al tolerance mechanisms in higher plants following their detection by inheritance studies and identification of desirable genes or loci (Bian et al., 2013). DNA-based molecular markers have acted as versatile tools for investigating various aspects of plant genomes including genetic variability, genome fingerprinting, genome mapping, gene localization, genome evolution, population genetics, taxonomy, plant breeding, and diagnostics (Joshi et al., 1999), which can enable the improvement of cereal crops. PCR-based markers are preferred and widely used as they use very small amounts of DNA, are highly efficient, straightforward, fast and easy to use which facilitates the process of genotyping

(Chikmawati et al., 2006; Inostroza-Blancheteau et al., 2010). Inter simple sequence repeat (ISSR) (Zietkiewicz et al., 1994) and random amplified polymorphic DNA (RAPD) (Williams et al., 1990) markers have previously been used to identify and assign distinguishing individuals, lines, populations and species. These PCR-based analytical tools use universal primers that anneal to multiple sites in different regions of the genome, allowing for the simultaneous amplification of several genetic loci (Luzio et al., 2015).

In this study, the Al tolerance screening of five wild rye species/subspecies and seven cultivated ryes being two of them used as testers, one tolerant (D. Zlote) and another sensitive (Riodeva) were done. Moreover, we used several RAPD and ISSR markers in order to identified potential DNA markers associated with Al tolerance in the *Secale* genus.

2. Materials and Methods

2.1. Plant material

Three species, *S. cereale* L., *S. strictum* (C. Persl) C. Persl and *S. vavilovii* Grossh. of the *Secale* genus were studied. Also, three subspecies of *S. cereale*: *S. cereale* ssp. *ancestrale* Zhuk., the cultivated *S. cereale* ssp. *cereale* and *S. cereale* ssp. *segetale* Zhuk.; and two of *S. strictum*: *S. strictum* ssp. *kuprijanovii* Grossh. and *S. strictum* ssp. *strictum* syn. *S. montanum* Guss. were also studied. These materials were kindly provided by Dr E. Larter, University of Winnipeg (Manitoba, Canada) (Dedio et al., 1969) and maintained in the germplasm bank of the Department of Genetics and Biotechnology at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Moreover, three rye varieties named Imperial (USA), JNK (Japan) and Alvão (Portugal) and two landraces from the northern Portugal (Lamego and Montalegre), all belonging to the cultivated *S. cereale* were also studied. For comparative study, we included two international testers frequently used: the Spanish consanguineous line Riodeva (sensitive) (Gallego and Benito, 1997; Matos et al., 2005; Fontecha et al., 2007) and the Polish variety Dankowskie Zlote (tolerant) (Pinto-Carnide and Guedes-Pinto, 1999; Kim et al., 2001; Jozefaciuk and Szatanik-Kloc, 2004).

2.2. Al tolerance screening assay

Al tolerance tests were carried out using the nutrient culture modified-pulse method described by Aniol (1984) and adapted by Gallego and Benito (1997). About thirty seeds

were sterilized with a solution containing distilled water and commercial bleach (3:1), rinsed well with water and germinated on wet filter paper in Petri dishes at 4 ºC for few hours being next incubated at 25 ºC for 48 h. Thereafter, seeds were put in a container with nutrient solution (0.4 mM $CaCl_2$, 0.65 mM KNO_3, 0.25 mM $MgCl_2 \cdot 6H_2O$, 0.01 mM $(NH_4)_2SO_4$ and 0.04 mM NH_4NO_3; pH 4.0) which was placed in a water bath at 25 ºC with a 16 h photoperiod and was continuously aerated, for four more days. Next, seedlings were moved for 24 h in a nutrient solution supplied with aluminum ($AlCl_3 \cdot 6H_2O$) at various concentration (5, 10, 20 and 30 ppm). After aluminum shock, roots were thoroughly washed in running tap water and stained with Eriochrome cyanine R for 10 min followed by another intense wash to remove the excess dye. Finally, seedlings were transferred to a fresh Al-free nutrient solution, for a further 48 h. The conditions of aeration, temperature and pH were maintained in all solutions. Root regrowth length were measured on the three main roots per plant. All the experiments were repeated twice. Statistical analysis was performed through two-way factorial analysis of variance (ANOVA) using the SPSS statistical package for Windows (v. 23.0; IBM Corp., Armonk, NY, USA).

2.3. Genomic DNA extraction

Young leaves from two distinct plants groups (the most tolerant and the most sensitive) of each rye (cultivated and wild) at study were collected. In the case of the inbred line Riodeva, which is a sensitive standard, shoots were collected from two different plants classified as sensitive. The tissues were frozen in liquid nitrogen and DNA extraction was carried out using a small-scale DNA isolation method (Dneasy Plant Mini Kit, form Qiagen, Hilden, Germany) according to the procedures described by the manufacturer.

2.4. RAPD markers

A total of nine 10-mer oligonucleotides (Operon Technologies, Alameda, USA) were selected (Supplementary Table S1) according to the degree of reproducibility, amplification and polymorphism. RAPD reactions were performed with minor modifications as described by Matos et al. (2001). Such reactions were carried out in 25 μL volume using T-Professional Thermocycler (Biometra, Göttingen, Germany). PCR products were analyzed on 1.8% TBE agarose gels stained with ethidium bromide. The

size of the PCR amplification products was estimated using a 100-bp DNA ladder (Thermo scientific, California, USA).

2.5. ISSR markers

Ten primers from UBC primer set 100/9 (University of British Columbia, Canada) based on dinucleotides repeats were chosen (Supplementary Table S1), with the same criteria as in RAPDs. ISSR reactions were performed in a 15 µL reaction volume containing 45 ng/µL of gDNA, 3 µL of primer (2.5 µM), and 7.5 µL of Taq PCR MasterMix (Qiagen), using PTC-100 Thermocycler (MJ Research Inc., Minnesota, USA) with the program described by Matos et al. (2001). High-resolution capillary electrophoresis was performed using a QIAxcel® DNA high-resolution gel cartridge (Qiagen) on a QIAxcel system (Qiagen), as per the manufacturer's instructions. Marker fragment sizes were calculated using the BioCalculator® software (Qiagen). This software produces a digital gel image and an electropherogram for fragment analysis.

3. Results and Discussion

3.1. Al tolerance characterization

Screening for Al tolerance using hydroponic assays is the most common methodology in cereals. This procedure is relatively rapid to perform, non-destructive and can evaluate many plants in a short time (Samac and Tesfaye, 2003). Indeed, it's a reliable method for Al tolerance characterization in crop species. Since Al toxicity is affected by many factors such as pH, concentration of Al, temperature, and concentrations of salts in solution (Ma et al., 1997), it is imperative to maintain the same conditions for an precise classification between different genotypes.

Several studies have confirmed the high ability of rye cultivars to grow under Al stress, mainly when compared to other related cereals with agronomic relevance as barley and wheat (Bona et al., 1993; Kim et al., 2001). Al tolerance experiments were carried out based on two major criteria: staining technique and root regrowth length measurement. The dye Eriochrome cyanine R forms a stable purple complex in the presence of Al, which allowed us to mark the root and observe if the seedlings have a recovery skill after Al shock (Fig. 1). Thereby, sensitive genotypes remained intensely stained, with any root regrowth, meaning that the apical meristem was injured. The inhibition of root elongation is the first and main visible injury triggered for Al toxicity

in susceptible plants (Kochian et al., 2004). These roots became inefficient in absorbing nutrients and water leading to plant damage. On the other hand, tolerant genotypes showed a root segment unstained (root regrowth), contrasting with the heavily stained root part exposed to Al, proving that root apical meristem was not destroyed and could recover. Root regrowth length was measured to determine the Al tolerance degree of the respective genotype.

Figure 1. Example of a tolerant (T) and a sensitive (S) genotype of *S. strictum*. Arrows shows the root regrowth in the tolerant rye and the lacking regrowth in the sensitive one.

According to this, we were able to classify all the ryes and wild ryes at study concerning Al tolerance behavior (Table 1, Fig. 2). At 5 ppm Al concentration, we could clearly classify as highly tolerant the variety JNK, as tolerant the wild ryes *S. ancestrale*, *S. segetale*, *S. kuprijanovii* and *S. vavilovii* as well as the varieties Alvão and Imperial and the Portuguese landraces Lamego and Montalegre, and finally, as moderately tolerant the wild subspecies *S. strictum*. Moreover, it was confirmed the tolerant and the sensitive pattern of the testers D. Zlote and Riodeva, respectively. The Spanish rye exhibited significant differences with all ryes except *S. strictum* at 5 ppm ($P < 0.001$). Since rye is one of the most tolerant cereal species, we decided to check his behavior in higher Al concentrations (10, 20 and 30 ppm).

We proved the great Al tolerance featuring cultivated ryes, with all the varieties and landraces showing higher resistance to Al stress than the tolerant tester D. Zlote at all concentrations tested ($P < 0.001$ at 5 ppm), except for Imperial at 20 and 30 ppm (Table 1, Fig. 2). Generally, the Al tolerance level of the different ryes decrease with the Al concentration increment, in particular from 5 to 10 ppm ($P < 0.001$). However, it is remarkable the high ability of these ryes to resist Al toxicity, being able to recover presenting root regrowth in most cases with the four Al concentrations used. At 5 ppm, any sensitive genotype were found in JNK (likewise at 10 ppm), Lamego and Montalegre and only one was detected in Alvão as well as three plants in Imperial. JNK exhibited the highest values of root regrowths with 63% reaching more than 15 mm at 5 ppm and almost 50% at 10 and 20 ppm. Even at 30 ppm two plants of JNK were ranked as highly tolerant and only one as sensitive. JNK presented significant differences with most ryes in all treatments ($P < 0.001$). The classification of this Japanese cultivar as highly tolerant agrees with the previous data of Gallego and Benito (1997). On the other hand, the Portuguese landraces Lamego and Montalegre were also quite tolerant at high Al concentrations in the earlier work of Pinto-Carnide and Guedes-Pinto (1999) whereas the tolerance of the cultivar Imperial was also verified by Silva-Navas et al. (2012). The high level of Al tolerance of these ryes can be due to its origin, since all come from regions where acidic soils are plentiful such as Japan (JNK), North America (Imperial) and Northeast Portugal (Alvão, Lamego and Montalegre). Natural selection and adaptation to local environmental conditions may have triggered the development of genotypes with Al-tolerance to ensure their survival.

Relatively to the wild ryes, there is no data on their behavior under Al stress. It has been well known that many wild plants show high tolerant phenotypes against abiotic stresses (Ellis et al., 2000; Ezaki et al., 2008). Thus, wild crop relatives could be a new and fruitful source of germplasm for crop improvement justifying the importance of their study relatively to their Al-tolerance as the Al-resistance mechanisms. In this work, we verified the great capability of the wild rye species/subspecies to tolerate the presence of Al at high concentrations. Except *S. strictum*, all the wild ryes were classified as tolerant obtaining higher levels of Al tolerance at 5 ppm than the tolerant tester D. Zlote ($P < 0.001$ except *S. ancestrale*), as occurred with the cultivars. *S. vavilovii* seems to be the species, along with JNK, that most tolerates the increment of Al concentration. Wild ryes displayed a similar behavior than the cultivated ryes, with most of the plants exhibiting root regrowths in all the Al concentrations tested. In turn, *S. strictum* was classified as

moderately Al-tolerant because, at 5 ppm, much of the tolerant plants presented tiny root regrowths when compared to the others and about half of his plants were ranked as sensitive. In higher Al concentrations, much of the genotypes were sensitive however, about 20% of them had the ability to recover contrarily to what occurred with the sensitive tester Riodeva which all the plants lacked root regrowth (Table 1, Fig. 2).

A great variability for Al tolerance was found in the genus *Secale*, differences were observed both between and within wild rye species/subspecies and cultivars analyzed. With the exception of the wild species *S. vavilovii* and the inbred line Riodeva, ryes and wild ryes are allogamous, thus heterozygous, which leads to the existence of a wide diversity of genotypes with tolerant and sensitive plants. This is a valuable characteristic in the genus *Secale* since it allows a better adaptation to adverse environments. The vast variability in rye for this trait was also witnessed by other authors (Pinto-Carnide and Guedes-Pinto, 1999; Kim et al., 2001). The germplasm studied is of great importance for rye breeding programs where tolerant genotypes can be selected as a potential genetic resource for introgression into related species more susceptible to Al stress and with high economic value such as wheat and barley.

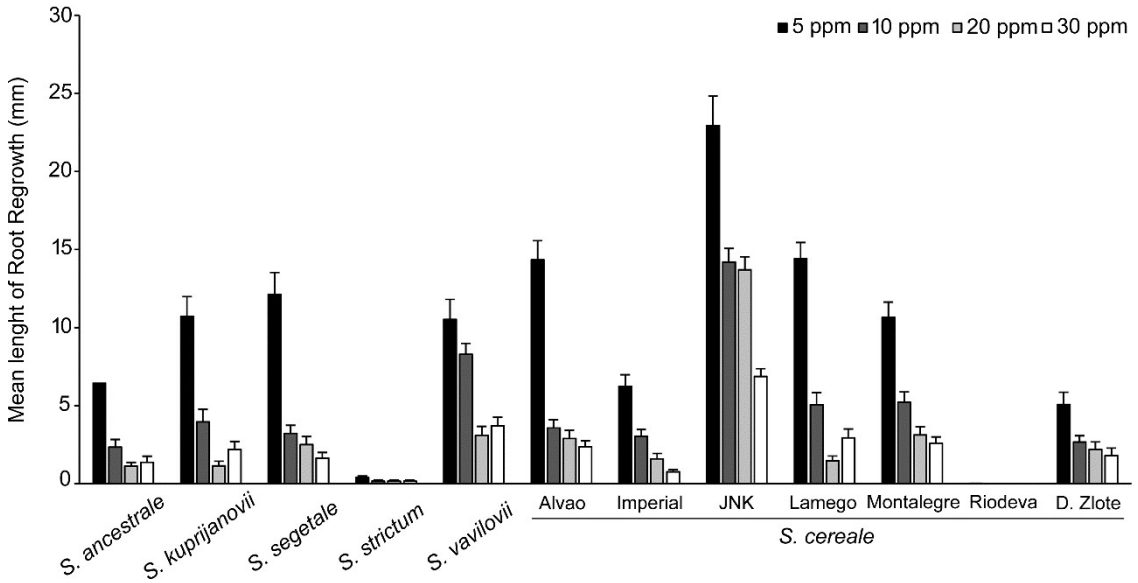


Figure 2. Mean lengths of root regrowth (mm) of several wild and cultivated ryes at different Al concentrations (5, 10, 20 and 30 ppm). Data are the means ±SE, $n = 60$. Significant statistical differences were found among ryes and treatments, between different ryes and between different treatments ($P < 0.001$). Relevant statistic data were inserted throughout the text.

Table 1. Percentage of plants without root regrowth and by classes of root regrowth of several wild and cultivated ryes at different Al concentrations.

	5 ppm				10 ppm				20 ppm				30 ppm			
	% plants without root regrowth	% plants with root regrowth			% plants without root regrowth	% plants with root regrowth			% plants without root regrowth	% plants with root regrowth			% plants without root regrowth	% plants with root regrowth		
		0 - 5 mm	5 - 15 mm	> 15 mm		0 - 5 mm	5 - 15 mm	> 15 mm		0 - 5 mm	5 - 15 mm	> 15 mm		0 - 5 mm	5 - 15 mm	> 15 mm
S. ancestrale	8,47	52,54	28,81	10,17	19,4	68,66	8,96	2,99	46,99	48,19	4,82	0	20,41	73,47	4,08	2,04
S. kuprijanovii	5,77	26,92	42,31	25	32,73	41,82	18,18	7,27	60,71	32,14	7,14	0	47,83	30,43	21,74	0
S. segetale	0	23,68	39,47	36,84	21,74	55,07	21,74	1,45	29,09	56,36	14,55	0	35,85	52,83	11,32	0
S. strictum	56,60	43,40	0	0	82,69	17,31	0	0	80	20	0	0	79,07	20,93	0	0
S. vavilovii	0	41,67	35	23,33	1,41	33,8	50,70	14,08	21,31	57,38	18,03	3,28	26,79	35,71	37,50	0
Alvão	1,49	16,42	44,78	37,31	6,38	61,70	31,91	0	20,37	61,11	16,67	1,85	17,24	67,24	15,52	0
Imperial	5,17	46,55	41,38	6,90	7,69	69,23	23,08	0	32,26	59,68	8,06	0	29,23	69,23	1,54	0
JNK	0	10,77	26,15	63,08	0	5,56	58,33	36,11	1,45	11,59	46,38	40,58	1,61	33,87	61,29	3,23
Lamego	0	4,84	56,45	38,71	17,74	46,77	25,81	9,68	13,51	83,78	2,70	0	23,53	54,90	19,61	1,96
Montalegre	0	31,75	46,03	22,22	9,43	50,94	35,85	3,77	7,84	74,51	15,69	1,96	7,02	70,18	22,81	0
Riodeva (Sensitive tester)	92,86	7,14	0	0	100	0	0	0	100	0	0	0	100	0	0	0
D.Zlote (Tolerant tester)	6,56	59,02	26,23	8,2	18,97	56,9	24,14	0	41,54	41,54	15,38	1,54	35,71	55,36	7,14	1,79

3.2. Identification of genetic markers associated with Al tolerance

Genomic DNA from two different plants often produces different amplification patterns and specific fragments generated from one individual but not for other represent DNA polymorphism and can be used as genetic markers (Jonah et al., 2011). RAPD and ISSR markers were selected for the high polymorphism degree described in numerous plant species and for their high genomic abundance as the good genome coverage.

The Al tolerance has been exhaustively studied for its genetic control and it was found that in rye this trait is controlled, at least, through four independent loci (*Alt1*, *Alt2*, *Alt3*, *Alt4*) located on chromosome arm *6RS*, *3RS*, *4RL* and *7RS*, respectively (Gallego and Benito, 1997; Ma et al., 2000; Miftahudin et al., 2002; Matos et al., 2005; Benito et al., 2010). In this work both RAPD and ISSR markers enabled to identify several Al tolerance/sensitivity associated fragments, 19 linked to tolerant and 15 to sensitive rye genotypes (Table 2).

In RAPD analysis, a total of 141 bands were obtained, with an average of 15.67 bands per RAPD primer being 103 polymorphic (73.05%). Six (OPA5, OPB13, OPC7, OPF9, OPN5 and OPO7) of the nine primers used, with a total of 75.51% of polymorphic bands, were identified as potential Al tolerance linked markers. These primers amplified 10 DNA fragments with differences between the sensitive and the tolerant genotypes of, at least, two distinct ryes. The polymorphic loci of the primers OPA5 and OPB13 were found in sensitive ryes ($OPA5_{550}$, $OPA5_{1500}$ and $OPB13_{510}$) whereas of OPN5 was in tolerant ones ($OPN5_{450}$). Likewise, with OPC7, OPF9 and OPO7 primers the polymorphic bands were related to both tolerant ($OPC7_{320}$, $OPF9_{2200}$ and $OPO7_{520}$) and sensitive ($OPC7_{1350}$, $OPF9_{680}$ and $OPO7_{710}$) genotypes. Interestingly, three of these DNA fragments ($OPA5_{550}$, $OPF9_{680}$ and $OPO7_{520}$) were found only in cultivated ryes, being possibly a marker restrict to the subspecies *S. cereale.*

Table 2. Amplification pattern of potential Al tolerance associated DNA fragments in Tolerant (T) and Sensitive (S) rye individuals, scored as Present (+) or Absent (-). ALV – Alvão; ANC – *S. ancestrale*; IMP – Imperial; JNK – JNK; KUP – *S. kuprijanovii*; LAM – Lamego; MTL – Montalegre; STR – *S. strictum*; RIO – Riodeva; SEG – *S. segetale*; VAV – *S. vavilovii*; ZLO – D. Zlote.

Primer	Marker (bp)	ALV T	ALV S	ANC T	ANC S	IMP T	IMP S	JNK T	JNK S	KUP T	KUP S	LAM T	LAM S	MTL T	MTL S	STR T	STR S	RIO S	RIO S	SEG T	SEG S	VAV T	VAV S	ZLO T	ZLO S
RAPDs																									
OPA5	550	-	+	-	-	-	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+
	1500	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
OPB13	510	-	-	-	+	-	+	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-
OPC7	320	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1350	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	+
OPF9	680	-	+	-	-	+	+	+	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	+
	2200	+	+	+	-	-	-	+	+	-	-	+	-	+	+	+	-	+	+	-	-	-	-	-	-
OPN5	450	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
OPO7	520	-	-	-	-	+	-	-	-	-	-	+	-	+	+	+	+	-	-	+	+	-	-	+	-
	710	-	+	-	-	+	+	-	-	-	+	-	-	-	+	-	-	-	-	+	+	-	-	+	+

Primer	Marker (bp)	ALV T	ALV S	ANC T	ANC S	IMP T	IMP S	JNK T	JNK S	KUP T	KUP S	LAM T	LAM S	MTL T	MTL S	STR T	STR S	RIO S	RIO S	SEG T	SEG S	VAV T	VAV S	ZLO T	ZLO S
ISSRs																									
810	570	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	+	-	+
	1100	+	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	1230	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
811	630	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	+	-	-
	640	+	-	+	+	+	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	+	-	-	-
	680	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+	+	-	-	+	+
	1600	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-
818	340	-	-	+	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-
	360	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
	600	-	-	+	-	+	+	-	-	-	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-
	1300	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
824	1005	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
	1550	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	-	-	-	-
	1792	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+
825	570	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	1560	+	-	+	-	-	-	+	+	-	-	-	-	-	-	+	+	-	-	+	+	-	-	+	-
827	550	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+
	587	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	650	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	+	-	+	-	+
	957	+	+	-	-	-	-	+	+	-	-	-	-	+	-	+	+	+	+	-	-	+	-	-	-
	970	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
835	880	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
	1835	+	-	+	-	+	-	+	+	-	-	+	+	+	-	-	-	+	+	+	+	-	-	-	-
836	1060	-	-	+	+	-	-	-	-	-	-	-	+	-	-	+	+	+	+	-	+	-	-	-	-

In turn, the ten ISSR primers analyzed achieved a greater polymorphism value (93.29%) with a total of 149 reproducible bands (14.9 bands/ISSR primer). An association with the Al tolerance trait was found with eight of these primers (UBC 810, 811, 818, 824, 825, 827, 835 and 836), with all reaching 94.62% of polymorphic bands. This high polymorphism degree could be the reason for an elevated number of Al stress linked fragments detected using ISSRs (24), with an average of three bands per primer. Most of these markers (15) were related to tolerant rye genotypes and were detected through all the referred primers (UBC 810_{1100}, 810_{1230}, 811_{640}, 811_{680}, 811_{1300}, 818_{340}, 818_{600}, 818_{1300}, 824_{1005}, 825_{570}, 825_{1560}, 827_{957}, 827_{970}, 835_{880} and 835_{1835}), except for UBC836. Only nine DNA fragments were related to susceptible rye genotypes (UBC 810_{570}, 811_{630}, 818_{360}, 824_{1550}, 824_{1792}, 827_{550}, 827_{587}, 827_{650} and 836_{1060}). As occurred with RAPD markers, four of the fragments found by ISSRs were exclusive to the *S. cereale* subspecies (UBC 810_{1100}, 824_{1005}, 824_{1550} and 824_{1792}).

Both RAPD and ISSR techniques proved to have a great potential to distinguish tolerant and sensitive genotypes based on DNA polymorphisms. Besides, both methods were effective in the search for promising markers linked to the Al tolerance trait. However, ISSRs were most promisor possibly due to the sequences targeted by them, the microsatellites, which are ubiquitous in eukaryotic genomes and evolve rapidly, allowing the development of a higher number of polymorphic fragments (Zietkiewicz et al., 1994; Bornet and Branchard, 2001).

These DNA-based molecular markers have been successfully used in cereals to develop specific DNA markers associated with other abiotic stresses like drought (Deshmukh et al., 2012), heat (Al-doss et al., 2009) and salt (Younis et al., 2007) tolerance. As regard Al tolerance trait, many linked markers were found in rye, over the years, using distinct techniques. Gallego et al. (1998a, 1998b) identified three RAPD markers, which have been converted to sequence-characterized amplified region (SCAR) markers, closely linked to the *Alt1* locus located on the short (*ScR01*$_{600}$ and *ScB15*$_{790}$) and long (*ScA08*$_{415}$) arm of chromosome *6R*. In turn, Miftahudin et al. (2002) reported five AFLP markers (*AMAL1* to *AMAL5*) linked to the *Alt3* gene on the *4RL*, where three of them were tightly linked and flanked the gene (*AMAL1*, *AMAL4* and *AMAL5*). Likewise, a RAPD marker transformed into a SCAR, *ScOPS14*$_{705,}$ was also localized on the *4RL* and linked to the *Alt3* gene (Benito et al., 2010). Furthermore, Matos et al. (2005) found ten RAPD, six SCIM (*Secale cereale* inter-microsatellite) and two SCM (*S. cereale* microsatellite) loci located on chromosome *7R* and linked to the *Alt4* gene. These markers

were used by Fontecha et al. (2007) to construct a map of the rye chromosome *7R* which allowed the finding of a complete co-segregation between *Alt4* and *ScALMTT1* gene. Also, Benito et al. (2010) linked to *Alt4* on chromosome *7R,* sixteen microsatellites (three on *7RS* and four on *7RL*) and five PCR-based markers (*B1*, *B4*, *B11*, *B26* and *BCD1230*). Finally, Camacho et al. (2005) located 24 SCIMs on the chromosomes 3R, 4R and 6R, which carry the Al-tolerance genes *Alt2*, *Alt3* and *Alt1*, respectively. Microsatellite (SSR) and ISSR markers have also been used to distinguish between Al tolerant and sensitive lines of *Brachypodium distachyon* and *Brachypodium hybridum* (Contreras et al., 2017).

Because of the allogamous feature of rye, Al tolerance is a very complex trait with polygenic inheritance which difficult its genetic improvement by traditional breeding strategies. Thus, to complement these conventional techniques, the identification of markers associated with Al tolerance may facilitate and speed up future breeding purposes aimed at improving this trait through marker assisted selection (MAS).

For a more accurate and reliable association of the molecular markers identified with Al tolerance trait, further research is needed. In a first approach, the chromosomal location of each linked marker will be necessary in order to confirm if they're localized in one of the chromosomes previously described as being involved in Al tolerance (*6RS*, *3RS*, *4RL* and *7RS*). Additionally, the loci whose location is confirmed on one of these chromosomes will be converted in SCAR markers, which is a more reliable methodology to obtain a specific amplification of a particular locus.

4. Conclusion

The high Al tolerance found in the *Secale* genus, even at very high Al concentrations, clearly prove that rye is one of the most tolerant cereals which makes it a valuable source of germplasm for breeding programs, such as improvement of related species more susceptible to this abiotic stress. The effectiveness of the current screening procedure to characterize rye genotypes for their performance on acidic soils was confirmed. Moreover, a vast genetic diversity was found both within and between different *Secale* species/subspecies/cultivars which enhances its agronomic value.

The present study demonstrated the potential of RAPDs and ISSRs, both DNA markers-based, in the identification of loci associated to the Al tolerance trait. The high polymorphisms revealed by these markers is promisor to the design of a test that will allow the distinction between sensitive and tolerant rye genotypes. Ten RAPD markers

distinguish between the sensitive and the tolerant genotypes of, at least, two distinct ryes. Fifteen ISSRs were associated with Al tolerant genotypes and nine with sensitive rye genotypes. Concluding both marker systems provide important resources that can be developed for molecular marker-assisted selection which has revolutionized breeding methods.

Acknowledgments

This work was supported by research PhD grant from Fundação para a Ciência e Tecnologia de Portugal (SFRH/BD/65040/2009).

References

Akhter, A., Wagastuma, T., Khan, M. S. H., and Tawaraya, K. (2009). Comparative Studies on Aluminum Tolerance Screening Technique for Sorghum, Soybean and Maize in Simple Solution Culture. *Am. J. Plant Physiol.* 4, 1–8.

Al-doss, A. A., Moustafa, K. A., and Ahmed, E. I. (2009). Assessment of genetic diversity in Saudi wheat genotypes under heat stress using molecular markers and agronomic traits. *Int. J. Plant Breed.* 3, 103–110.

Aniol, A. (1984). Induction of aluminum tolerance in wheat seedlings by low doses of aluminum in the nutrient solution. *Plant Physiol.* 76, 551–5. doi:10.1104/pp.76.3.551.

Aniol, A., and Gustafson, J. P. (1984). Chromosome location of genes controlling aluminium tolerance in wheat, rye and triticale. *Can. J. Genet. Cytol.* 26, 701–705.

Benito, C., Silva-Navas, J., Fontecha, G., Hernández-Riquer, M. V., Eguren, M., Salvador, N., et al. (2010). From the rye *Alt3* and *Alt4* aluminum tolerance loci to orthologous genes in other cereals. *Plant Soil* 327, 107–120. doi:10.1007/s11104-009-0035-9.

Bian, M., Zhou, M., Sun, D., and Li, C. (2013). Molecular approaches unravel the mechanism of acid soil tolerance in plants. *Crop J.* 1, 91–104. doi:10.1016/j.cj.2013.08.002.

Bona, L., Wright, R. J., Baligar, V. C., and Matuz, J. (1993). Screening wheat and other small grains for acid soil tolerance. *Landsc. Urban Plan.* 27, 175–178. doi:10.1016/0169-2046(93)90046-G.

Bornet, B., and Branchard, M. (2001). Nonanchored Inter Simple Sequence Repeat (ISSR) markers: Reproducible and specific tools for genome fingerprinting. *Plant Mol. Biol. Report.* 19, 209–215. doi:10.1007/BF02772892.

Bushuk, W. (2001). Rye production and uses worldwide. *Cereal Foods World* 46, 70–73.

Camacho, M. V., Matos, M., González, C., Pérez-Flores, V., Pernaute, B., Pinto-Carnide, O., et al. (2005). *Secale cereale* inter-microsatellites (SCIMs): Chromosomal location and genetic inheritance. *Genetica* 123, 303–311. doi:10.1007/s10709-004-5553-z.

Chikmawati, T., Ma, X., Ross, K., and Gustafson, J. P. (2006). "Secale," in *Genome Mapping and Molecular Breeding in Plants - Cereals and Millets*, ed. C. Kole (Springer-Verlag Berlin Heidelberg), 243–255.

Contreras, R., Figueiras, A. M., Gallego, F. J., Benavente, E., Manzaneda, A. J., and Benito, C. (2017). Neutral molecular markers support common origin of aluminium tolerance in three congeneric grass species growing in acidic soils. *AoB Plants* 9, 1–16. doi:10.1093/aobpla/plx060.

Contreras, R., Figueiras, A. M., Gallego, F. J., and Benito, C. (2014). *Brachypodium distachyon*: A model species for aluminium tolerance in Poaceae. *Funct. Plant Biol.* 41, 1270–1283. doi:10.1071/FP13362.

Dedio, W., Kaltsikes, P. J., and Larter, E. N. (1969). Numerical chemotaxonomy in the genus *Secale*. *Can. J. Bot.* 47, 1175–1180.

Deshmukh, R., Tomar, N. S., Tripathi, N., and Tiwari, S. (2012). Identification of RAPD and ISSR markers for drought tolerance in wheat (*Triticum aestivum* L.). *Physiol. Mol. Biol. Plants* 18, 101–104. doi:10.1007/s12298-011-0096-0.

Ellis, R. P., Forster, B. P., Robinson, D., Handley, L. L., Gordon, D. C., Russell, J. R., et al. (2000). Wild barley: a source of genes for crop improvement in the 21st century? *J. Exp. Bot.* 51, 9–17. doi:10.1093/JEXBOT/51.342.9.

Ezaki, B., Nagao, E., Yamamoto, Y., Nakashima, S., and Enomoto, T. (2008). Wild plants, *Andropogon virginicus* L. and *Miscanthus sinensis* Anders, are tolerant to multiple stresses including aluminum, heavy metals and oxidative stresses. *Plant Cell Rep.* 27, 951–961. doi:10.1007/s00299-007-0503-8.

Fontecha, G., Silva-Navas, J., Benito, C., Mestres, M. A., Espino, F. J., Hernández-Riquer, M. V., et al. (2007). Candidate gene identification of an aluminum-activated organic acid transporter gene at the *Alt4* locus for aluminum tolerance in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 114, 249–260. doi:10.1007/s00122-006-0427-7.

Gallego, F. J., and Benito, C. (1997). Genetic control of aluminium tolerance in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 95, 393–399.

Gallego, F. J., Calles, B., and Benito, C. (1998a). Molecular markers linked to the aluminium tolerance gene *Alt1* in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 97, 1104–1109. doi:10.1007/s001220050997.

Gallego, F. J., López-Solanilla, E., Figueiras, A. M., and Benito, C. (1998b). Chromosomal location of pcr fragments as a source of dna markers linked to aluminium tolerance genes in rye. *Theor. Appl. Genet.* 96, 426–434. doi:10.1007/s001220050759.

Inostroza-Blancheteau, C., Soto, B., Ibáñez, C., Ulloa, P., Aquea, F., Arce-Johnson, P., et al. (2010). Mapping aluminum tolerance loci in cereals: A tool available for crop breeding. *Electron. J. Biotechnol.* 13, 1–12. doi:10.2225/vol13-issue4-fulltext-4.

Jonah, P. M., Bello, L. L., Lucky, O., Midau, A., Moruppa, S. M., and Moruppa, Ω. S. M. (2011). Review : The importance of molecular markers in plant breeding programmes. *Glob. J. Sci. Front. Res.* 11, 5–12. Available at: https://globaljournals.org/GJSFR_Volume11/2-Review-The-Importance-of-Molecular.pdf.

Joshi, S. P., Ranjekar, P. K., and Gupta, V. S. (1999). Molecular markers in plant genome analysis. Current Science 77 (2):230-240. *Curr. Sci.* 77, 230–240.

Jozefaciuk, G., and Szatanik-Kloc, A. (2004). Decrease in variable charge and acidity of root surface under Al treatment are correlated with Al tolerance of cereal plants. *Plant Soil* 260, 137–145. doi:10.1023/B:PLSO.0000030175.23904.5a.

Kim, B. Y., Baier, A. C., Somers, D. J., and Gustafson, J. P. (2001). Aluminium tolerance in triticale, wheat and rye. *Euphytica* 120, 329–337.

Kochian, L. V., Pence, N. S., Letham, D. L. D., Pineros, M. A., Magalhaes, J. V., Hoekenga, O. A., et al. (2002). Mechanisms of metal resistance in plants: Aluminum and heavy metals. *Plant Soil* 247, 109–119. doi:10.1023/A:1021141212073.

Kochian, L. V, Hoekenga, O. A., and Piñeros, M. A. (2004). How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. *Annu. Rev. Plant Biol.* 55, 459–493. doi:10.1146/annurev.arplant.55.031903.141655.

Luzio, A., Coimbra, A. M., Benito, C., Fontaínhas-Fernandes, A. A., and Matos, M. (2015). Screening and identification of potential sex-associated sequences in *Danio rerio*. *Mol. Reprod. Dev.* 82, 756–764. doi:10.1002/mrd.22508.

Ma, J. . F., Taketa, S., and Yang, Z. M. (2000). Aluminum tolerance genes on the short arm of chromosome *3R* are linked to organic acid release in triticale. *Plant Physiol* 122, 687–694. doi:10.1104/pp.122.3.687.

Ma, J. F., and Furukawa, J. (2003). Recent progress in the research of external Al detoxification in higher plants: A minireview. *J. Inorg. Biochem.* 97, 46–51. doi:10.1016/S0162-0134(03)00245-9.

Ma, J. F., Ryan, P. R., and Delhaize, E. (2001). Aluminium tolerance in plants and the complexing role of organic acids. *Trends Plant Sci.* 6, 273–278. doi:10.1016/S1360-1385(01)01961-6.

Ma, J. F., Shen, R., Zhao, Z., Wissuwa, M., Takeuchi, Y., T, E., et al. (2002). Response of rice to Al stress and identification of quantitative trait loci for Al tolerance. *Plant Cell Physiol* 43, 652–659.

Ma, J. F., Zheng, S. J., Li, X. F., Takeda, K., and Matsumoto, H. (1997). A rapid hydroponic screening for aluminium tolerance in barley. *Plant Soil* 191, 133–137. doi:10.1023/A:1004257711952.

Matos, M., Camacho, M. V., Pérez-Flores, V., Pernaute, B., Pinto-Carnide, O., and Benito, C. (2005). A new aluminum tolerance gene located on rye chromosome arm *7RS*. *Theor. Appl. Genet.* 111, 360–369. doi:10.1007/s00122-005-2029-1.

Matos, M., Pinto-Carnide, O., and Benito, C. (2001). Phylogenetic relationships among Portuguese rye based on isozyme, RAPD and ISSR markers. *Hereditas* 134, 229–236. doi:10.1111/j.1601-5223.2001.00229.x.

Miftahudin, T., Scoles, G., and Gustafson, J. (2002). AFLP markers tightly linked to the aluminum tolerance gene *Alt3* in rye. *Theor. Appl. Genet.* 104, 626–631. doi:10.1007/s00122-001-0782-3.

Pinto-Carnide, O., and Guedes-Pinto, H. (1999). Aluminium tolerance variability in rye and wheat Portuguese germplasm. *Genet. Resour. Crop Ev.* 46, 81–85.

Samac, D. A., and Tesfaye, M. (2003). Plant improvement for tolerance to aluminum in acid soils – a review. *Plant Cell. Tissue Organ Cult.* 75, 189–207.

Silva-Navas, J., Benito, C., Téllez-Robledo, B., Abd El-Moneim, D., and Gallego, F. J. (2012). The *ScAACT1* gene at the *Qalt5* locus as a candidate for increased aluminum tolerance in rye (*Secale cereale* L.). *Mol. Breed.* 30, 845–856. doi:10.1007/s11032-011-9668-5.

Simões, C. C., Melo, J. O., Magalhaes, J. V, and Guimarães, C. T. (2012). Genetic and molecular mechanisms of aluminum tolerance in plants. *Genet. Mol. Res.* 11, 1949–1957. doi:10.4238/.

Singh, D., Rai, A. K., and Panyang, O. (2009). Hematoxylin staining as a potential screening technique for aluminium tolerance in pea (*Pisum sativum* l.). *Curr. Sci.* 96, 1029–1030.

Von Uexkull, H. R., and Mutert, E. (1995). Global extent , development and economic impact of acid soils. *Plant Soil* 171, 1–15.

Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V (1990). DNA polymorphisms amplified by arbitrary primers are usefil as genetic markers. 18, 6531–6535.

Younis, R. A. A., Ahmed, M. F., and EL-Menshawi, M. M. (2007). Molecular genetic markers associated with salt tolerance in grain sorghum. *Arab J. Biotech.* 10, 249–258.

Zietkiewicz, E., Rafalski, A., and Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20, 176–183. doi:10.1006/geno.1994.1151.

Supplementary data

Table S1. Sequences of RAPD and ISSR primers used in this study.

Primer	5' – 3'	Primer	5' – 3'
RAPD		**ISSR**	
OPA5	AGGGGTCTTG	UBC810	$(GA)_8T$
OPB13	TTCCCCCGCT	UBC811	$(GA)_8C$
OPC7	GTCCCGACGA	UBC812	$(GA)_8A$
OPC13	AAGCCTCGTC	UBC818	$(CA)_8G$
OPF9	CCAAGCTTCC	UBC823	$(TC)_8C$
OPN5	ACTGAACGCC	UBC824	$(TC)_8G$
OPO4	AAGTCCGCTC	UBC825	(AC)8T
OPO7	CAGCACTGAC	UBC827	$(AC)_8G$
OPR13	GGACGACAAG	UBC835	$(AG)_8YC$
		UBC836	$(AG)_8YA$

Y= (C, T).

CHAPTER III

Aluminum tolerance in rye: internal and external resistance mechanisms

SUBCHAPTER III-1

Characterization, genetic diversity, phylogenetic relationships, and expression of the aluminum tolerance *MATE1* gene in *Secale* species

E. Santos[1], C. Benito[2], J. Silva-Navas[2,3], F. J. Gallego[2], A. M. Figueiras[2], O. Pinto-Carnide[4] and M. Matos[1]

[1]*Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, 500-801 Vila Real, Portugal; Biosystems & Integrative Sciences Institute, Faculty of Sciences, University of Lisboa, 1749-016 Lisboa, Portugal*
[2]*Department of Genetics, Faculty of Biology, Complutense University of Madrid, E-28040 Madrid, Spain*
[3]*Centre for Plant Biotechnology and Genomics, Institute for Agricultural and Food Research and Technology, E-28223 Madrid, Spain*
[4]*Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, 500-801Vila Real, Portugal*

Keywords: Al-activated citrate transporter; citrate exudation; cultivated and wild rye.

*Published in *Biologia Plantarum* (2018) DOI: 10.1007/s10535-017-0749-0

Abstract

Aluminum (Al) is the main limiting factor for crop production in acidic soils. Efflux of organic acids is one of the mechanisms that determine Al-tolerance and Al-activated citrate transporter (multidrug and toxic compound extrusion) *MATE1* gene is involved in different species. The contribution of the rye *MATE1* gene (*ScMATE1*) depends on the rye (*Secale cereale* L.) cultivars and the crosses analyzed; there is no information about different rye species. The cDNA sequences, phylogenetic relationships, Al-tolerance, citrate exudation and expression of *ScMATE1* gene were analyzed in several cultivars and wild species/subspecies of the *Secale* genus. Genotypes highly tolerant to Al were found within this genus. For the first time, sequences of the cDNA of the *ScMATE1* gene were isolated and characterized in wild ryes. At least two copies of this gene were found likely to be related to Al-tolerance. The sequence comparison of 13 exons of *ScMATE1* revealed variability between species, but also inter- and intra-cultivars. Variations were found in the Al-induced expression of *ScMATE1* gene, as well as its contribution to Al-tolerance. The pattern of citrate exudation was inducible in most of the species/subspecies studied and constitutive in few. The phylogenetic analysis indicated that *ScMATE1* is orthologous of two genes (*HvMATE1* and *TaMATE1*) involved in the Al stress response in barley and wheat, respectively, but not orthologous of *SbMATE*, implicated in Al-tolerance in sorghum. *ScMATE1* is involved in the response to Al stress in ryes, but its contribution to Al-tolerance is complex, and like in other species, there are tolerant and sensitive alleles in the different cultivars and species studied.

1. Introduction

Acidic soils are a worldwide problem for agriculture. One of reasons is that aluminum (Al) is solubilized at acid pH producing the toxic cation Al^{3+}, which can restrict plant growth. Some plant species have developed different mechanisms to tolerate Al^{3+} toxicity. Several physiological mechanisms of Al-tolerance have been proposed, but the agronomical efficacy of promoting yield stability on acidic soils remains uncertain (Hoekenga and Magalhães, 2011). One of the mechanisms that determines the resistance of some species is the efflux of one or more organic anions (*e.g*. citrate and malate) from the root tips to the soil. The genes controlling this trait are members of the Al-activated malate transporters (*ALMT*) and citrate transporters (*MATE*) families, which encode membrane proteins that facilitate organic anion efflux across the plasma membrane. Identification of these and other resistance genes provides an opportunity to enhance the Al-tolerance of plants by marker-assisted breeding or other biotechnological methods (Ryan et al., 2010).

Some members of MATE protein family, including AtMATE (from *Arabidopsis thaliana*), HvMATE1 (from *Hordeum vulgare*), OsFRDL4 (from *Oryza sativa*), SbMATE (from *Sorghum bicolor*), TaMATE (from *Triticum aestivum*) and ZmMATE (from *Zea mays*), are involved in Al-activated citrate secretion (Furukawa et al., 2007; Magalhaes et al., 2007; Liu et al., 2009; Ryan et al., 2009; Maron et al., 2010; Tovkach et al., 2013).

Rye (*Secale cereale* L.), one of the most Al-tolerant cereal crops, secretes both citrate and malate from roots in response to Al, and the exudation pattern described is inducible (Li et al., 2000). Previous studies reported that the Al-tolerance of rye is high when compared with barley and wheat, even the relatively Al-sensitive rye inbred line Riodeva (Gallego and Benito, 1997), is more Al-tolerant than the tolerant cultivars of barley and wheat.

Rye *ScALMT1* gene (Al-malate activated transporter) has been reported to be a candidate gene, for the previously reported *Alt4* tolerance locus on chromosome *7RS* (Matos et al., 2005: Fontecha et al., 2007; Collins et al., 2008; Benito et al., 2010). Another candidate gene for Al-tolerance in rye is *ScMATE1* (homologue of *HvMATE1*), and although in some analyses its implication was not evident (Collins et al., 2008), experiments with different crosses reported cosegregation of this gene with a new QTL for Al- tolerance (Silva-Navas et al., 2012). Yokosho et al. (2010) isolated two genes,

named *ScFRDL1* and *ScFRDL2*, also involved in Al-tolerance in rye. Both genes were mainly expressed in roots, besides their different expression patterns. These authors found that *ScFRDL2* gene might be involved in Al-induced secretion of citrate (ScFRDL2 has 80.6% identity with OsFRDL2, a putative Al-responsive protein in rice), whereas the *ScFRDL1* gene should be implicated in the efflux of citrate into the xylem (*ScFRDL1* has 94.2% identity with *HvMATE1* an Al-activated citrate transporter in barley). The comparison between both DNA and protein sequences, corresponding to *ScAACT1* and *ScFRDL1,* revealed a 100% identity. The chromosomal location and identity of the sequences, support that *ScMATE1*, *ScAACT1*, and *ScFRDL1* are the same genes. Thus, in order to avoid confusion with the nomenclature, we have decided hereafter to use the first name utilized for this gene in rye: *ScMATE1* (Collins et al., 2008).

Previous studies about the implication of the *MATE1* gene on Al-tolerance in rye indicate that some alleles confer tolerance and that others do not. Moreover, the variability and the expression of this gene has not been examined in the wild species/subspecies of the genus *Secale,* whose germplasm could be of interest for breeding programs. Therefore, in this work, we conducted different analyses to characterize the Al- tolerance and the *ScMATE1* gene expression in several species/subspecies of the genus *Secale*.

2. Materials and Methods

2.1. Plants

In this work, three wild species of the genus *Secale*, *S. strictum* (Persl) Persl ssp. *strictum* (R1211), *S. sylvestre* Host (R892) and *S. vavilovii* Grossh. (PI618682) were analyzed, as well as three subspecies of *Secale cereale* L.: *S. cereale* ssp. *ancestrale* Zhuk. (PI445975), the cultivated *S. cereale* ssp. *cereale* and *S. cereale* ssp. *segetale* Zhuk. (PI326284). Ryes of accessions with codes beginning with "PI" were kindly provided by The National Small Grains Collection (NSGC) of the United States Department of Agriculture - Agricultural Research Service (USDA-ARS) and those with "R" by Genebank Gatersleben, Institute of Plant Genetic and Crop Plant Research (IPK). Within the cultivated ryes two landraces (Lamego and Montalegre), the cultivar Imperial, and the inbred lines Riodeva (germplasm bank of the Department of Genetics and Biotechnology at the University of Trás-os-Montes and Alto Douro collection, Vila Real, Portugal) and P105 (kindly supplied by Dr. A. Börner from IPK) were studied. In order to make it easier,

the subspecies designation will be abbreviated in the text by *S. ancestrale*, *S. cereale, S. segetale* and *S. strictum*.

2.2. Al-tolerance screening tests

The screening method described by Aniol and Gustafson (1984) and adapted by Gallego and Benito (1997) was used for the Al-tolerance characterization. Twenty seeds were disinfected in 0.1% (m/v) $HgCl_2$ solution for 10 min and rinsed with de- ionized water. The seeds were germinated in Petri dishes in the dark at 4 °C overnight and then incubated at 25 °C for two days. Germinated seeds were transferred to a nylon mesh floating on a continuously aerated nutrient solution containing 0.4 mM $CaCl_2$, 0.65 mM KNO_3, 0.25 mM $MgCl_2 \cdot 6H_2O$, 0.01 mM $(NH_4)_2SO_4$, and 0.04 mM NH_4NO_3 (pH 4.0) and grown in a chamber at a temperature of 20 °C, an air humidity of 65%, a 16-h photoperiod, and an irradiance of 150 µmol m^{-2} s^{-1}. Four days after sowing, the seedlings were incubated for 24 h in nutrient solution with 150 µM aluminum in the form of $AlK(SO_4)_2 \cdot 12H_2O$ (pH 4.0). After Al exposure, the seedlings were thoroughly washed with de-ionized water. Then, roots were stained with 0.1% aqueous solution of Eriochrome cyanine R for 10 min, and the excess of dye washed with de-ionized water. Subsequently, the seedlings were transferred to a fresh Al-free nutrient solution for 48 h (renewed daily). After recovery, the plants were classified as tolerant or sensitive according to their root regrowth.

2.3. RNA extraction, cDNA synthesis and sequencing

Seedlings of Lamego and Montalegre landraces, Riodeva inbred line, and of the five wild species/subspecies (7-d-old) were exposed to nutrient solution with 300 µM Al for 8 or 24 h. Root apices (1 cm) and leaves either exposed or not to Al, were collected, immediately frozen in liquid nitrogen, and stored at -80 °C until use. Homogenization was carried out using TissueLyser II (Qiagen, Hilden, Germany) and 5 mm stainless steel beads (Qiagen). Total RNA was extracted from both roots and leaves of about 20 different plants per genotype and per Al exposure time (0, 8, and 24 h) using TRIzol® kit (Invitrogen, Carlsbad, CA, USA). RNA quality was checked by gel electrophoresis and then quantified with a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Total RNA (2 µg) was reverse transcribed with a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA)

according to the conditions specified by the supplier. For *ScMATE1* gene sequencing only cDNAs obtained from roots of plants exposed to Al for 24 h were used. The full-length ScMATE1 open reading frame (cDNA) was isolated with two primer pairs designed from the genomic ScMATE1 sequence of rye lines Ailés and Riodeva (Silva-Navas et al., 2012; Supplementary Table S1). The resultant PCR products were cloned into pGEM-T-Easy cloning kit (Promega, Madison, USA) following the manufacturer's protocol.

2.4. Sequence analyses

Sequences were analyzed with Chromas Lite 1.0 (Technelysium, Brisbane, Australia). A BLASTN search (http: //www.ncbi.nlm. nih.gov/) was performed to confirm the DNA and amino acid sequences predicted from the analysis. Alignments between different *ScMATE1* sequences were made using the ClustalW algorithm (http://www.ebi.ac.uk/Tools/clustalw). The sequences obtained in this work and other sequence data from different rye cultivars previously obtained (Silva-Navas et al., 2012) were compared. DnaSP v. 5.1 (Librado and Rozas, 2009) was used to calculate sequence diversity parameters. Several software programs were used to predict the secondary structures and membrane topologies of the different ScMATE1 proteins (Supplementary Table S2).

2.5. Phylogenetic analyses

Phylogenetic relationships among different MATE1 proteins and cDNA sequences were analyzed using MEGA 4.0 (Tamura et al., 2007) probing different evolutionary distances (number of differences, *p*-distance, Jukes-Cantor, Kimura 2-para- meter, Tamura 3-parameter, and maximum composite likelihood), amino acid substitution models (number of differences, *p*-distance, and Poisson correction) and clustering methods (neighbor-joining, minimum evolution, maximum parsimony, and UPGMA). Bootstraps with 10 000 replicates were performed to test the robustness of the dendrograms. LOFT 2.2 software (Van der Heijden et al., 2007) was used to identify levels of orthology from trees (LOFTs).

2.6. Gene expression analysis by semi-quantitative (sq) and quantitative (q) real-time PCR

For expression studies, we used cDNAs obtained from roots and leaves of plants not exposed to Al (0 h) and exposed to Al for 8 or 24 h as described above. *ScMATE1* gene expression was determined by real-time qPCR (Silva-Navas et al., 2012; Supplementary Table S1) using a primer pair, designed with Primer Express® 2.0 software (Applied Biosystems). Primers for the housekeeping *18S* rye gene, described by Fontecha et al. (2007), were used as control. PCR reactions were performed in a total volume of 0.02 cm^3 containing 0.01 cm^3 of Fast SYBR® Green Master Mix (Applied Biosystems), 6.0 pmol of each primer and cDNA dilutions made from ~ 200 ng of RNA. Reactions were carried out using a 7900 HT fast real-time PCR system (Applied Biosystems) with the following program: one step at 95 °C for 20 s and 40 cycles at 95 °C for 1 s and 60 °C for 20 s. All PCR samples and controls were prepared in duplicate in 0.1 cm^3 MicroAmp™ optical plates (Applied Biosystems). Normalizations and standard deviations calculations of the samples were made according to relative standard curve.

The *ScMATE1* expression was also determined by sqPCRs, using *18S* gene as reference. The analyses were conducted using mRNAs from the five wild species/ subspecies of the genus *Secale*. To detect *ScMATE1* and *18S* mRNA, the primers utilized were the same as used in the qPCR. The sqPCR was performed in a 0.02 cm^3 reaction volume containing 0.002 cm^3 of cDNA, 0.002 cm^3 of each gene-specific primer, and 0.01 cm^3 of Taq PCR MasterMix (Qiagen), using the following program: an initial step at 95 °C for 3 min, 30 cycles at 94 °C for 20 s, at 60 °C for 30 s, and at 72 °C for 35 s, followed by a final extension at 72°C for 7 min. PCR products were visualized on 1 - 2% Tris-acetate-EDTA (TAE) agarose gels.

2.7. Citrate exudation

Citric acid was determined using enzymatic methods described by Dagley (1974) and Delhaize et al. (1993). Citrate efflux from intact roots of seedlings of the five wild species/subspecies (*S. ancestrale*, *S. segetale*, *S. strictum*, *S. sylvestre* and *S. vavilovii*) and three cultivated ryes (Imperial, Riodeva and P105) was assayed with and without Al stress. Briefly, 20 seeds from each rye were sterilized with NaClO + distilled H_2O (1:1) for 40 min. Then, seeds were washed three times with sterile water, added to flasks containing 0.2 mM $CaCl_2$ (pH 4.3) and, finally, incubated on a rotary shaker (95 rpm) at 23 °C, a 16-h photoperiod, and an irradiance of 150 μmol m^{-2} s^{-1} for 6 d. Thereafter, the

solution from flasks was decanted and the seedlings were rinsed three times with the same solution as mentioned above. Later, 0.05 mM $AlCl_3$ (pH 4.3) was added to half of the seedlings. Root exudates were collected after 3, 6, and 24 h.

2.8. Number of repetitions and statistics

Screening for Al tolerance was repeated two times with the same conditions. Expression studies and citrate quantification were performed with three biological replicates for each rye sample and treatment. Analysis of variance (ANOVA) was performed using the SPSS statistical package for Windows (v. 23.0; IBM Corp., Armonk, NY, USA). Significant differences between means were determined using the Tukey test.

3. Results

A great variability for aluminum tolerance was found both between and within the rye species/subspecies and cultivars studied. The Al-tolerance screening method (Table 1) allowed the classification of the different rye plants as tolerant or sensitive to Al stress. Within *S. cereale*, the genotypes Montalegre, Lamego, Imperial, and P105 were classified as Al-tolerant similarly as the wild ryes *S. ancestrale*, *S. segetale*, and *S. vavilovii*. Compared with these ryes, *S. strictum* was classified as moderately Al-tolerant. On the other hand, the inbred line Riodeva and *S. sylvestre* were classified as Al-sensitive (Supplementary Fig. S1).

Table 1. Mean lengths of root regrowth (MLRR) as a measure of Al-tolerance of the plant material used in this work. Means ±SD, $n = 20$. Different letters indicate significant differences at $P < 0.05$, according to Tukey's test.

Rye species	Classification	MLRR (mm)
S. ancestrale	wild subspecies	5.49 ± 3.87b
S. segetale	wild subspecies	6.62 ± 4.15bc
S. strictum	wild subspecies	3.14 ± 3.08ab
S. sylvestre	wild species	0.13 ± 0.25a
S. vavilovii	wild species	5.13 ± 4.17b
S. cereale		
Imperial	cultivar	6.04 ± 5.51b
Lamego	landrace	14.03 ± 8.06d
Montalegre	landrace	10.43 ± 7.79cd
P105	inbred line	12.30 ± 4.10d
Riodeva	inbred line	0.03 ± 0.07a

We obtained six different cDNA sequences in *S. cereale* (four from Lamego and two from Montalegre) and 13 sequences corresponding to wild *Secale* species/subspecies (two from each of *S. segetale*, *S. strictum*, and *S. sylvestre*, three from *S. ancestrale* and four from *S. vavilovii*). The sequences of the *ScMATE1* gene obtained in this study were deposited in Genbank (accessions Nos. KX632094 - KX632112). The sequence comparisons revealed intra-cultivar, inter-cultivar, intraspecific and interspecific variability. In all the ryes analyzed, a 1 665 bp coding region (including stop codon) was found for the *ScMATE1* gene. In *S. sylvestre*, in addition of the 1 665 bp sequence, another sequence of 1 668 bp was found.

We have compared 11 different cDNA sequences of *ScMATE1* in *S. cereale*, the six mentioned above and five (one from each of Ailés, Imperial, Linea V, Petkus, and Riodeva) from the previous work of Silva-Navas et al. (2012) (Supplementary Table S3A). Comparisons among these different *ScMATE1* sequences revealed two INDELs (insertion/deletion) of three bp each that were only observed in the exon 1 of Riodeva. Taking into account the values of nucleotide diversity (ND - 0.00755), haplotype diversity (Hd - 1) and average number of nucleotide differences (k - 12.564), the most variable exons were 1, 4, and 8, with exon 1 displaying clearly the highest values for these diversity indexes, whereas the less variable exons were 7 and 13. In order to study the variability of the *ScMATE1* gene and its exons among different species/subspecies of *Secale*, six cDNA sequences were compared: five from wild species/subspecies and one from cultivated rye (*S. cereale*), represented by the cultivar Imperial (Supplementary Table S3B). The results obtained indicated that, considering the values of ND (0.01842), Hd (1), and k (30.667), the *ScMATE1* most diverse exons in the *Secale* genus were 1, 8, and 11 and the least variable exons were 7, 1, 2 and 13. A unique INDEL, with 3 bp, was observed in the exon 1 of *S. sylvestre,* while variation for the same INDEL was found among *S. cereale* cultivars. Taking only into account the changes in the coding region, exon 1 was the most variable, both in *S. cereale* as in whole *Secale* genus.

The variability analysis of the proteins encoded by *MATE1* gene was made using amino acid sequences from the five wild species/subspecies and two *S. cereale* cultivars (Imperial and Riodeva). The deduced proteins comprised 554 (*S. ancestrale*, *S. segetale*, *S. strictum*, *S. vavilovii* and cv. Imperial), 555 (*S. sylvestre*), and 556 (Riodeva) amino acid residues, with a molecular mass between 58.3 and 58.4 kDa. The proteins were hydrophobic, containing the characteristic MatE domain of MATE family. Depending on the protein structure prediction software used, seven to eleven putative transmembrane

helix regions (TMH1 to TMH11) were found (Supplementary Table 2 and Fig. S2). Seven of these transmembrane helices (TMH3, TMH4, TMH5, TMH6, TMH7, TMH8, and TMH10) were predicted by all programs used.

The phylogenetic relationships among 46 MATE proteins from different plant species were established. ScMATE1 predicted proteins shared 93% identity with HvAACT1 barley protein, 90% with OsFRDL1 and 68% with OsFRDL4 rice proteins, 66% with SbMATE sorghum protein, 60% with AtMATE and AtFRD3 *Arabidopsis* proteins. The predicted ScFRDL1 rye protein of Yokosho et al. (2010) is identical (100% identity) to the predicted ScMATE1 protein in this study; therefore, both are included in the dendrogram with the same name (ScMATE1). The different methods of distance calculations for protein sequences and the different clustering methods used to obtain the phylogenetic trees of the MATE protein family, gave dendrograms with identical structure and very high bootstrap values. In all cases, two main clusters (A and B) were defined, both including proteins from monocot and dicot species (Fig. 1). Cluster A appears divided into two subclusters: A1 and A2, and the former subdivided again into several groups and subgroups. The MATE proteins of the families MATE1 (FRDL1) and MATE2 (FRDL2) belong to different groups of subclusters A1, whereas MATE3 (FRDL3) proteins are grouped in the subcluster A2. Subcluster B is also divided in two groups: B1, including MATE proteins from six different species, and B2, including MATE proteins from ten different species. This analysis revealed the existence of seven different groups of orthologous genes. With the construction of a more complete dendrogram, including 74 additional MATE protein sequences from the plant molecular database Phytozome.net (Supplementary Fig. S3), the structure obtained was similar to the previous one (Fig. 1).

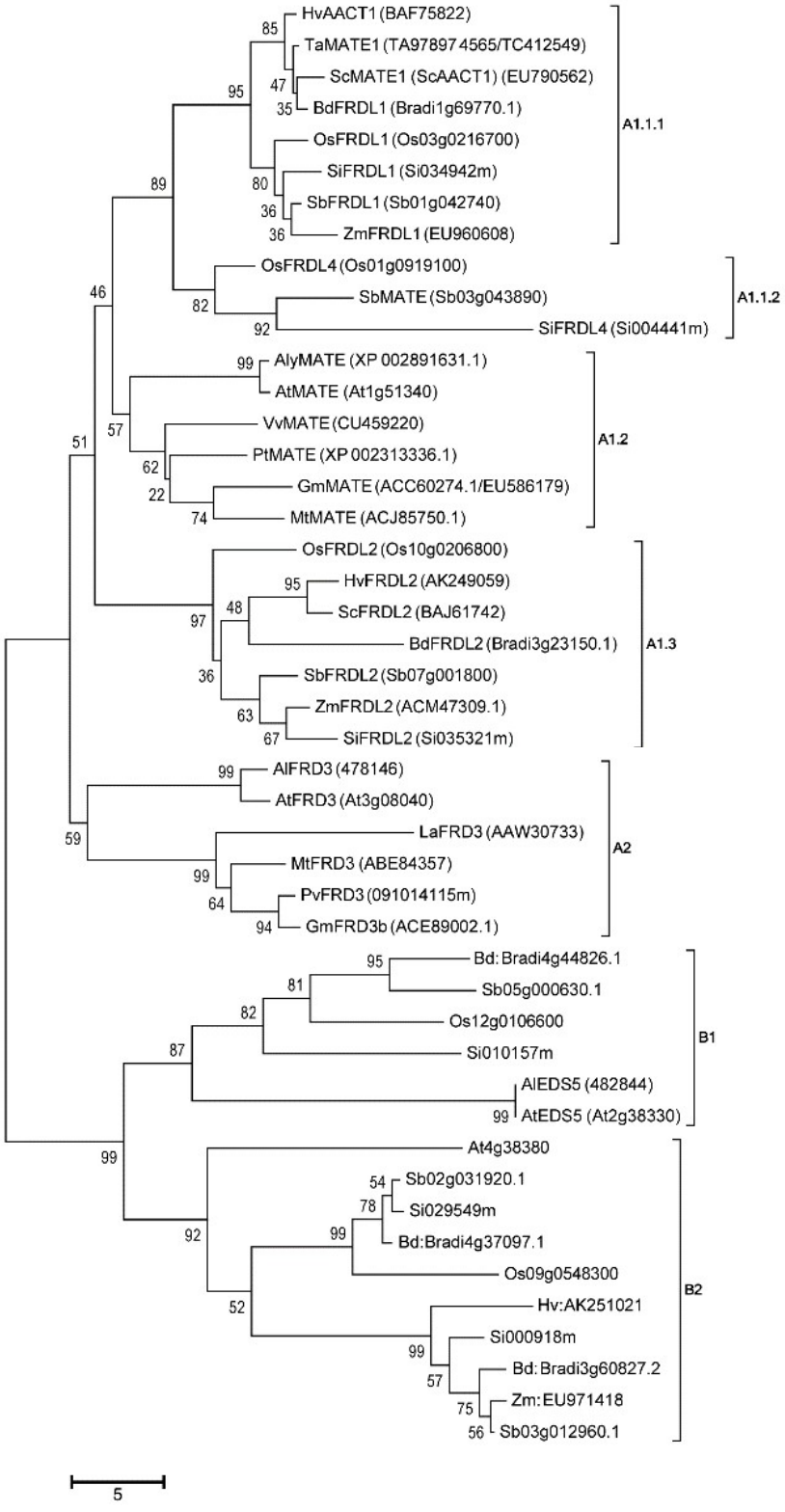


Figure 1. Phylogenetic relationships obtained with hypothetical proteins from MATE family. The evolutionary distance and the cluster method used were *p*-distance and neighbor-joining, respectively. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) are shown next to the branches. The brackets indicate groups of orthologous genes obtained using LOFT 2.2 software. Abbreviations: Al – *Arabidopsis lyrata*; At – *Arabidopsis thaliana*; Bd – *Brachypodium distachyon*; Gm – *Glycine max*; Hv – *Hordeum vulgare*; La – *Lupinus albus*; Mt – *Medicago truncatula*; Os – *Oryza sativa*; Pv – *Phaseolus vulgaris*; Pt – *Populus trichocarpa*; Sc – *Secale cereale*; Sb – *Sorghum bicolor*; Si –*Setaria italica*; Ta – *Triticum aestivum*; Vv –*Vitis vinifera*; Zm – *Zea mays*.

In order to facilitate the *ScMATE1* phylogenetic analysis among the species/subspecies of the genus *Secale*, only one cDNA or protein sequence from each species/subspecies was used (Fig. 2). As all *S. cereale* plants gave identical results, the cultivar Imperial was used as representative of the cultivated species. The cDNAs and amino acid sequences from *B. distachyon*, *H. vulgare*, *O. sativa*, *S. bicolor*, *T. aestivum*, and *Z. mays* were used as an outgroup. The dendrograms obtained from cDNAs and the ones obtained from proteins showed the same structure and, repeatedly, bootstrap values were very high. All the species/subspecies of the genus *Secale* have grouped in the same cluster. In addition, wheat, barley, and *Brachypodium* grouped together in the same cluster as the genus *Secale*.

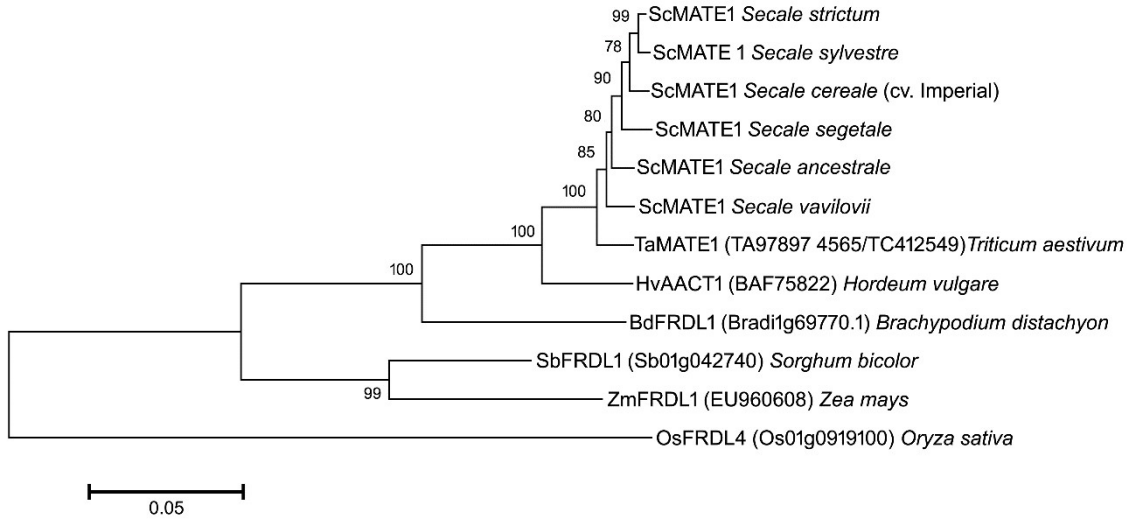


Figure 2. Phylogenetic relationships obtained for coding region of the *ScMATE1* gene from different species/subspecies of the genus *Secale* compared with the orthologous cDNA from *H. vulgare*, *O. sativa*, *S. bicolor*, *T. aestivum*, and *Z. mays*. The evolutionary distance and the cluster method used were Kimura two parameters and neighbor-joining, respectively. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) are shown next to the branches.

On the first approach, the expression of *ScMATE1* gene in roots of *S. cereale* plants (Lamego, Montalegre and Riodeva), not treated (0 h) and exposed to Al for 8 h and 24 h, was studied. Previous studies (data not shown) showed a little amount of *ScMATE1* mRNA in the leaves of *S. cereale* cultivars so that we have not included leaves in this early study. Furthermore, the same expression values were obtained at 0, 8, and 24 h without Al which made us to only use the 0 h time point. The results indicate that there is no significant induction of the *ScMATE1* expression at 8 h in the roots of the cultivars. However, after 24 h of Al exposure, this gene was clearly expressed in Lamego, Montalegre and inbred line Riodeva with a 20, 13.5, and 12-fold difference (fd) compared

to control without Al treatment (0 h) (Fig. 3). Riodeva showed the highest amount of mRNA (59-fold) and Lamego showed the smallest one (20-fold).

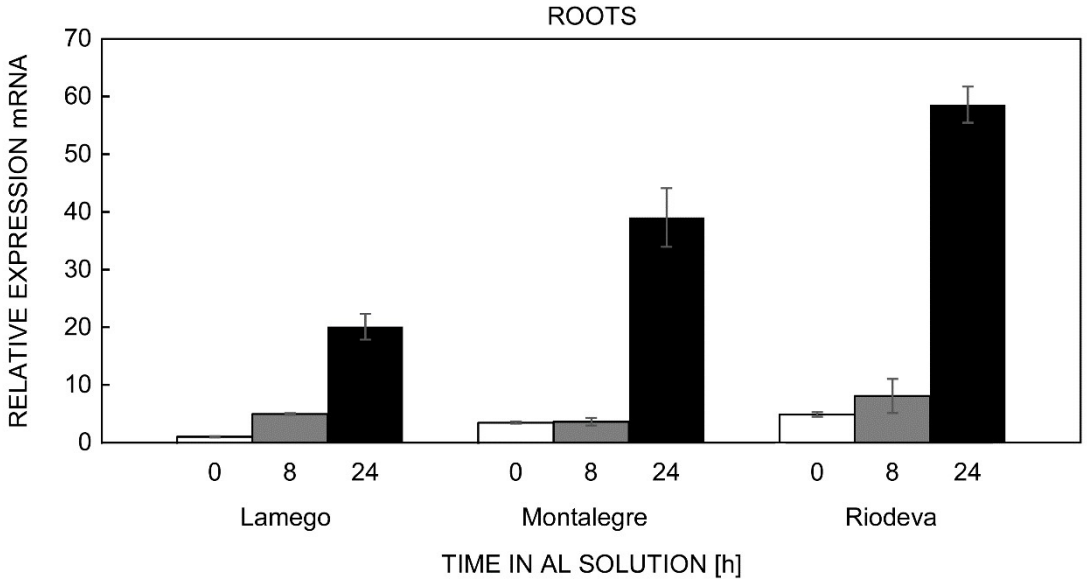


Figure 3. Real-time qPCR showing expression patterns of root tip cDNA transcripts of *ScMATE1* gene in *S. cereale* two landraces (Lamego and Montalegre) and the inbred line Riodeva after 300 µM Al treatment. Change (fold difference) at each time point (0, 8, and 24 h) is expressed as the relative expression compared to less fluorescence signal (Lamego without Al stress, 0 h). Significant differences among cultivars were found ($P < 0.05$), according to Tukey test (Two-way ANOVA).

Taking into account the great differences observed between plants not treated (0 h) and plants exposed to Al for 24 h, the study of the *ScMATE1* expression in the other five species/subspecies of *Secale* was done. Since there is no data of this gene expression in the wild ryes, both leaves and roots were analyzed. The amount of *ScMATE1* mRNA was significantly higher in roots than in leaves, both in absence (0 h) and in presence of Al (24 h) in the five wild species/subspecies of *Secale*. Similar results were observed by the semi-quantitative analyses (data not shown). The ratio root/leaf detected without Al was 3.33 (*S. ancestrale*), 8.92 (*S. segetale*), 2.70 (*S. strictum*), 5.43 (*S. sylvestre*), and 8.30 (*S. vavilovii*), whereas after the treatment with Al it was 5.30 (*S. ancestrale*), 11.99 (*S. segetale*), 1.99 (*S. strictum*), 8.75 (*S. sylvestre*), and 6.57 (*S. vavilovii*) (data not shown). The results obtained comparing plants without Al, as controls, and plants exposed to Al for 24 h indicated that the mRNA of *ScMATE1* was not induced in the roots of *S. ancestrale* (1.18 fd), *S. strictum* (1.15 fd), and *S. vavilovii* (1.19 fd) and moderately induced in *S. sylvestre* (2.10 fd) and *S. segetale* (1.73 fd) (data not shown). The data of real-time qPCR agrees with the results of sqPCR analyses (Fig. 4). The amount of mRNA of *ScMATE1* in the roots of the different rye species/subspecies, with and without Al, was

very similar, except in *S. sylvestre* and *S. segetale* where a clear increase is observed in the mRNA amount, when exposed to Al (Fig. 4).

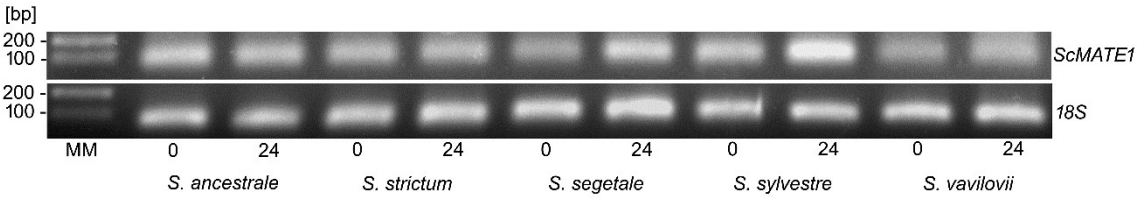


Figure 4. Semi-quantitative PCR results showing temporal expression patterns of root tip cDNA transcripts of *ScMATE1* gene from *S. ancestrale*, *S. strictum*, *S. segetale*, *S. sylvestre*, and *S. vavilovii* without Al (0 h) and with 300 µM Al for 24 h. Below is the expression of the housekeeping gene *18S* used as a reference.

The exudation of citrate (Fig. 5) started after a lag phase of several hours of Al exposure (usually started after 6 h – data not shown) in *S. ancestrale*, *S. vavilovii* and *S. cereale* cv. Imperial. On the other hand, *S. sylvestre* and *S. segetale* began to exudate citrate immediately after contact with Al. After 24 h of Al exposure, induced root citrate exudation was observed in all species/subspecies. In the case of the inbred lines Riodeva and P105, the amount of citrate exudates, with and without Al, were the same over time being the citrate exudation clearly constitutive in both lines. *S. strictum* has a constitutive citric acid exudation too since the amount of his exudates is higher without Al.

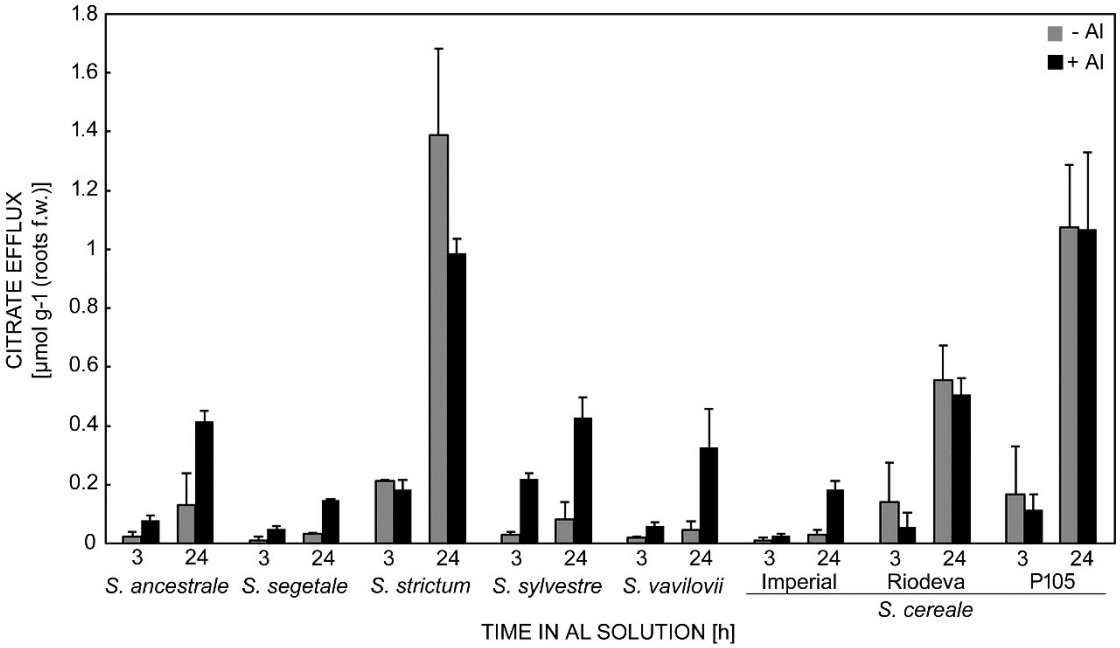


Figure 5. Citrate exudation of *S. ancestrale*, *S. segetale*, *S. strictum*, *S. sylvestre*, *S. vavilovii*, *S. cereale* cv. Imperial and two inbred lines (Riodeva and P105). The seedlings were incubated in hydroponic culture with 0 or 0.05 mM $AlCl_3$. Data of root exudates collected at 3 and 24 h. Data are the means ±SD, $n = 10$ per treatment and time in Al solution. Significant statistical differences were found between rye species ($P < 0.001$). Comparing with and without Al in each rye, significant differences were found in *S. ancestrale* ($P < 0.05$), *S. segetale*, *S. sylvestre* and Imperial ($P < 0.01$) (Three-way ANOVA; Tukey test).

4. Discussion

Soil acidity is a worldwide problem, and when associated with Al toxicity, it is one of the main factors limiting crop production. The first injury of Al toxicity is the inhibition of the development of the root system. The root regrowth observed for Riodeva and Imperial agree with the previous data obtained by Gallego and Benito (1997) and Silva-Navas et al. (2012), respectively. As in previous studies (Pinto-Carnide and Guedes-Pinto, 1999; Silva et al., 2012), the landraces Montalegre and Lamego exhibited a great Al-tolerance. The wild ryes and the inbred line P105, not previously studied, mostly showed a high degree of Al-tolerance with the exception of the *S. sylvestre*.

A great variability was observed for Al-tolerance in cultivated rye and wild rye, being intra- and inter-cultivar and intra- and interspecific. With the exception of *S. sylvestre* and *S. vavilovii*, all the rye species/subspecies are allogamous, with heterozygous genotypes, which leads to the existence of a wide diversity of tolerant and sensitive genotypes. In the autogamous species *S. sylvestre* and the inbred lines of *S. cereale* this diversity was not found. All the plants of *S. sylvestre* were sensitive and all the plants of inbred line P105 were tolerant. Pinto-Carnide and Guedes-Pinto (1999) also observed variability in Al-tolerance among their rye as well as wheat cultivars.

Rye has a huge importance in Al-tolerance approaches, due to its high capacity to tolerate Al, as shown in the present study. The selection of crops well adapted to acidic soils is important, therefore, tolerant cultivated ryes and wild ryes prove to be a potential resource for genetic improvement programs, especially the autogamous species and the inbred lines.

Nineteen sequences isolated (13 from wild ryes and six from cultivated ryes) were similar to those previously obtained by Silva-Navas et al. (2012), who also found a 1 665 bp coding region of the *ScMATE1* gene in various cultivars. However, one of the sequences of *S. sylvestre* obtained had 1 668 bp coding region. Silva-Navas et al. (2012) also found in Riodeva an additional cDNA with a coding region of 1 671 bp. Both results suggest that *ScMATE1* has, at least, two copies in the wild species *S. sylvestre* and in the inbred line Riodeva. Several copies of the *ZmMATE1* gene were also detected in tolerant lines of maize (Maron et al., 2013).

The difference in the cDNA length is found in the exon 1 where Riodeva and *S. sylvestre* show two and one 3 bp insertions, respectively. The INDEL found in *S. sylvestre* is the same as one of the INDELs found in Riodeva. As none of these insertions changes

the reading frame, the only difference in the predicted proteins is the presence of two additional amino acids in the inbred line and only one in the wild species. The detection of these two different cDNAs cannot be attributed to heterozygosity, since both types of plants are homozygous: Riodeva is an inbred line with more than forty generations of self-pollination and *S. sylvestre* is an autogamous species. Curiously, both ryes are classified as Al-sensitive, and these insertions may have a potential relation with Al sensitivity/tolerance. Santos et al. (2016) also found a sequence with two 3 bp insertion (the same as in the exon 1 of the *ScMATE1* gene in Riodeva) in a sensitive genotype of *S. vavilovii*, which reinforces this point. Maron et al. (2013) found a correlation between the three copies of the *ZmMATE1* gene and the Al-tolerance in maize.

A great genetic variability was observed in the *ScMATE1* cDNA sequences within the subspecies *S. cereale* and even higher in the whole *Secale* genus (Supplementary Table S3). This variability is important to reduce the vulnerability to biotic and abiotic stresses, which allows rye adaptation to adverse environments. Moreover, it has a high selection value that can be exploited through breeding programs to improve the yield/performance of related crops, and even within *Secale*, since this genus is one of the most tolerant to Al stress.

The results of the variability analysis of the deduced proteins from *ScMATE1* gene agrees with those previously obtained by Silva-Navas et al. (2012) with cultivated ryes. The deduced protein sequences of the *ScMATE1* alleles differed at 76 residues (28 TNSCs – total numbers of synonymous changes, 46 TNRC – total numbers of replacement changes and two insertions). All the changes detected in the different ScMATE1 proteins do not alter the transmembrane structure of this protein. Therefore, the main function of putative proteins (the citrate transport) is, probably, not affected.

The phylogenetic relationships among the deduced MATE1 proteins grouped the genes involved in the Al-tolerance of sorghum (*SbMATE*), rice (*OsFRDL4*), and barley (*HvMATE1* - *HvAACT1*) in the cluster A1.1 (Fig. 1) (Furukawa et al., 2007; Magalhaes et al., 2007; Wang et al., 2007; Yokosho et al., 2011). However, the intron-exon structure of these three genes is quite different. The *OsFRDL1* gene of rice and the *ScMATE1* gene of rye share the same intron-exon structure as their orthologous barley gene (13 exons and 12 introns). Moreover, according to this, the phylogenetic relationships obtained indicate that sorghum and barley genes are not orthologous, since they are clustered in different subgroups (A1.1). The orthologous of *HvMATE1* would be the sorghum *SbFRDL1* as both genes are in the same subgroup (A1.1.1) and share the same exon-

intron structure. The *TaMATE1*, *ZmMATE1* (*ZmFRDL1*), *ScMATE1* and *BdMATE1* (*BdFRDL1*) genes have also been implicated in the Al-tolerance in wheat, maize, cultivated rye, and *Brachypodium distachyon*, respectively (Ryan et al., 2009; Maron et al., 2010; Silva-Navas et al., 2012; Contreras et al., 2014). However, there are no publications about the implication of the *SbFRDL1*, *SiFRDL1*, and *OsFRDL1* genes in the tolerance of their respective species. All the *MATE* genes from the A1.1.1 subgroup are probably orthologous of *HvMATE1*, and therefore have a similar function. This hypothesis is also supported by the synteny relationships. The region of the *B. distachyon* chromosome 1*,* where is the *BdFRDL1* gene located, is syntenic with the region of the rice chromosome *3,* that harbors the *OsFRDL1* gene. In addition, there are synteny relationships with the chromosome arms 4HL of barley (*HvMATE1*), 4BL of wheat (*TaMATE1*, putative location), and 7RS of rye (*ScMATE1*) (Naranjo et al., 1987; Gale and Devos, 1998; Collins et al., 2008; Silva-Navas et al., 2012). The genes *OsFRDL4*, *SiFRDL4*, and *SbMATE* appear in a different subgroup (A1.1.2). In this case, the synteny also agrees with the orthology relationships; chromosome 1 of rice (*OsFRDL4*) is syntenic to the chromosome 3 of sorghum (*SbMATE*).

On the other hand, in the subcluster A1.3, the genes *OsFRDL2* (rice), *ScFRDL2*/*ScMATE2* (rye), *ZmFRDL2*/*ZmMATE2*) (maize), and *BdFRDL2*/*BdMATE2* (*B. distachyon*) were related to Al-tolerance, but only the rice and rye genes were involved in Al-induced citrate secretion (Maron et al., 2010; Yokosho et al., 2010, 2016; Contreras et al., 2014). Therefore, all the genes of this group are probably orthologous and could have a similar function. The *Arabidopsis AtMATE* gene (A1.2) has been related with the release of citric acid in response to Al-stress and is, probably, orthologous of the genes of the same group. Also, this group is more similar to the MATE1 group (A1.1), rather than the one of FRDL2 proteins (A1.3). The genes of subcluster A2 are probably orthologous to the *AtFRD3* gene of *A. thaliana*.

The taxonomy of the genus *Secale* is questioned, as different phylogenetic relationships have been obtained depending on the markers used. For this reason, the number of species proposed for this genus ranged from three to 14 (Stutz, 1972; Chikmawati et al., 2005). In our case, all the *Secale* species/subspecies studied appear as monophyletic (Fig. 2). The wild species *S. sylvestre* and *S. strictum* are the most closely related and are both close related to the cultivated *S. cereale*. The wild rye *S. vavilovii* is the phylogenetically more distant species. Our data agrees with previous reports on various aspects: *1*) a close relationship between *S. sylvestre* and *S. strictum* has been

described in different works (De Bustos and Jouve, 2002; Chikmawati et al., 2005; Shang et al., 2006; Ren et al., 2011). *2*) *S. strictum* is considered the direct antecessor of *S. cereale* (Vences et al., 1987; De Bustos and Jouve, 2002; Zhou et al., 2010; Santos et al., 2016). *3*) The wild subspecies *S. segetale* and *S. ancestrale* showed proximity among them and with *S. cereale* in the studies of De Bustos and Jouve (2002), Chikmawati et al. (2005), and Shang et al. (2006). These wild ryes are considered subspecies of *S. cereale* by Khush (1962) and Cuadrado and Jouve (2002). *4*) Although *S. vavilovii* is an autogamous species, different studies revealed the existence of intraspecific variability and their closely relationship with *S. cereale* (Shang et al., 2006; Fu et al., 2010; Santos et al., 2016). The phylogenetic relationships obtained, among the different members of the family *Poaceae*, (Fig. 2) agree with the previous data (Fig. 1) from these grasses with rye, wheat, barley, and *Brachypodium* grouped in the same cluster.

The exudation of organic acids, the most recognized mechanism responsible for Al-tolerance, is mediated by transporters that belongs to the two different gene families *ALMT* (malate) and *MATE* (citrate), both located in the plasma membrane. As referenced in the introduction of this work, the *MATE1* gene has been related to Al-tolerance in several crops. Since there is no data in wild ryes concerning *MATE1* gene, and different alleles of cultivated ryes showed a different contribution to Al-tolerance, we decided to study the expression of this gene and the citrate exudation.

Citric acid is more efficient than malic acid in preventing Al-induced inhibition of root elongation (Delhaize et al., 1993; Basu et al., 1994; Ma et al., 1997). However, the most effective gene involved in Al-tolerance, up to date, codifies an Al-activate malate transporter protein (ALMT) in wheat, rye, and *Arabidopsis*. Organic acids can be exudate immediately after the onset of Al treatment – type I (Li et al. 2000, Furukawa et al. 2007, Ryan et al. 2009) or after a lag phase of several hours – type II (Magalhaes et al., 2007; Liu et al., 2009;Yokosho et al., 2011), as described in rye (Li et al. 2000).

One important finding of our research is the pattern of citrate exudation being variable in *S. cereale* and in the genus *Secale* (Fig. 5). The Riodeva and P105 inbred lines of *S. cereale* and the wild subspecies *S. strictum* showed a constitutive pattern (their roots exudate citrate with as well as without Al) whereas the citrate exudation of the rye cv. Imperial and the other *Secale* wild species/subspecies is Al-inducible. However, Riodeva and *S. sylvestre* are Al-sensitive and the remaining *Secale* species/subspecies and cultivars are tolerant. These results mean that in *Secale* Al does not always induce the exudation of citrate, as it has been previously described. Furthermore, there is not a clear

correlation between the Al-tolerance and the amount of citrate exuded in these ryes (Fig. 5). The amount of organic acids released varies per crop species, which makes complex the comparison of exudates amounts between the tolerant and sensitive ryes at study, since most of them belong to different *Secale* species/subspecies.

The two different citrate exudation timing patterns were observed: in *S. sylvestre* and *S. segetale* it started immediately after Al exposure (pattern I), whereas in *S. ancestrale*, *S. vavilovii* and cv. Imperial it was delayed for at least 6 h (pattern II). It was described that plants belonging to pattern II may require genes for the enhanced Al-tolerance. Probably, this possible lack of correlation between the Al-tolerance and the citrate exudation, could be explained considering that the *ALMT1* gene is the most important for Al-tolerance in rye, and its presence can mask the action of the *MATE1* gene, or else, another *MATE* gene can be involved in the citrate transporter induced by Al.

The expression of *ScMATE1* in the genus *Secale* was significantly higher in roots than in leaves. In the same way, the *ScMATE1* (*ScFRDL1*), *HvMATE1* (*HvAACT1*), *BdMATE1*, and *TaMATE1* genes are also mainly expressed in the roots of cultivated rye, barley, *B. distachyon*, and wheat, respectively (Yokosho et al., 2010; Fujii et al., 2012; Tovkach et al., 2013; Contreras et al., 2014). This fact may be a sign that the *ScMATE1* gene is involved in the Al-tolerance of ryes since roots are the main target for Al toxicity and this region is expected to express the genes contributing in Al resistance.

Silva-Navas et al. (2012) found in one F_2 that an allele of the *ScMATE1* contributed to Al-tolerance. In addition, they detected that this gene was clearly Al-inducible in Imperial, Riodeva, 2672/4, and Petkus and that it was not induced in Ailés, all cultivated ryes. Our results concerning *ScMATE1* induction in Riodeva (Fig. 3) agree with results obtained by those authors. The *ScMATE1* gene is clearly induced in Lamego (20 fd) and Montalegre (13.5 fd) (Fig. 3), poorly induced in *S. sylvestre* (2.10 fd) and *S. segetale* (1.73 fd) and not induced in *S. ancestrale* (1.18 fd), *S. strictum* (1.15 fd) and *S. vavilovii* (1.19 fd) (Fig. 4). However, a different allele of *ScMATE1* does not contribute to Al-tolerance in the rye lines analyzed by Collins et al. (2008). Another allele from an inbred line, is induced by Fe deficiency rather than the presence of Al, as Yokosho et al. (2010) detected; these authors suggested that *ScMATE1* is involved in the citrate efflux into the xylem important for Fe translocation. Different works with different species indicate that the induction or non-induction of a gene is not necessarily indicative of its implication in the Al- tolerance. *TaALMT1* gene of wheat, related to Al- tolerance, is constitutively

expressed in roots of Al-tolerant and Al-sensitive lines (Sasaki et al., 2004; Delhaize et al., 2007).

There is not a direct relation between Al-tolerance and the expression of the *ScMATE1* gene, as there is not the relation between the expression of this gene and the citrate exudation. Citrate exudation is relatively high and constitutive in the sensitive Riodeva, but the expression of *ScMATE1* is higher in Al presence, indicating that this gene is not the only one involved in citrate exudation. The tolerant Imperial and *S. segetale* show an inducible citrate exudation, being coincident with an increase in *ScMATE1* expression. This leads us to suggest that the *ScMATE1* gene is involved in the Al-tolerance in these two ryes. Both ryes exuded a little amount of citrate but was highly induced by Al, and, as previously stated, citric acid is efficient at minor quantities (Ma et al., 1997). Conversely, the tolerant *S. ancestrale* and *S. vavilovii* have an inducible citrate secretion, but the *ScMATE1* gene is not induced, indicating that this gene is not the only one involved in it. The constitutive expression of the gene can interfere with the Al-tolerance of these wild ryes. The tolerant *S. strictum* has a constitutive citrate exudation pattern, with no alteration in expression of the *ScMATE1* gene, whereas the sensitive *S. sylvestre* has an inducible citrate exudation and a low increment in the *ScMATE1* gene expression. *S. strictum* exhibited the highest amount of citrate exudates both with and without Al, which could be related to its tolerance to Al stress.

The data obtained indicates that there exists a great variability in the expression of *ScMATE1* gene within *S. cereale*, as among different *Secale* species/subspecies. This could be related with the high variability found for this gene. In *S. cereale* plants, this gene showed a larger expression than in wild species. This could be due to an adaptation of the cultivated ryes to acid soils that allows the evolution to genotypes with better Al-tolerance. Lamego and Montalegre are landraces that come from the Northeast Portugal and the Imperial cultivar derived from North America where, in both cases, acid soils are abundant.

It was found that Al-induced expression of Al resistance genes was positively regulated by a transcription factor member of C_2H_2-type zinc-finger family, sensitive to proton rhizotoxicity 1 (AtSTOP1) in *Arabidopsis* (Iuchi et al., 2007) and Al resistance transcription factor (OsART1) in rice (Yamaji et al., 2009). Although *AtSTOP1* is not induced by Al, it is very important in the Al-tolerance, since when it is not expressed in the *AtSTOP1* mutant, the Al-tolerance of the plant decreases drastically. In turn, *OsART1* is constitutively expressed in the roots, and its expression is not affected by Al treatment.

The expression of the Al-tolerance genes *OsFRDL2* and *AtMATE* were regulated by OsART1 and AtSTOP1, respectively (Liu et al., 2009; Yokosho et al., 2016).

Variations in the promoter and downstream regions of *MATE* genes could be associated with enhanced Al-tolerance. The insertion of multiple miniature inverted-repeat transposable elements (MITEs) in the sorghum *SbMATE* promoter (Magalhães *et al.* 2007) and a 1-kb insertion in the upstream of the *HvMATE1* coding region in barley (Fujii *et al.* 2012) has been correlated with Al-tolerance. In addition, insertion of MITEs in downstream region of *ScMATE1* gene has been described in rye, but their implication in tolerance has not been demonstrated (Silva-Navas et al., 2012).

There are at least three ways to recognize if a gene is implicated in Al-tolerance: one is the observation of an increase in Al-tolerance of transgenic plants; the second, to have a QTL or a major locus with Mendelian inheritance for Al-tolerance co-segregating with the candidate gene in a cross; and at last, to possess a knockout mutant. Silva-Navas et al. (2012) have detected a QTL for Al-tolerance co-segregating with *ScMATE1* gene. Our results, together with previous data obtained in rye by other authors, suggest that the *ScMATE1* gene is involved in cultivated rye and wild rye Al-tolerance, however, not all alleles of *ScMATE1* contribute to Al-tolerance like in other species.

Acknowledgements

This work was supported by research grants AGL 2008-03049/AGR from the Ministerio de Educación y Ciencia de España, PR34/07-1581 from the Santander/Complutense, Acción Integrada España-Portugal (PT2009-0096 y E-171/10) and PHD grant from the Fundação para a Ciência e Tecnologia de Portugal (SFRH/ BD/ 65040/ 2009). J. Silva-Navas was a recipient of the Contratos de Personal Investigador de Apoyo (Comunidad de Madrid).

References

Aniol, A., and Gustafson, J. P. (1984). Chromosome location of genes controlling aluminium tolerance in wheat, rye and triticale. *Can. J. Genet. Cytol.* 26, 701–705.

Basu, U., Godbold, D., and Taylor, G. J. (1994). Aluminium resistance in *Triticum aestivum* L. associated with enhanced exudation of malate. *J. Plant Physiol.* 144, 747–753.

Benito, C., Silva-Navas, J., Fontecha, G., Hernández-Riquer, M. V., Eguren, M., Salvador, N., et al. (2010). From the rye *Alt3* and *Alt4* aluminum tolerance loci to orthologous genes in other cereals. *Plant Soil* 327, 107–120. doi:10.1007/s11104-009-

0035-9.

Chikmawati, T., Skovmand, B., and Gustafsson, J. P. (2005). Phylogenetic relationships among *Secale* species revealed by amplified fragment length polymorphisms. *Genoma* 48, 792–801. doi:10.1139/g05-043.

Collins, N. C., Shirley, N. J., Saeed, M., Pallotta, M., and Gustafson, J. P. (2008). An *ALMT1* gene cluster controlling aluminum tolerance at the *Alt4* Locus of Rye (*Secale cereale* L.). *Genetics* 179, 669–682. doi:10.1534/genetics.107.083451.

Contreras, R., Figueiras, A. M., Gallego, F. J., and Benito, C. (2014). *Brachypodium distachyon*: A model species for aluminium tolerance in Poaceae. *Funct. Plant Biol.* 41, 1270–1283. doi:10.1071/FP13362.

Cuadrado, A., and Jouve, N. (2002). Evolutionary trends of different repetitive DNA sequences during speciation in the genus *Secale*. *J. Hered.* 93, 339–345. doi:10.1093/jhered/93.5.339.

De Bustos, A., and Jouve, N. (2002). Phylogenetic relationships of the genus *Secale* based on the characterisation of rDNA ITS sequences. *Plant Syst. Evol.* 235, 147–154. doi:10.1007/s00606-002-0215-z.

Delhaize, E., Gruber, B. D., and Ryan, P. R. (2007). The roles of organic anion permeases in aluminium resistance and mineral nutrition. *FEBS Lett.* 581, 2255–2262.

Delhaize, E., Ryan, P. R., and Randall, P. J. (1993). Aluminum Tolerance in Wheat (*Triticum aestivum* L.) (II. Aluminum-Stimulated Excretion of Malic Acid from Root Apices). *Plant Physiol* 103, 695–702. doi:103/3/695 [pii].

Fontecha, G., Silva-Navas, J., Benito, C., Mestres, M. A., Espino, F. J., Hernández-Riquer, M. V., et al. (2007). Candidate gene identification of an aluminum-activated organic acid transporter gene at the *Alt4* locus for aluminum tolerance in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 114, 249–260. doi:10.1007/s00122-006-0427-7.

Fu, S., Tang, Z., Ren, Z., Zhang, H., and Yan, B. (2010). Isolation of rye-specific DNA fragment and genetic diversity analysis of rye genus *Secale* L. using wheat SSR markers. *J. Genet.* 89, 489–492. doi:10.1007/s12041-010-0070-6.

Fujii, M., Yokosho, K., Yamaji, N., Saisho, D., Yamane, M., Takahashi, H., et al. (2012). Acquisition of aluminium tolerance by modification of a single gene in barley. *Nat. Commun.* 3, 713. doi:10.1038/ncomms1726.

Furukawa, J., Yamaji, N., Wang, H., Mitani, N., Murata, Y., Sato, K., et al. (2007). An aluminum-activated citrate transporter in barley. *Plant Cell Physiol.* 48, 1081–1091.

Gale, M. D., and Devos, K. M. (1998). Comparative genetics in the grasses. *Proc. nat. Acad. Sci. USA* 95, 1971–1974.

Gallego, F. J., and Benito, C. (1997). Genetic control of aluminium tolerance in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 95, 393–399.

Hoekenga, O., and Magalhães, J. (2011). "Mechanisms of aluminum tolerance" in *Root Genomics*, eds. A. C. Oliveira and R. Varshney (Springer-Verlag, Berlin - Heidelberg .), 133–153.

Iuchi, S., Koyama, H., Iuchi, A., Kobayashi, Y., Kitabayashi, S., Kobayashi, Y., et al. (2007). Zinc finger protein STOP1 is critical for proton tolerance in *Arabidopsis* and coregulates a key gene in aluminum tolerance. *Proc. Natl. Acad. Sci.* 104, 9900–9905. doi:10.1073/pnas.0700117104.

Khush, G. S. (1962). Cytogenetic and evolutionary studies in *Secale* II. Interrelationships in the wild species. *Evolution (N. Y).* 16, 484–496.

Li, X. F., Ma, J. F., and Matsumoto, H. (2000). Pattern of aluminum-induced secretion of

organic acids differs between rye and wheat. *Plant Physiol.* 123, 1537–1544. doi:10.1104/pp.123.4.1537.

Librado, P., and Rozas, J. (2009). DnaSP v5.1: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451–1452.

Liu, J., Magalhaes, J. V., Shaff, J., and Kochian, L. V. (2009). Aluminum-activated citrate and malate transporters from the *MATE* and *ALMT* families function independently to confer *Arabidopsis* aluminum tolerance. *Plant J.* 57, 389–399. doi:10.1111/j.1365-313X.2008.03696.x.

Ma, J. F., Zheng, S. J., and Matsumoto, H. (1997). Specific secretion of citric acid induced by Al Stress in *Cassia tora* L. *Plant Cell Physiol.* 38, 1019–1025.

Magalhaes, J. V, Liu, J., Guimarães, C. T., Lana, U. G. P., Alves, V. M. C., Wang, Y.-H., et al. (2007). A gene in the multidrug and toxic compound extrusion (*MATE*) family confers aluminum tolerance in sorghum. *Nat. Genet.* 39, 1156–1161. doi:10.1038/ng2074.

Maron, L. G., Guimaraes, C. T., Kirst, M., Albert, P. S., Birchler, J. A., Bradbury, P. J., et al. (2013). Aluminum tolerance in maize is associated with higher *MATE1* gene copy number. *Proc. Natl. Acad. Sci.* 110, 5241–5246. doi:10.1073/pnas.1220766110.

Maron, L. G., Piñeros, M. A., Guimarães, C. T., Magalhaes, J. V., Pleiman, J. K., Mao, C., et al. (2010). Two functionally distinct members of the *MATE* (multi-drug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. *Plant J.* 61, 728–740. doi:10.1111/j.1365-313X.2009.04103.x.

Matos, M., Camacho, M. V., Pérez-Flores, V., Pernaute, B., Pinto-Carnide, O., and Benito, C. (2005). A new aluminum tolerance gene located on rye chromosome arm 7RS. *Theor. Appl. Genet.* 111, 360–369. doi:10.1007/s00122-005-2029-1.

Naranjo, T., Roca, A., Goicoechea, P. G., and Giráldez, R. (1987). Arm homoeology of wheat and rye chromosomes. *Genome* 29, 873–882.

Pinto-Carnide, O., and Guedes-Pinto, H. (1999). Aluminium tolerance variability in rye and wheat Portuguese germplasm. *Genet. Resour. Crop Ev.* 46, 81–85.

Ren, T. H., Chen, F., Zou, Y. T., Jia, Y. H., Zhang, H. Q., Yan, B. J., et al. (2011). Evolutionary trends of microsatellites during the speciation process and phylogenetic relationships within the genus *Secale*. *Genome* 54, 316–326. doi:10.1139/g10-121.

Ryan, P. R., Raman, H., Gupta, S., Horst, W. J., and Delhaize, E. (2009). A Second Mechanism for Aluminum Resistance in Wheat Relies on the Constitutive Efflux of Citrate from Roots. *Plant Physiol.* 149, 340–351. doi:10.1104/pp.108.129155.

Ryan, P. R., Tyerman, S. D., Sasaki, T., Furuichi, T., Yamamoto, Y., Zhang, W. H., et al. (2010). The identification of aluminium-resistance genes provides opportunities for enhancing crop production on acid soils. *J. Exp. Bot.* 62, 9–20. doi:10.1093/jxb/erq272.

Santos, E., Matos, M., Silva, P., Figueiras, A. M., Benito, C., and Pinto-Carnide, O. (2016). Molecular diversity and genetic relationships in *Secale*. 95, 273–281. doi:10.1007/s12041-016-0632-3.

Sasaki, T., Yamamoto, Y., Ezaki, B., Katsuhara, M., Ahn, S., Ryan, P., et al. (2004). A wheat gene encoding an aluminum-activated malate transporter. *Plant J* 37, 645–653.

Shang, H. Y., Wei, Y. M., Wang, X. R., and Zheng, Y. L. (2006). Genetic diversity and phylogenetic relationships in the rye genus *Secale* L. (rye) based on *Secale cereale* microsatellite markers. *Genet. Mol. Biol.* 29, 685–691. doi:10.1590/S1415-

47572006000400018.

Silva-Navas, J., Benito, C., Téllez-Robledo, B., El-Moneim, D. A., and Gallego, F. J. (2012). The *ScAACT1* gene at the *Qalt5* locus as a candidate for increased aluminum tolerance in rye (*Secale cereale* L.). *Mol. Breed.* 30, 845–856. doi:10.1007/s11032-011-9668-5.

Silva, S., Santos, C., Matos, M., and Pinto-Carnide, O. (2012). Al toxicity mechanism in tolerant and sensitive rye genotypes. *Environ. Exp. Bot.* 75, 89–97. doi:10.1016/j.envexpbot.2011.08.017.

Stutz, H. C. (1972). On the origin of cultivated rye. *Am. J. Bot.* 59, 59–70.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. . (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599.

Tovkach, A., Ryan, P. R., Richardson, A. E., Lewis, D. C., Rathjen, T. M., Ramesh, S., et al. (2013). Transposon-mediated alteration of *TaMATE1B* expression in wheat confers constitutive citrate efflux from root apices. *Plant Physiol.* 161, 880–92. doi:10.1104/pp.112.207142.

Van der Heijden, R. T., Snel, B., Van Noort, V., and Huynen, M. A. (2007). Orthology prediction at scalable resolution by phylogenetic tree analysis. *BMC Bioinformatics* 8, 83.

Vences, F. J., Vaquero, F., and Pérez de la Vega, M. (1987). Phylogenetic relationships in *Secale* (Poaceae): An isozymatic study. *Plant Syst. Evol.* 157, 33–47. doi:10.1007/BF00939179.

Wang, J., Raman, H., Zhou, M., Ryan, P. R., Delhaize, E., Hebb, D. M., et al. (2007). High-resolution mapping of the Alp locus and identification of a candidate gene *HvMATE* controlling aluminium tolerance in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.* 115, 265–276. doi:10.1007/s00122-007-0562-9.

Yamaji, N., Huang, C. F., Nagao, S., Yano, M., Sato, Y., Nagamura, Y., et al. (2009). A Zinc Finger Transcription Factor ART1 Regulates Multiple Genes Implicated in Aluminum Tolerance in Rice. *Plant Cell* 21, 3339–3349. doi:10.1105/tpc.109.070771.

Yokosho, K., Yamaji, N., Fujii-Kashino, M., and Ma, J. F. (2016). Functional Analysis of a MATE Gene *OsFRDL2* Revealed its Involvement in Al-Induced Secretion of Citrate, but a Lower Contribution to Al Tolerance in Rice. *Plant Cell Physiol.* 57, 976–985. doi:10.1093/pcp/pcw026.

Yokosho, K., Yamaji, N., and Ma, J. F. (2010). Isolation and characterisation of two *MATE* genes in rye. *Funct. Plant Biol.* 37, 296–303. doi:10.1071/FP09265.

Yokosho, K., Yamaji, N., and Ma, J. F. (2011). An Al-inducible *MATE* gene is involved in external detoxification of Al in rice. *Plant J.* 68, 1061–1069. doi:10.1111/j.1365-313X.2011.04757.x.

Zhou, J., Yang, Z., Li, G., Liu, C., Tang, Z., Zhang, Y., et al. (2010). Diversified chromosomal distribution of tandemly repeated sequences revealed evolutionary trends in *Secale* (Poaceae). *Plant Syst. Evol.* 287, 49–56.

Supplementary data

Table S1. Sequences of the primer pairs employed in this study. Ta - annealing temperature. Primers based on rye *ScMATE1* (*ScAACT1*) gene reported by Silva-Navas *et al.* (2012).

Primer	Sequence (5' → 3')	Ta	Purpose
AACT1-qPCR-1F	CCCACCGAGCCTTAAATCC	60 °C	quantitative RT-PCR
AACT1-qPCR-1R	GAGCAGCAGGAATCCACACC		
cDNA-1F	CGAGCGAACCATCGGCTGATCGAC	56 °C	full-length cDNA
cDNA-1R	TCACTTCCGGAGGAAAGCCCATGG		
cDNA-2F	GACCTCTCAGGCAAGCATCGATCC		

Table S2. Software programs used to deduce the structure of ScMATE1 proteins.

Program	URL address	Reference
TopPred	http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=toppred	Claros and Von Heijne (1994)
SOSUI	http://bp.nuap.nagoya-u.ac.jp/sosui/	Hirokawa et al. (1998)
PSIpred	http://bioinf.cs.ucl.ac.uk/psipred/psiform.html	McGuffin et al. (2000)
TMHMM	http://www.cbs.dtu.dk/services/TMHMM/	Krogh et al. (2001)
HMMTOP	http://www.enzim.hu/hmmtop	Tusnády and Simon (2001)
DAS	http://mendel.imp.univie.ac.at/sat/DAS/DAS.html	Cserzo et al. (2002)

References

Claros, M. G., and Von Heijne, G. (1994). TopPred II: an improved software for membrane protein structure predictions. *CABIOS* 10, 685–686.

Cserzo, M., Eisenhaber, F., Eisenhaber, B., and Simon, I. (2002). On filtering false positive transmembrane protein predictions. *Protein Eng.* 15, 745–752.

Hirokawa, T., Boon-Chieng, S., and Mitaku, S. (1998). SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* 14, 378–379.

Krogh, A., Larsson, B., Von Hiejne, G., and Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. mol. Biol.* 305, 567–580.

McGuffin, L. J., Bryson, K., and Jones, D. T. (2000). The PSIPRED protein structure prediction server. *Bioinformatics* 16, 404–405.

Tusnády, G. E. and Simon, J. (2001). The HMMTOP transmembrane topology prediction server. *Bioinformatics* 17, 849–850.

Table S3. Diversity parameters obtained comparing (a) eleven different sequences of cDNA from different rye cultivars (four from Lamego, two from Montalegre, and one from each of Ailés, Imperial, Linea V, Petkus, and Riodeva) and (b) six different sequences of cDNA from six different species of *Secale* (one from each of *S. ancestrale*, *S. segetale*, *S. strictum*, *S. sylvestre*, *S. vavilovii*, and *S.cereale* cv. Imperial using the DnaSP v. 5.0 software.

(a)

Secale cereale	**Ex1**	**Ex2**	**Ex3**	**Ex4**	**Ex5**	**Ex6**	**Ex7**	**Ex8**	**Ex9**	**Ex10**	**Ex11**	**Ex12**	**Ex13**	**cDNA**
Exon size [bp]	200	122	207	194	136	97	101	164	72	117	96	122	43	1671
SWAG	6	0	0	0	0	0	0	0	0	0	0	0	0	6
IMS	179	118	204	185	135	95	101	157	70	114	93	120	43	1614
VPS	15	4	3	9	1	2	0	7	2	3	3	2	0	51
TNM	15	4	3	9	1	2	0	7	3	3	3	2	0	52
SVS	7	3	3	7	1	2	0	2	2	3	3	2	0	35
PIS	8	1	0	2	0	0	0	5	0	0	0	0	0	16
TNSC	6	0	0	1	0	2	0	1	3	3	3	2	0	21
TNRC	9	4	3	8	1	0	0	6	0	0	0	0	0	31
Hd	0.964	0.345	0.345	0.727	0.182	0.345	0	0.491	0.491	0.491	0.182	0.345	0	1
ND (Pi)	0.02418	0.00715	0.00264	0.00993	0.00134	0.00375	0	0.0122	0.00732	0.00466	0.00568	0.00298	0	0.00755
k	4.691	0.873	0.545	1.927	0.182	0.364	0	2	0.527	0.545	0.545	0.364	0	12.564

(b)

***Secale* genus**	**Ex1**	**Ex2**	**Ex3**	**Ex4**	**Ex5**	**Ex6**	**Ex7**	**Ex8**	**Ex9**	**Ex10**	**Ex11**	**Ex12**	**Ex13**	**cDNA**
Exon size [bp]	197	122	207	194	136	97	101	164	72	117	96	122	43	1668
SWAG	1	0	0	0	0	0	0	0	0	0	0	0	0	1
IMS	184	116	201	188	131	92	99	147	70	112	89	121	43	1593
VPS	10	6	6	6	5	5	2	17	2	5	7	1	0	72
TNM	10	6	6	6	5	5	2	17	2	5	7	1	0	72
SVS	8	5	3	6	2	3	2	5	1	4	3	1	0	43
PIS	2	1	3	0	3	2	0	12	1	1	4	0	0	29
TNSC	5	1	1	2	5	1	0	2	1	3	5	1	0	27
TNRC	5	5	5	4	0	4	2	15	1	2	2	0	0	45
Hd	1	1	1	0.8	0.933	0.933	0.6	0,933	0.733	0.8	1	0.333	0	1
ND (Pi)	0.01993	0.01858	0.0132	0.01031	0.01765	0.02131	0.0066	0.05081	0.01204	0.01595	0.03403	0.00273	0	0.01842
k	3.867	2.267	2.733	2	2.4	2.067	0.667	8.333	0.867	1.867	3.267	0.333	0	30.667

Ex – exon; SWAG – sites with alignments gaps; IMS – invariable (monomorphic) sites; VPS – variable (polymorphic) sites; TNM – total number of mutations; SVS – singleton variable sites; PIS – parsimony informative sites; TNSC – total number of synonymous changes; TNRC – total number of replacement changes; Hd – haplotype (gene) diversity; ND (Pi) – nucleotide diversity; k – average number of nucleotide differences.

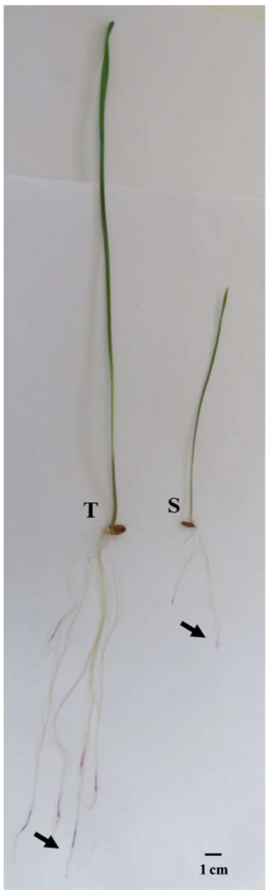


Figure S1. Example of a tolerant (T) (*S. segetale*) and a sensitive (S) (*S. sylvestre*) rye genotype. Arrows shows the root regrowth in the tolerant rye and the lacking regrowth in the sensitive one.

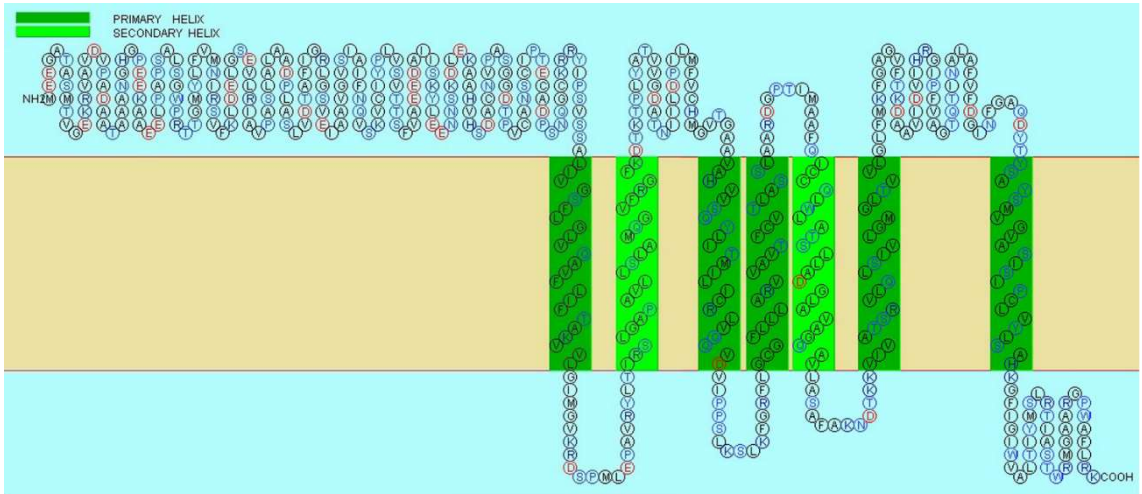


Figure S2. Transmembrane domains of the ScMATE1 proteins predicted by SOSUI software (Hirokawa et al. 1998). This software predicted seven transmembrane domains for all ScMATE1 protein analyzed except for *S. strictum* and *S. sylvestre*, with a prediction of eight transmembrane domains. The synonymous changes (blue circles), the conserved amino acids (yellow circles) and the non-synonymous changes (white circles) detected between the 12 hypothetical proteins analyzed are indicated.

SUBCHAPTER III-2

Biochemical, physiological and genetic analysis of aluminum tolerance of different rye species

E. Santos[1], O. Pinto-Carnide[2], A. M. Figueiras[3], C. Benito[3] and M. Matos[1]

[1]*Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, Quinta de Prados, 5001-801 Vila Real, Portugal;BioISI- Biosystems & Integrative Sciences Institute, University of Lisboa, Faculty of Sciences, Campo Grande, Lisboa, Portugal*

[2]*CITAB -Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal*

[3]*Departamento de Genética, Facultad de Biologia, Universidad Complutense, C/ José Antonio Novais, 12, 28040 Madrid, Spain*

Keywords: Al toxicity; *Secale* spp.; histochemical staining; Al resistance mechanisms; genes expression

*Under review in *Environmental and Experimental Botany* (EEB_2018_874)

Abstract

Aluminum (Al) toxicity is the major limitation for crop productivity in acid soils which are widespread throughout the world. Plant species differ in their responses to this abiotic stress having developed resistance mechanisms to detoxify and tolerate Al both internally and externally. Rye (*Secale cereale* L.) is one of the most Al-tolerant cereal with a valuable genetic background for breeding purposes. Wild relatives (*Secale* spp.) have great importance once they can provide new sources of genes related to this trait. Different cellular disorders possibly associated to Al tolerance/toxicity were observed through histochemical root staining methods in cultivated and wild ryes and a correlation was found. Moreover, expression studies of seven candidate Al-tolerance genes (*ScALMT1*, *ScMATE2*, *ScSTOP1*, *ScMDH1*, *ScMDH2*, *ScCu/ZnSOD* and *ScMnSOD*) were performed in roots and shoots of five wild ryes. All of them seems to have an active contribution on Al resistance mechanisms of these ryes, however, *ScALMT1* and *ScMATE2* genes clearly have a key role in the Al-tolerance increment. Al tolerance in *Secale* genus seems to be a genetically complex trait where different resistance mechanisms coexist, due to several genes whose cumulative effects improves the ability to withstand Al phytotoxicity.

1. Introduction

Acid soils are found throughout the world and their aluminum toxicity represents the major limitation for crop production. Soil acidity is rising due to many factors as soil composition, environmental issues and farming practices (Samac and Tesfaye, 2003; Ma and Ryan, 2010). Aluminum (Al) is a ubiquitous metal being the third most abundant element at Earth's crust which has been documented as the main inhibitor of plant growth in acid soils. When soil pH decreases becoming acidic, Al solubilizes in the soil solution prevailing the phytotoxic form Al^{3+} which rapidly inhibits the growth and function of roots, reducing crop yields (Kochian et al., 2004). The increasing area of arable land affected by acidity is a serious threat in world food production. With a fast growing global population, an increase in crop productivity is needed to support this demand. So, a better understanding of the mechanisms and genes involved in plants Al tolerance is imperative to continue improving crop species better adapted to acid soils.

It is well known that some plants have mechanisms that allow them to tolerate toxic levels of metals and to subsist in acid soils. Two main resistance mechanisms have been proposed: one that exclude Al from the root tips (exclusion mechanisms) and other that enable plants to accommodate Al safely once it enters the symplast (tolerance mechanisms) (Ryan et al., 2010; Kochian et al., 2015).

Several mechanisms of exclusion have been reported with a special focus in the release of organic acids from roots of many plant species in response to Al which are mediated by membrane transporters, resulting in harmless Al-complexes formation (Li et al., 2000; Ma et al., 2001). Genes encoding organic acid transporters have been associated with Al tolerance in plants with emphasis on two gene families that encode Al-activated malate (*ALMT - Aluminum-activated Malate Transporter*) and citrate (*MATE - Multidrug and Toxic compound Extrusion*) transporters localized to the plasma membrane. Both have been identified in several gramineous plants as wheat (Sasaki et al., 2004; Ryan et al., 2009), rye (Fontecha et al., 2007; Silva-Navas et al., 2012), maize (Maron et al., 2010; Ligaba et al., 2012), barley (Furukawa et al., 2007; Gruber et al., 2010), sorghum (Magalhaes et al., 2007), rice (Yokosho et al., 2011, 2016) and Yorkshire fog (*Holcus lanatus*; Chen et al., 2013b). In different studies it was found that Al-induced expression of *ALMT* and *MATE* genes were positively regulated by a transcription factor member of C_2H_2-type zinc-finger family, AtSTOP1 (*Sensitive to Proton rhizotoxicity 1*) in *Arabidopsis* (Iuchi et al., 2007) and OsART1 in rice (Yamaji et al., 2009).

Tolerance mechanisms involves chelation of cytosolic Al by carboxylate anions with the subsequent sequestration into the vacuole and can, as well, minimize oxidative stress and repair Al^{3+}-induced damage (Kochian et al., 2004; Ryan and Delhaize, 2012). As a consequence of Al input at cell level, reactive oxygen species (ROS) such as O_2^-, hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2) are produced and a disorder of plasma membrane stability and function occurs (Yamamoto et al., 2002). Increased ROS production stimulate the development of oxidative stress in roots which can induce genes encoding antioxidant enzymes such as superoxide dismutase (SOD) (Matsumoto and Motoda, 2012). Several isoforms of SOD exists according to their metal prosthetic group (Fe, Cu/Zn and Mn) and subcellular localization (Bowler et al., 1992).

Plant species differ considerably in their ability to tolerate the toxicity of this metal common in acid soils which results in a wide genetic intra- and inter-specific variability to Al tolerance. Among cereals of the *Triticeae* tribe, rye (*Secale cereale* L.) has been described as the most tolerant species (Aniol and Gustafson, 1984; Bona et al., 1993; Pinto-Carnide and Guedes-Pinto, 1999; Kim et al., 2001). Members of the *Poaceae* family have a high agronomic interest being the world´s primary nutritional source whose production has to keep up the demographic growth. Rye comprises an important pool of genetic resources that can be used to improve related species economically significant and more susceptible to this abiotic stress, and thus, contribute to enhance crop yields on acid soils. Wild plant species related to cultivars have a great importance once they can provide new sources of medicines, nutrition and genes (Schoen and Brown, 2001). Therefore, the conservation of genetic resources such as wild plants has been implemented in seed banks to allow his study and to ensure the sustainable provision of food in the long term (FAO, 2018).

Several species/subspecies of the *Secale* genus were studied in this work in order to reach the following objectives: 1) to observe different cellular disorders possibly related to Al tolerance/toxicity through histochemical root staining methods; 2) to study the expression level of several genes possibly involved in Al stress response; 3) to determinate the exudation pattern of malate relating it to gene expression.

2. Material and Methods

2.1. Plant materials

Three wild species/subspecies of the genus *Secale*, *S. strictum* (Persl) Persl ssp. *strictum* (R1211), *S. sylvestre* Host (R892) (autogamous) and *S. vavilovii* Grossh. (PI618682) (autogamous) were analyzed, as well as three subspecies of *Secale cereale* L.: *S. cereale* ssp. *ancestrale* Zhuk. (PI445975), the cultivated *S. cereale* ssp. *cereale* and *S. cereale* ssp. *segetale* Zhuk. (PI326284). Ryes of accessions with codes beginning with "PI" were kindly provided by The National Small Grains Collection (NSGC) of the United States Department of Agriculture - Agricultural Research Service (USDA-ARS) and those with "R" by Genebank Gatersleben, Institute of Plant Genetic and Crop Plant Research (IPK). Within the cultivated taxon the landrace Montalegre (Northern Portugal), the inbred line Riodeva (Spain) and the variety Imperial (USA), from the germplasm bank of the Department of Genetics and Biotechnology at the University of Trás-os-Montes and Alto Douro collection, Vila Real, Portugal, were studied. In order to make it easier, the subspecies designation will be abbreviated in the text by *S. ancestrale*, *S. cereale, S. segetale* and *S. strictum*. The Al-tolerance screening assays and the subsequent Al characterization of plants were described and carried out in Santos et al. (2018).

2.2. Histochemical root staining

The hydroponic method described by Santos et al. (2018) was used for rye seed germination and growth. Seven-day-old seedlings were incubated for 24 h in nutrient solution with 300 µM aluminum in the form of $AlKSO_4 \cdot 12H_2O$ (pH 4.0). Hence, four histochemical root staining methods were carried out. As control, we used barley (*Hordeum vulgare* L.), highly sensitive to Al, and a sample of each rye without Al exposure. A minimum of five individual roots from different seedlings were examined for each assay. Following the protocol described by Yin et al. (2010), two fluorescent dyes were used, Morin and DCF-DA (2',7'-dichlorofluorescein diacetate). The first allowed the visualization of root Al accumulation where root tips were excised, incubated in 5 mM ammonium acetate buffer (NH_4OAc; pH 5.0), stained with this same buffer containing 100 µM Morin and washed with the former. Besides, with the second one, it was possible to observe the H_2O_2 distribution after placing the excised root apices into a solution containing 200 µM $CaCl_2$ (pH 4.4) and 10 µM of DCF-DA. According the methodology used by Yamamoto et al. (2001), cell death was detected by staining root

tips with Evans Blue solution (0.025% [w/v] Evans Blue in 100 µM $CaCl_2$, pH 5.6) which were then washed three times with 100 µM $CaCl_2$. Furthermore, the detection of lipid peroxidation was performed using Schiff's reagent where stained roots were rinsed with a freshly sulphite solution (0.5% [w/v] $K_2S_2O_5$ in 0.05 M HCl) and kept in it until observation. Root tips excised and stained with Morin and DCF-DA were observed under fluorescence microscope (Model CX31, Olympus, Tokyo, Japan) and root tips stained with Evans blue and Schiff's reagent under light stereomicroscope (Model SMZ 168, MOTIC, Barcelona, Spain).

2.3. RNA extraction and cDNA synthesis

Seven-day-old seedlings of the five wild species/subspecies were exposed to Al at 300 µM for 24 h. Root apices (1 cm) and shoots either untreated (0 h) or Al treated (24 h) were collected being immediately frozen in liquid nitrogen. For homogenization, the TisseLyser II (Qiagen, Hilden, Germany) and 5 mm Stainless Steel Beads (Qiagen) were used. Total RNA was extracted from both roots and shoots of about 20 different plants per genotype and per Al exposure time, using TRIzol® kit columns (Invitrogen, California, USA) as described by the supplier. RNA quality was checked by gel electrophoresis and then quantified with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). The first-strand cDNA was synthetized with the "high capacity cDNA reverse transcription kit" (Applied Biosystems, Madrid, Spain) according to the manufacturer's protocol.

2.4. Quantitative RT-PCR

For expression analysis, mRNAs obtained from roots and leaves of each wild rye (0 h and 24 h) were used. Quantitative real time RT-PCR (qRT-PCR) was performed for seven Al tolerance candidate genes: *ScALMT1*, *ScMATE2*, *ScSTOP1*, *ScMDH1* and *ScMDH2* (mitochondrial Malate dehydrogenase), *ScCu/ZnSOD (*Cu and Zn dependent Superoxide dismutase) and *ScMnSOD* (Mn dependent Superoxide dismutase). The list of primer pairs used in this study are shown in the Supplementary Table S1. The ribosomal *18S* gene was used as housekeeping to normalize the results obtained in the assay. The qRT-PCR was carried out in the 7900HT fast real-time PCR system (Applied Biosystems), under the following conditions: 10 min at 95 ºC activation, 40 cycles of 15 sec at 95 ºC and 1 min at 60 ºC. Melting curve analyses were performed to validate the specificity of PCR and

to exclude the interference of primer-dimers and gDNA. All reactions (with samples and controls) were prepared in duplicates in 96-well plates including two standard curves (target gene and endogenous control) in order to apply the relative standard curve method, supported by Applied Biosystems, to analyze the data. In this approach, unknown sample values are interpolated from the standard curves. Duplicated control reactions for every sample without reverse transcription were included to ensure that PCR products were not due to amplification of contaminant genomic DNA.

2.5. Organic acids assay

Citrate efflux was estimated in our group previous work (Santos et al., 2018) and malic acid was determined using enzymatic methods previously described by Gutmann and Wahlefeld (1974) and Delhaize et al. (1993), with some modifications. Malate efflux from intact root seedlings of the five wild species/subspecies and two cultivated ryes (Imperial and Riodeva) was assayed with and without Al. All the procedure was carried out in sterile conditions. Briefly, 20 seeds from each rye were sterilized with a cleaning solution [1 NaClO: 1 ddH_2O] and soaked for 40 min. Then, seeds were washed three times with sterile water, added to flasks (each fifteen) containing 0.2 mM $CaCl_2$ (pH 4.3) and, finally, incubated at 23 °C on a rotary shaker (95 rpm) for 6 days. Thereafter, the solution from flasks was decanted and the seedlings were rinsed three times with the same solution mentioned above. Later, seedlings were incubated at the same previous conditions but in half of them were added 0.05 mM $AlCl_3$ (pH 4.3) to the solution. Root exudates were collected at 3, 6 and 24 h time point and the fresh weight of roots from each pot was measured. All experiments were conducted with two replicates.

2.6. Statistical analysis

Analysis of variance (ANOVA) was performed using the SPSS statistical package for Windows (v. 23.0; IBM Corp., Armonk, NY, USA). Significant differences between means were determined using Duncan test.

3. Results

3.1. Evaluation of Al tolerance with histochemical dyes

In our previous work, we did the Al-tolerance characterization of all the wild and cultivated ryes of this present study by the estimation of the relative root growth (Santos et al., 2018). In short, *S. ancestrale*, *S. segetale*, *S. vavilovii*, Imperial and Montalegre were classified as Al-tolerant, *S. strictum* as moderately Al-tolerant and, finally, *S. sylvestre* and Riodeva as Al-sensitive.

Barley root samples, used as sensitive control, stained intensively with the four histochemical dyes after Al treatment (Fig. 1). On the other hand, any staining was observed in the rye roots not exposed to Al of each assay, used as tolerant control. After Al exposure, *S. sylvestre* and Riodeva roots showed an intense fluorescence with Morin throughout the apices indicative of their high Al accumulation (Fig. 1A). Contrarily, *S. ancestrale*, *S. segetale*, *S. vavilovii*, Imperial and Montalegre roots did not accumulate Al since only a tiny fluorescence was observed in the cap. In turn, *S. strictum* roots evidenced low Al accumulation with higher and lower fluorescence than the tolerant and sensitive ryes, respectively. An intense fluorescence was also observed along the Al-stressed root apices of *S. sylvestre* and Riodeva with DCF-DA indicating strong H_2O_2 production as opposed to what happened in *S. ancestrale*, *S. segetale*, *S. vavilovii*, Imperial and Montalegre roots where this ROS was not produced since no fluorescence was detected (Fig. 1B). In *S. strictum* roots a slight fluorescence was observed being in low stress oxidative. Furthermore, a pale magenta color was observed with Schiff´s reagent over the Al-treated roots of *S. sylvestre* and Riodeva which means that great lipid peroxidation was produced (Fig. 1C). In the other ryes a very faint staining appeared in a small zone of the root apex, the distal part of the transition zone (DTZ) where passage from cell division to cell elongation occurs, being larger in *S. strictum*. Thus, a little amount of lipid peroxidation happened in this specific root region of these ryes. In addition, an extensive blue staining (Evans blue) revealed that wide cell death was induced by Al in the roots of *S. sylvestre* and Riodeva whereas in the other ryes only the DTZ of the roots was poorly affected presenting minor cell death (Fig. 1D).

In all assays, *S. sylvestre* and Riodeva evidenced warped roots with crack formation and behaved according the control barley, although with less intensity, being clearly susceptible to Al toxicity and the most Al-affected ryes. On the other hand, *S. ancestrale*, *S. segetale*, *S. vavilovii* and the *S. cereale* cultivars Imperial and Montalegre were able to

avoid Al toxicity. Finally, most of the *S. strictum* roots analyzed showed an intermediate behavior.

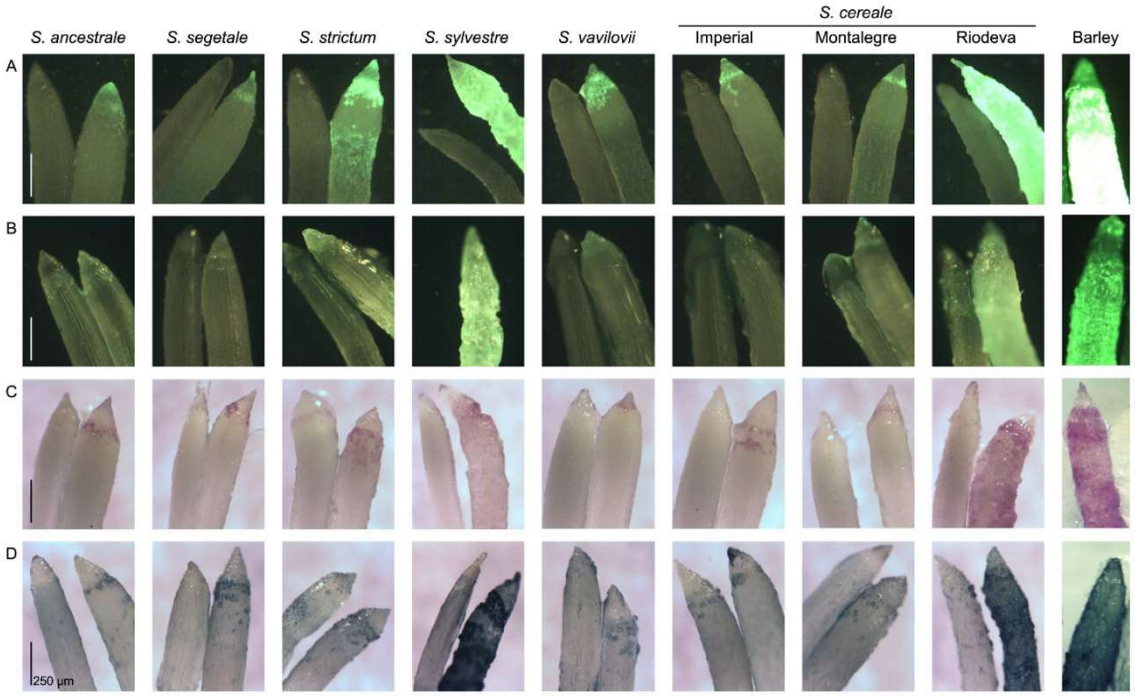


Figure 1. Histochemical root dye techniques in different rye species: A) Al accumulation, B) H_2O_2 production, C) lipid peroxidation and D) cell death. Roots with (right) and without (left) Al treatment were stained with Morin (A), DCF-DA (B), Schiff's reagent (C), and Evans Blue (D). Barley was used as an Al-sensitive control.

3.3. Expression pattern of several Al tolerance candidate genes

Most genes were mainly expressed in roots, either exposed or not to Al, with special emphasis to *ScALMT1* and *ScMATE2* genes (Supplementary Fig. S1). Some exceptions occurred with the genes *ScMDH1* and *ScMnSOD* in *S. strictum*, after 24 h of Al exposure, which expressed around the same level in both tissues. Curiously, *ScSTOP1* was the only gene which expressed about two fold difference (fd) more in shoots than in roots, except for *S. sylvestre* (Supplementary Fig. S1). This species is the only Al-sensitive and presented the least amount of mRNA in roots with all the candidate genes after 24 h of Al contact (Fig. 2). The same occurred in shoots not including the genes *ScALMT1* and *ScMATE2* (Supplementary Fig. S2). The differences of the transcript amount among *S. sylvestre* and the other rye species/subspecies in both tissues (0 and 24 h) were significant in most genes ($P < 0.05$).

Both *ScALMT1* and *ScMATE2* genes were clearly Al-upregulated in the roots of *S. segetale* (2.57 and 3.03 fd relatively to control, respectively), *S. strictum* (3.06 and 4.15 fd) and *S. vavilovii* (1.90 and 4.24 fd) and not in *S. sylvestre* (Fig. 2). In *S. ancestrale* roots only the gene *ScALMT1* presented an induction (2.74 fd). Differences in root expression were significant in *S. ancestrale*, *S. segetale* and *S. strictum* with *ScALMT1* and in *S. vavilovii* with *ScMATE2* ($P < 0.05$). In leaves, *ScALMT1* was Al-induced in all ryes whereas *ScMATE2* expression was constitutive (i.e., both in absence and presence of Al) except in *S. sylvestre* which was quite repressed (Supplementary Fig. S2). The expression of *ScSTOP1* and of the antioxidant genes (*ScCu/ZnSOD* and *ScMnSOD*) had a constitutive pattern in both tissues (Fig. 2 and Supplementary Fig. S2). Furthermore, *ScMDH1* and *ScMDH2* genes expression was constitutive in the roots of *S. ancestrale* and *S. segetale*, and repressed in the remaining, chiefly in *S. sylvestre* that reached almost 3 fd (relatively to control) (Fig. 2). In leaves, the expression of these genes was constitutive in *S. segetale*, *S. strictum* and *S. vavilovii* and downregulated in *S. ancestrale* but especially in *S. sylvestre* (~ 5 fd) (Supplementary Fig. S2). The repression behavior in *S. sylvestre* both in roots as in shoots was statistically significant ($P < 0.05$).

3.4. Malate efflux from intact roots

Root malate secretion (Fig. 3) was clearly induced by Al in all ryes except *S. strictum* which showed a constitutive pattern with significant differences among 3 and 24 h time point ($P < 0.05$). In addition, *S. strictum* exhibited the highest amount of malate exudates after 24 h being in stress along with Imperial whereas *S. segetale* had the least. The malate exudation started after a lag phase of several hours of Al exposure (after 6 h – data not shown) in *S. ancestrale*, *S. sylvestre*, Imperial and Riodeva (Pattern II). Contrarily, *S. segetale* and *S. vavilovii* began to release malate immediately after contact with Al, displaying 5.23 ($P < 0.05$) and 5.73 fd to no treatment at 3 h, respectively (Pattern I). Furthermore, malic acid release increases significantly over time in the presence of Al in most ryes ($P < 0.05$).

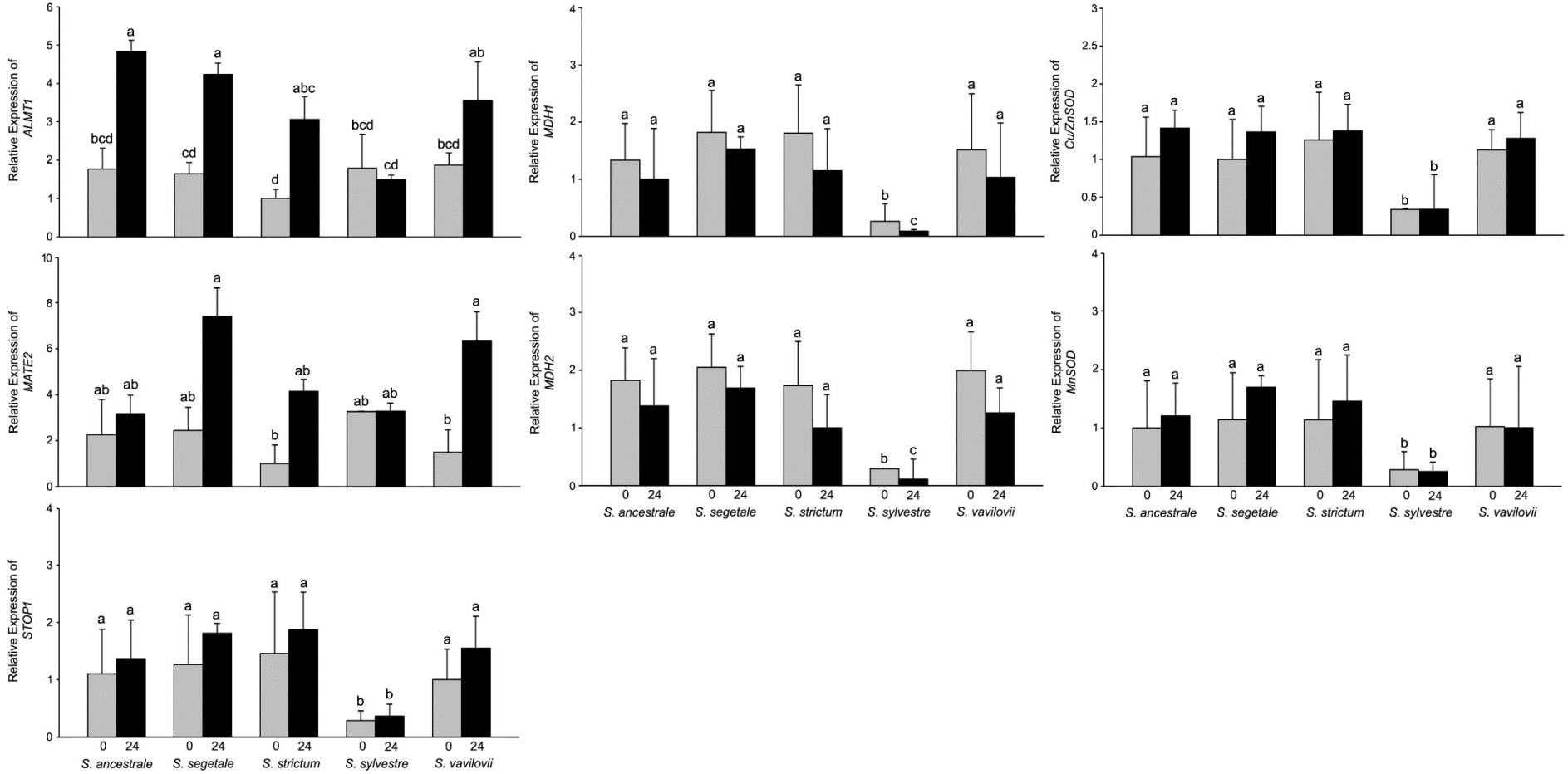


Figure 2. Quantitative RT-PCR showing expression patterns of root tip transcripts of several genes in five wild ryes treated with 300 µM $AlK(SO_4)_2$ (24 h) and without treatment (0 h). Data were normalized to *18S* gene. Change (fold difference) at each time point is expressed as the relative expression compared to the sample with lowest expression. Different lower case letters above bars represent differences between samples by Duncan ($P < 0.05$). The columns represent the mean of three replicates per time point and bars show ±SD.

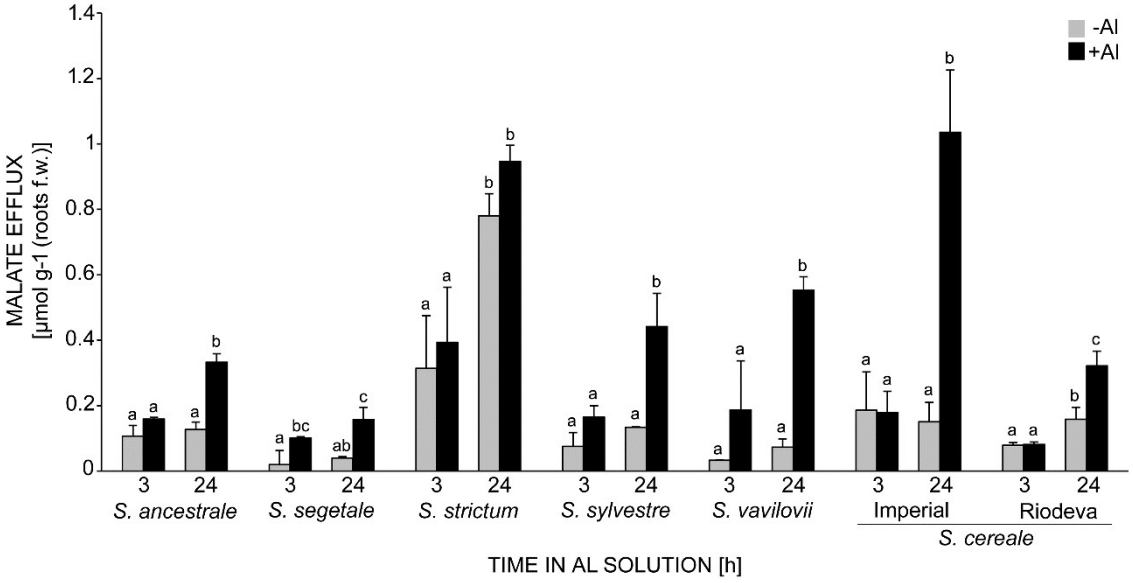


Figure 3. Malate exudation of different rye species. Seedlings were incubated in hydroponic culture with 0 or 50 µM $AlCl_3$. Data of root exudates collected at 3 and 24 h. Data are the means ±SD, $n = 10$. Different lower case letters above bars represent significant differences between treatments and time points ($P < 0.05$) considering the same rye.

4. Discussion

Since roots are the major target for Al toxicity, it is quite obvious that the primary site for Al accumulation is the root apex (root cap, meristem and elongation zone). Al accumulation causes changes in the root cell membrane structure and function, affecting aggregation, fusion and changes in the permeability of liposomes and packaging of fatty acids of the plasma membranes (Inostroza-Blancheteau et al., 2012). Al entry causes various adverse effects such as generation of ROS (Yamamoto et al., 2001, 2002). This cellular process seems to be a determining factor of Al-inhibition of root elongation which, consequently, enhance the level of lipid peroxidation in the root system (Yamamoto et al., 2002). The peroxidation of lipids is a relatively early sign induced by Al accumulation and the most prominent symptom under oxidative stress (Yamamoto et al., 2001). Al-induced ROS production, such as hydrogen peroxide (H_2O_2), and Al-enhanced lipid peroxidation, have the capacity to oxidize cellular compounds, as lipids, proteins, enzymes and nucleic acids, leading to the loss of membrane integrity and, eventually, cell death (Matsumoto and Motoda, 2012). This is a late symptom caused by cracks in the roots due to inhibition of root elongation (Yamamoto et al., 2001, 2002).

All the cellular disturbances observed through the different histochemical root staining performed seems to be correlated (Fig. 1). The Al-sensitive *S. sylvestre* and Riodeva presented the highest rate of root growth inhibition, accumulated more Al on the

root apical meristem and were under oxidative stress (H_2O_2 accumulation) which possibly leaded to high lipid peroxidation formation and, subsequently, cell death. On the other hand, the Al-tolerant ryes (*S. ancestrale*, *S. segetale*, *S. vavilovii*, Imperial and Montalegre) did not accumulate Al in their roots but Al presence was detected in the root tip mainly in the DTZ which was described to be the most active region of Al uptake (Matsumoto and Motoda, 2012). Our results showed that in this particular area Al injury occurs with more intensity being firstly affected as was demonstrated by Sivaguru et al. (2013). Indeed, a minimal amount of both lipid peroxidation and cell death was observed only in this specific region. Moreover, no H_2O_2 accumulation was detected in these rye roots meaning that they were not under oxidative stress since no significant ROS was produced. In turn, *S. strictum* classified as moderately Al-tolerant, always maintained an intermediate behavior with bigger and smaller symptoms than the tolerant and sensitive ryes, respectively. It was described in several works that Al-sensitive genotypes accumulated more aluminum in root tissues than the Al-tolerant ones (Delhaize et al., 1993; Contreras et al., 2014; El-Moneim et al., 2014a; Sánchez-Parra et al., 2014). Furthermore, Al-sensitive plants generated a stronger ROS signal than the tolerant ones in tobacco cells (Devi et al., 2003), *Melaleuca* (Tahara et al., 2008), *Brachypodium* (Contreras et al., 2014) and rye cultivars (El-Moneim et al., 2014a; Sánchez-Parra et al., 2014). Devi et al. (Devi et al., 2003) indicated that a high ROS scavenging ability resulted in enhanced Al-tolerance.

These four histochemical dye methods can be employed to classify Al tolerance of rye genotypes since a great correlation with the estimation of root growth was found. Al-tolerant ryes were able to exclude Al of their root apices and to prevent the formation of stress oxidative in it which suggest both Al-exclusion and internal Al-detoxification mechanisms to overcome Al toxicity.

Plants have developed several resistance mechanisms to raise Al tolerance and detoxification and avoid Al toxicity damages (Fig. 4). Exudation of organic acids (OA) from roots is the most recognized Al resistance mechanism. A strong correlation exists between Al tolerance and Al-activated OA release in numerous plant species (Kochian et al., 2004). Crop species differ in the kind of OA anions, amount and secretion patterns (Ma et al., 2014). Rye secretes both citrate and malate from its roots in response to Al stress and both are able to chelate Al, thereby detoxifying Al in the rhizosphere (Li et al., 2000; Ma et al., 2001; El-Moneim et al., 2014b). *ALMT* and *MATE* genes which encodes OA transporters have been associated with Al tolerance in several plants and both seems

to be regulated by the transcriptional factor STOP1 or ART1 in *Arabidopsis* and rice, respectively (Liu et al., 2009; Sawaki et al., 2009; Yokosho et al., 2011). Other genes involved in the OA metabolism (Krebs cycle) can also be implicated in Al tolerance such as *MDH1* and *MDH2*. Furthermore, internal Al-resistance mechanisms depends partially of many antioxidant enzymes, such as superoxide dismutase (SOD). This enzyme system is the first line of defense against Al-induced ROS production and one of the most effective antioxidant in preventing cellular damage (Alscher et al., 2002). In the present work, estimation of malate exudation and expression studies of these genes were done since no data are known in wild ryes.

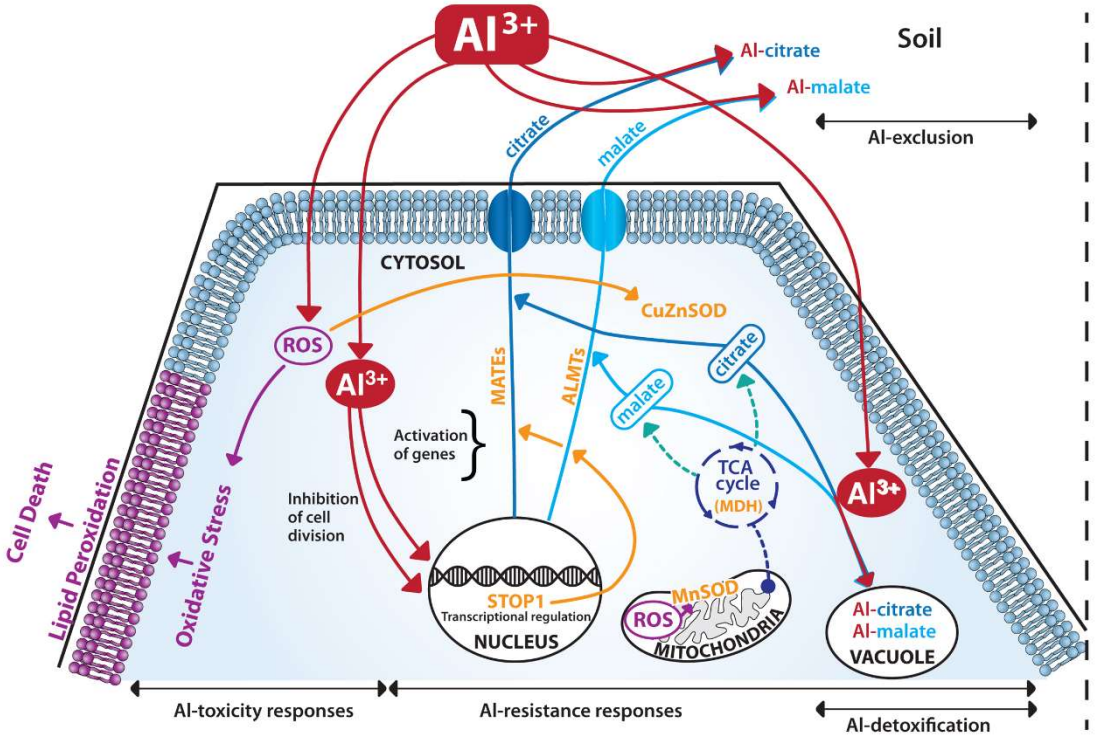


Figure 4. Model illustrating the Al resistance mechanisms proposed: Al exclusion and Al tolerance/detoxification. Genes at study possibly involved in Al-resistance responses are included such as cellular processes implicated in Al-toxicity responses. Adapted from Kochian et al. (2004) with modifications.

Malate exudation was constitutive in *S. strictum* and induced by Al in all the other ryes (Fig. 3). Since the amount of OA exudates varies per plant species (Ma et al., 2014), it is complex to compare the quantities released by the different rye species/subspecies and to relate it with Al tolerance. Ishikawa et al. (2000) did not found a correlation between Al tolerance and the amount of OA efflux of different plant species. Within *S.*

cereale, the tolerant Imperial exuded more malate than the sensitive Riodeva as expected. Based on the timing of malate secretion, rye cultivars have been reported to behave according to Pattern II (Li et al., 2000; El-Moneim et al., 2014a) as we observed in *S. ancestrale*, *S. sylvestre*, Riodeva and Imperial, however, a different pattern (Pattern I) was found in the wild ryes *S. segetale* and *S. vavilovii*. It has been suggested that in Pattern I, Al may just activate a preexisting transporter in the plasma membrane to initiate OA anion secretion, and thus, induction of genes is not required. On the other hand, in Pattern II, Al can induce the expression of genes and the synthesis of proteins involved in both metabolism and transport of OA anions (Ma et al., 2001).

Expression data revealed that all the candidate genes expressed more in roots than in shoots with the exception of *ScSTOP1*. Since the root apex is the critical site for Al toxicity, it is in that region that genes for Al tolerance are likely to be expressed (Delhaize and Ryan, 1995). This first evidence suggests the implication of these genes in the Al tolerance in wild ryes. Most *ALMT* and *MATE* genes are expressed in the root tips of gramineous plants such as wheat (Sasaki et al., 2004; Ryan et al., 2009), barley (Fujii et al., 2012), rye (Fontecha et al., 2007; Collins et al., 2008; Yokosho et al., 2010) and *Brachypodium* (Contreras et al., 2014). Expression in this tissue localization can efficiently detoxify Al externally (Ma et al., 2014). El-Moneim et al. (2014a) and Sánchez-Parra et al. (Sánchez-Parra et al., 2014) reached the same results and conclusion with the two of each *MDH* and *SOD* genes, respectively, in rye cultivars including Riodeva and Imperial.

Both *ScALMT1* and *ScMATE2* genes were Al-upregulated in almost all the tolerant ryes whereas no induction was observed in any of them in the sensitive *S. sylvestre* (Fig. 2). Also the number of transcripts after 24 h of Al exposure was lower in *S. sylvestre* except for the gene *ScMATE2* in *S. ancestrale*. Both genes seems to be a source of greater Al tolerance in rye. The lowest (1.90 fd) and highest (4.24 fd) upregulation rates of, respectively, *ScALMT1* and *ScMATE2* genes were observed in *S. vavilovii*. This species displayed a Pattern I in malate release and II in citrate (Santos et al., 2018), meaning that *S. vavilovii* did not require the expression of *ScALMT1* but needed the *ScMATE2* expression ($P < 0.05$) to enhance Al tolerance. *S. segetale* exhibited a Pattern I in both malic and citric acid exudation and both genes were Al-induced, moreover, in our former work (Santos et al., 2018) *ScMATE1* gene was slightly induced. This subspecies reached the lowest amount of both OA but great differences to no treatment at each time point were observed ($P < 0.05$). Even with a Pattern I OA release, *S. segetale* seems to need

genes induction for Al-enhanced tolerance. Likewise, *S. ancestrale* exuded low quantity of both OA but with significant differences with control at 24 h time exposure ($P < 0.05$) being the secretion of both OA increasingly induced by Al over time ($P < 0.05$). A Pattern II was observed in both OA but only *ScALMT1* was induced ($P < 0.05$) contributing to the raise of Al-tolerance of this subspecies. Another gene involved in the metabolism or transport of citric acid might be related to Al tolerance of *S. ancestrale*. Contrarily, *S. strictum* showed a constitutive pattern of both malate and citrate exudation (Santos et al., 2018) and exhibited the biggest amount of both OA. Moreover, a great induction was observed with both *ScALMT1* and *ScMATE2* genes. All of this suggests the involvement of both malate and citrate pathways to increase Al resistance in this subspecies. Taken together, *ScALMT1* can be involved in the malate exudation of *S. ancestrale*, *S. segetale* and *S. strictum* while *ScMATE2* can be involved in the citrate exudation of *S. segetale*, *S. strictum* and *S. vavilovii*.

In Fontecha et al. (2007), *ScALMT1* gene transcripts were induced by the presence of Al and were more abundant in the tolerant rye cultivar than the sensitive inbred line Riodeva. Likewise, in Collins et al. (2008) *ScALMT1* expression of root rye cultivars was upregulated by Al. Homolog genes from wheat (Sasaki et al., 2004), *Arabidopsis* (Hoekenga et al., 2006), rape (Ligaba et al., 2006), soybean (Liang et al., 2013), *Medicago sativa* (Chen et al., 2013a) and *Holcus lanatus* (Chen et al., 2013b) were also involved in Al-tolerance responses mediated by malate exudation. On the other hand, *ScFRDL2* (*ScMATE2*) gene expression of a rye inbred line roots was greatly Al-upregulated being involved in Al-induced secretion of citrate in Yokosho et al. (2010). Also in rice, *OsFRDL2* is involved in the Al-induced secretion of citrate but with poor contribution to high Al tolerance (Yokosho et al., 2016). Contrarily, *ZmMATE2* may not mediate root citrate exudation but it is implicated in Al tolerance in maize (Maron et al., 2010). As *ALMTs* and *MATEs* both confer Al resistance via root OA release, this appears to be a striking example of functional coevolution of Al tolerance by two transporters that are structurally and functionally quite different (Kochian et al., 2015).

We have seen that the amount of OA exudates is a quantitative trait that is controlled by *ALMT* and *MATE* but it can also be by MDH in the case of malate. Both *ScMDH1* and *ScMDH2* revealed much more mRNA in the tolerant ryes than the sensitive *S. sylvestre*, either with or without Al exposure ($P < 0.05$), which reinforce their involvement in Al tolerance (Fig. 2). Besides, a clear and significant repression was observed with both genes in *S. sylvestre* ($P < 0.05$). El-Moneim et al. (2014a) obtained the same results and

suggested that this repression could be indirectly related to the exudation of malate since Al induced its release. On the other hand, expression of both *ScMDH1* and *ScMDH2* genes was constitutive in *S. ancestrale* and *S. segetale* meaning that they also have a role in their strategy for Al tolerance increase. Previous works analyzed the enzymatic activity of MDH in roots of wheat and rye cultivars and no changes were observed in either species, suggesting that this enzyme was not affected by Al (Ryan et al., 1995; Li et al., 2000). Taking into account that there are active isoforms of MDH in some organelles and cytosol, it is very difficult to establish a correlation between MDH activities and the expression of a specific MDH mRNA (El-Moneim et al., 2014a).

Al-induced expression of *ALMT* and *MATE* genes was reported to be mediated by the transcription factor STOP1. Curiously, in this work *ScSTOP1* gene expressed more in shoots than in roots in all the tolerant ryes whereas the sensitive one (*S. sylvestre*) showed an equal expression level in both tissues without Al and a higher amount in roots after Al treatment. This suggests an Al tolerance mechanism acting in shoots in the case of this regulatory gene. Moreover, expression in both tissues was constitutive in all ryes with much less amount of transcripts in *S. sylvestre* ($P < 0.05$) demonstrating a role in Al-tolerance in these ryes (Fig. 2). A constitutive expression pattern indicates that the involvement of this transcription factor in the Al induction of gene expression must involve posttranslational processes (Kochian et al., 2015). Likewise, *AtSTOP1* presented a constitutive expression pattern in *Arabidopsis* in the work of Iuchi et al. (2007). These authors suggested that Al exposure may lead to AtSTOP1 phosphorylation which activates it to participate in the transcription of Al tolerance genes such as *AtALMT1*. This transcription factor was studied in two other species of the Poaceae family, *Holcus lanatus* (Chen et al., 2013b) and *Triticum aestivum* (Garcia-Oliveira et al., 2013). In the first, *HlALMT1* gene expression was enhanced increasing number of cis-acting elements for ART1 in the promoter region. In the second, a time-dependent Al-responsive expression of TaSTOP1 homologues observed in the root tissues of a tolerant and a sensitive wheat genotype suggested a putative role for TaSTOP1 in Al tolerance. AtSTOP2 is a homolog of AtSTOP1 identified in *Arabidopsis*, which may partner with AtSTOP1 in regulating the expression of some of the Al and low-pH resistance genes (Kobayashi et al., 2014). As we could observe, crop species differ in how gene expression is regulated being difficult to reach a conclusion; further research is required to understand the specific role of the *ScSTOP1* in the *Secale* genus.

The antioxidant genes of this study also seems to have an active role in the Al-tolerance of these wild ryes. *ScCu/ZnSOD* and *ScMnSOD* could possibly take part on the Al-detoxification of the tolerant ryes allowing ROS production circumventing, and therefore, enhancing Al tolerance. So, both genes can contribute to suppress the lipid peroxidation of these ryes caused by H_2O_2 thus decreasing oxidative damages as observed with the histochemical root dyes. The little mRNA amount of *S. sylvestre* compared to the other ryes ($P < 0.05$) and the constitutive expression of all of them strengthens this theory (Fig. 2). In Sánchez-Parra et al. (2014) studies, a higher expression of *ScCu/ZnSOD* and *ScMnSOD* genes was observed in the tolerant rye cultivar roots compared to the sensitive line Riodeva. In turn, Richards et al. (1998) found a link between Al and oxidative stress where *Cu/ZnSOD* gene was induced by Al stress in *Arabidopsis*. An association between Al tolerance and SOD enzyme activity was found in rye cultivars (Silva et al., 2013) and soybean (Du et al., 2010) where tolerant plants exhibited a larger SOD activity after Al exposure than sensitives. The correlation between the transcript amount of *SOD* genes and SOD enzyme activity is hard to establish because of the different existing isoforms (Cu/Zn, Mn and Fe) (Sánchez-Parra et al., 2014).

All these candidate Al-tolerance genes seems to have an active contribution on the Al resistance mechanisms of these rye species/subspecies. However, *ScALMT1* and *ScMATE2* genes clearly have a key role in the increment of Al tolerance. On the other hand, *S. sylvestre* proceeded always in the same way than other sensitive plants in all the genes characterized emphasizing his sensitivity feature to Al toxicity.

The struggle in studying Al-related processes in plants can be attributed to the complex chemistry of this metal (Delhaize and Ryan, 1995). Some plants appear to display one type of mechanism only (barley) but others display resistance mechanisms (exclusion and tolerance) which may be additive (*Arabidopsis*, buckwheat and rice) (Ryan and Delhaize, 2010). It becomes clear that species having multiple Al-tolerance genes are more tolerant to Al than species having single tolerance gene (Ma et al., 2014). In rye, Al tolerance seems to be a genetically complex trait where different resistance mechanisms coexist. Thus, the high Al tolerance that characterize cultivated and wild ryes may be due to several genes whose cumulative effects allow to a better Al toxicity circumventing.

Al tolerance is unlikely to have been an early trait for plants that did not evolve on acidic environments (Ryan and Delhaize, 2010). Crop species would have evolved after acid soils exposition to overtake this abiotic adversity. It has been demonstrated that *S.*

sylvestre, among the other rye species/subspecies, was the most ancient species that split off first from the common ancestor and so, phylogenetically, the most divergent rye (Chikmawati et al., 2005; Shang et al., 2006 Santos et al., 2018). It is possible that this Al-susceptible wild species still possesses ancestral mechanisms and the other rye species/subspecies generally tolerant have evolved adapting to acid soils with efficient Al resistance mechanisms. Interestingly, *S. strictum* that was classified as moderately tolerant, separate from the rest soon after *S. sylvestre* (Chikmawati et al., 2005; Shang et al., 2006; Santos et al., 2016, 2018).

5. Conclusion

Research on this thematic is pertinent for crop breeding programs to keep up with the increase food demand in nearby future. *Secale* genus owns a vast genetic diversity which provides wide variety of Al-tolerance genes that can be explored in order to clarify the Al-resistance mechanisms involved. In this work, seven candidate genes (*ScALMT1*, *ScMATE2*, *ScSTOP1*, *ScMDH1*, *ScMDH2*, *ScCu/ZnSOD* and *ScMnSOD*) seems to have an active contribution on Al tolerance improvement in different ways in each rye species/subspecies. Clear evidences were found for an Al-exclusion mechanism complemented by an internal Al-detoxification mechanism in the tolerant ryes. OA release seems to be implicated in the Al tolerance of these ryes but other mechanisms can also be acting, especially in *S. ancestrale* and *S. segetale* which exuded a little OA amount. The search of new candidate genes and the study of the regulatory processes implicated are crucial for a better understanding of this approach.

Acknowledgments

This work was supported by research PhD grant from Fundação para a Ciência e Tecnologia de Portugal (SFRH/BD/65040/2009). We are grateful to the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) and to The National Small Grains Collection (NSGC) of the United States Department of Agriculture - Agricultural Research Service (USDA-ARS) for supplying us with seeds

References

Alscher, R. G., Erturk, N., and Heath, L. S. (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J. Exp. Bot.* 53, 1331–1341.

doi:10.1093/jexbot/53.372.1331.

Aniol, A., and Gustafson, J. P. (1984). Chromosome location of genes controlling aluminium tolerance in wheat, rye and triticale. *Can. J. Genet. Cytol.* 26, 701–705.

Bona, L., Wright, R. J., Baligar, V. C., and Matuz, J. (1993). Screening wheat and other small grains for acid soil tolerance. *Landsc. Urban Plan.* 27, 175–178. doi:10.1016/0169-2046(93)90046-G.

Bowler, C., Montagu, M. Van, and Inze, D. (1992). Superoxide dismutase and stress tolerance. *Annu. Rev. Plant Biol.* 34, 83–116. doi:10.1146/annurev.pp.43.060192.000503.

Chen, Q., Wu, K. H., Wang, P., Yi, J., Li, K. Z., and Yu, Y. X. (2013a). Overexpression of *MsALMT1*, from the aluminum-sensitive *Medicago sativa* enhances malate exudation and aluminum resistance in tobacco. *Plant Mol. Biol. Rep.* 31, 769–774. doi:10.1007/s11105-012-0543-2.

Chen, Z. C., Yokosho, K., Kashino, M., Zhao, F. J., Yamaji, N., and Ma, J. F. (2013b). Adaptation to acidic soil is achieved by increased numbers of cis-acting elements regulating *ALMT1* expression in *Holcus lanatus*. *Plant J.* 76, 10–23. doi:10.1111/tpj.12266.

Chikmawati, T., Skovmand, B., and Gustafson, J. P. (2005). Phylogenetic relationships among *Secale* species revealed by amplified fragment length polymorphisms. *Genome* 48, 792–801. doi:10.1139/g05-043.

Collins, N. C., Shirley, N. J., Saeed, M., Pallotta, M., and Gustafson, J. P. (2008). An *ALMT1* gene cluster controlling aluminum tolerance at the *Alt4* locus of rye (*Secale cereale* L.). *Genetics* 179, 669–682. doi:10.1534/genetics.107.083451.

Contreras, R., Figueiras, A. M., Gallego, F. J., and Benito, C. (2014). *Brachypodium distachyon*: A model species for aluminium tolerance in Poaceae. *Funct. Plant Biol.* 41, 1270–1283. doi:10.1071/FP13362.

Delhaize, E., and Ryan, P. R. (1995). Aluminum Toxicity and Tolerance in Plants. *Plant Physiol* 107, 315–321.

Delhaize, E., Ryan, P. R., and Randall, P. J. (1993). Aluminum Tolerance in Wheat (*Triticum aestivum* L.) (II. Aluminum-Stimulated Excretion of Malic Acid from Root Apices). *Plant Physiol* 103, 695–702. doi:103/3/695 [pii].

Devi, S. R., Yamamoto, Y., and Matsumoto, H. (2003). An intracellular mechanism of aluminum tolerance associated with high antioxidant status in cultured tobacco cells. *J. Inorg. Biochem.* 97, 59–68. doi:10.1016/S0162-0134(03)00182-X.

Du, B., Nian, H., Zhang, Z., and Yang, C. (2010). Effects of aluminum on superoxide dismutase and peroxidase activities, and lipid peroxidation in the roots and calluses of soybeans differing in aluminum tolerance. *Acta Physiol. Plant.* 32, 883–890. doi:10.1007/s11738-010-0476-z.

El-Moneim, D. A., Contreras, R., Silva-Navas, J., Gallego, F. J., Figueiras, A. M., and Benito, C. (2014a). On the consequences of aluminium stress in rye: Repression of two mitochondrial malate dehydrogenase mRNAs. *Plant Biol.* 17, 123–133. doi:10.1111/plb.12219.

El-Moneim, D. A., Contreras, R., Silva-Navas, J., Gallego, F. J., Figueiras, A. M., and Benito, C. (2014b). Pectin methylesterase gene and aluminum tolerance in *Secale* cereale. *Environ. Exp. Bot.* 107, 125–133. doi:10.1016/j.envexpbot.2014.06.006.

Fontecha, G., Silva-Navas, J., Benito, C., Mestres, M. A., Espino, F. J., Hernández-Riquer, M. V., et al. (2007). Candidate gene identification of an aluminum-activated

organic acid transporter gene at the *Alt4* locus for aluminum tolerance in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 114, 249–260. doi:10.1007/s00122-006-0427-7.

Fujii, M., Yokosho, K., Yamaji, N., Saisho, D., Yamane, M., Takahashi, H., et al. (2012). Acquisition of aluminium tolerance by modification of a single gene in barley. *Nat. Commun.* 3, 713. doi:10.1038/ncomms1726.

Furukawa, J., Yamaji, N., Wang, H., Mitani, N., Murata, Y., Sato, K., et al. (2007). An aluminum-activated citrate transporter in barley. *Plant Cell Physiol.* 48, 1081–1091.

Garcia-Oliveira, A. L., Benito, C., Prieto, P., de Andrade Menezes, R., Rodrigues-Pousada, C., Guedes-Pinto, H., et al. (2013). Molecular characterization of *TaSTOP1* homoeologues and their response to aluminium and proton (H^+) toxicity in bread wheat (*Triticum aestivum* L.). *BMC Plant Biol.* 13, 134. doi:10.1186/1471-2229-13-134.

Gruber, B., Ryan, P., Richardson, A., Tyerman, S., Ramesh, S., Hebb, D., et al. (2010). *HvALMT1* from barley is involved in the transport of organic anions. *J Exp Bot* 61, 1455–1467.

Gutmann, I., and Wahlefeld, A. (1974). "L-malate: determination with malate dehydrogenase and NAD.," in *Methods of enzymatic analysis*, eds. H. Bergmeyer and K. Gawehn (New York: Academic Press), 1585–1589.

Hoekenga, O. A., Maron, L. G., Pineros, M. A., Cancado, G. M. A., Shaff, J., Kobayashi, Y., et al. (2006). *AtALMT1*, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in *Arabidopsis*. *Proc. Natl. Acad. Sci.* 103, 9738–9743. doi:10.1073/pnas.0602868103.

Inostroza-Blancheteau, C., Renge, Z., Alberdi, M., De La Luz Mora, M., Aquea, F., Arce-Johnson, P., et al. (2012). Molecular and physiological strategies to increase aluminum resistance in plants. *Mol. Biol. Rep.* 39, 2069–2079. doi:10.1007/s11033-011-0954-4.

Ishikawa, S., Wagatsuma, T., Sasaki, R., and Ofei-Manu, P. (2000). Comparison of the amount of citric and malic acids in Al media of seven plant species and two cultivars each in five plant species. *Soil Sci. Plant Nutr.* 46, 751–758.

Iuchi, S., Koyama, H., Iuchi, A., Kobayashi, Y., Kitabayashi, S., Kobayashi, Y., et al. (2007). Zinc finger protein STOP1 is critical for proton tolerance in *Arabidopsis* and coregulates a key gene in aluminum tolerance. *Proc. Natl. Acad. Sci.* 104, 9900–9905. doi:10.1073/pnas.0700117104.

Kim, B. Y., Baier, A. C., Somers, D. J., and Gustafson, J. P. (2001). Aluminium tolerance in triticale, wheat and rye. *Euphytica* 120, 329–337.

Kobayashi, Y., Ohyama, Y., Kobayashi, Y., Ito, H., Iuchi, S., Fujita, M., et al. (2014). STOP2 activates transcription of several genes for Al- and low pH-tolerance that are regulated by STOP1 in *Arabidopsis*. *Mol. Plant* 7, 311–322. doi:10.1093/mp/sst116.

Kochian, L. V., Hoekenga, O. A., and Piñeros, M. A. (2004). How Do Crop Plants Tolerate Acid Soils? Mechanisms of Aluminum Tolerance and Phosphorous Efficiency. *Annu. Rev. Plant Biol.* 55, 459–493. doi:10.1146/annurev.arplant.55.031903.141655.

Kochian, L. V., Piñeros, M. A., Liu, J., and Magalhaes, J. V. (2015). Plant Adaptation to Acid Soils: The Molecular Basis for Crop Aluminum Resistance. *Annu. Rev. Plant Biol.* 66, 571–598. doi:10.1146/annurev-arplant-043014-114822.

Li, X. F., Ma, J. F., and Matsumoto, H. (2000). Pattern of aluminum-induced secretion of organic acids differs between rye and wheat. *Plant Physiol.* 123, 1537–1544.

doi:10.1104/pp.123.4.1537.

Liang, C., Piñeros, M. A., Tian, J., Yao, Z., Sun, L., Liu, J., et al. (2013). Low pH, aluminum, and phosphorus coordinately regulate malate exudation through *GmALMT1* to improve soybean adaptation to acid soils. *Plant Physiol.* 161, 1347–1361. doi:10.1104/pp.112.208934.

Ligaba, A., Katsuhara, M., Ryan, P., Shibasaka, M., and Matsumoto, H. (2006). The *BnALMT1* and *BnALMT2* genes from rape encode aluminum-activated malate transporters that enhance the aluminum resistance of plant cells. *Plant Physiol.* 142, 1294–1303.

Ligaba, A., Maron, L., Shaff, J., Kochian, L., and Piñeros, M. (2012). Maize ZmALMT2 is a root anion transporter that mediates constitutive root malate efflux. *Plant Cell Env.* 35, 1185–1200.

Liu, J., Magalhaes, J. V., Shaff, J., and Kochian, L. V. (2009). Aluminum-activated citrate and malate transporters from the *MATE* and *ALMT* families function independently to confer *Arabidopsis* aluminum tolerance. *Plant J.* 57, 389–399. doi:10.1111/j.1365-313X.2008.03696.x.

Ma, J. F., Chen, Z. C., and Shen, R. F. (2014). Molecular mechanisms of Al tolerance in gramineous plants. *Plant Soil* 381, 1–12. doi:10.1007/s11104-014-2073-1.

Ma, J. F., and Ryan, P. R. (2010). Foreword: Understanding how plants cope with acid soils. *Funct. Plant Biol.* 37, 2008–2011. doi:10.1071/FPv37n4-FO.

Ma, J. F., Ryan, P. R., and Delhaize, E. (2001). Aluminium tolerance in plants and the complexing role of organic acids. *Trends Plant Sci.* 6, 273–278. doi:10.1016/S1360-1385(01)01961-6.

Magalhaes, J. V, Liu, J., Guimarães, C. T., Lana, U. G. P., Alves, V. M. C., Wang, Y.-H., et al. (2007). A gene in the multidrug and toxic compound extrusion (*MATE*) family confers aluminum tolerance in sorghum. *Nat. Genet.* 39, 1156–1161. doi:10.1038/ng2074.

Maron, L. G., Piñeros, M. A., Guimarães, C. T., Magalhaes, J. V., Pleiman, J. K., Mao, C., et al. (2010). Two functionally distinct members of the *MATE* (multi-drug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. *Plant J.* 61, 728–740. doi:10.1111/j.1365-313X.2009.04103.x.

Matsumoto, H., and Motoda, H. (2012). Aluminum toxicity recovery processes in root apices. Possible association with oxidative stress. *Plant Sci.* 185–186, 1–8. doi:10.1016/j.plantsci.2011.07.019.

Pinto-Carnide, O., and Guedes-Pinto, H. (1999). Aluminium tolerance variability in rye and wheat Portuguese germplasm. *Genet. Resour. Crop Ev.* 46, 81–85.

Richards, K. D., Schott, E. J., Sharma, Y. K., Davis, K. R., and Gardner, R. C. (1998). Aluminum Induces Oxidative Stress Genes in *Arabidopsis thaliana*. *Plant Physiol.* 116, 409–418.

Ryan, P., Delhaize, E., and Randall, P. (1995). Malate efflux from root apices and tolerance aluminum are highly correlated in wheat. *Aust. J. Plant Physiol.* 22, 531 – 536.

Ryan, P. R., and Delhaize, E. (2010). The convergent evolution of aluminium resistance in plants exploits a convenient currency. *Funct. Plant Biol.* 37, 275–284. doi:10.1071/FP09261.

Ryan, P. R., and Delhaize, E. (2012). "Adaptations to Aluminum Toxicity," in *Plant*

Stress Physiology, ed. S. Shabala (Boston, MA, USA: CAB International), 171–193.

Ryan, P. R., Raman, H., Gupta, S., Horst, W. J., and Delhaize, E. (2009). A Second Mechanism for Aluminum Resistance in Wheat Relies on the Constitutive Efflux of Citrate from Roots. *Plant Physiol.* 149, 340–351. doi:10.1104/pp.108.129155.

Ryan, P. R., Tyerman, S. D., Sasaki, T., Furuichi, T., Yamamoto, Y., Zhang, W. H., et al. (2010). The identification of aluminium-resistance genes provides opportunities for enhancing crop production on acid soils. *J. Exp. Bot.* 62, 9–20. doi:10.1093/jxb/erq272.

Samac, D. A., and Tesfaye, M. (2003). Plant improvement for tolerance to aluminum in acid soils – a review. *Plant Cell. Tissue Organ Cult.* 75, 189–207.

Sánchez-Parra, B., Figueiras, A. M., El-Moneim, D. A., Contreras, R., Rouco, R., Gallego, F. J., et al. (2014). The role of two superoxide dismutase mRNAs in rye aluminium tolerance. *Plant Biol.* 17, 694–702. doi:10.1111/plb.12281.

Santos, E., Benito, C., Silva-Navas, J., Gallego, F. J., Figueiras, A. M., Pinto-Carnide, O., et al. (2018). Characterization, genetic diversity, phylogenetic relationships, and expression of the aluminum tolerance *MATE1* gene in *Secale* species. *Biol. Plant.* 62, 109–120. doi:10.1007/s10535-017-0749-0.

Santos, E., Matos, M., Silva, P., Figueiras, A. M., Benito, C., and Pinto-Carnide, O. (2016). Molecular diversity and genetic relationships in *Secale*. *J. Genet.* 95, 273–281. doi:10.1007/s12041-016-0632-3.

Sasaki, T., Yamamoto, Y., Ezaki, B., Katsuhara, M., Ahn, S. J., Ryan, P. R., et al. (2004). A wheat gene encoding an aluminium-activated malate transporter. *Plant J.* 37, 645–653.

Sawaki, Y., Iuchi, S., Kobayashi, Y., Kobayashi, Y., Ikka, T., Sakurai, N., et al. (2009). STOP1 Regulates Multiple Genes That Protect *Arabidopsis* from Proton and Aluminum Toxicities. *Plant Physiol.* 150, 281–294. doi:10.1104/pp.108.134700.

Schoen, D. J., and Brown, A. H. D. (2001). The Conservation of Wild Plant Species in Seed Banks. *Bioscience* 51, 960. doi:10.1641/0006-3568(2001)051[0960:TCOWPS]2.0.CO;2.

Shang, H. Y., Wei, Y. M., Wang, X. R., and Zheng, Y. L. (2006). Genetic diversity and phylogenetic relationships in the rye genus *Secale* L. (rye) based on *Secale cereale* microsatellite markers. *Genet. Mol. Biol.* 29, 685–691. doi:10.1590/S1415-47572006000400018.

Silva-Navas, J., Benito, C., Téllez-Robledo, B., Abd El-Moneim, D., and Gallego, F. J. (2012). The *ScAACT1* gene at the *Qalt5* locus as a candidate for increased aluminum tolerance in rye (*Secale cereale* L.). *Mol. Breed.* 30, 845–856. doi:10.1007/s11032-011-9668-5.

Silva, S., Pinto, G., Correia, B., Pinto-Carnide, O., and Santos, C. (2013). Rye oxidative stress under long term Al exposure. *J. Plant Physiol.* 170, 879–889. doi:10.1016/j.jplph.2013.01.015.

Sivaguru, M., Liu, J., and Kochian, L. V. (2013). Targeted expression of *SbMATE* in the root distal transition zone is responsible for sorghum aluminum resistance. *Plant J.* 76, 297–307. doi:10.1111/tpj.12290.

Tahara, K., Yamanoshita, T., Norisada, M., Hasegawa, I., Kashima, H., Sasaki, S., et al. (2008). Aluminium distribution and reactive oxygen species accumulation in root tips of two *Melaleuca* trees differing in aluminium resistance. *Plant Soil* 307, 167–178. doi:10.1007/s11104-008-9593-5.

Yamaji, N., Huang, C. F., Nagao, S., Yano, M., Sato, Y., Nagamura, Y., et al. (2009). A Zinc Finger Transcription Factor ART1 Regulates Multiple Genes Implicated in Aluminum Tolerance in Rice. *Plant Cell* 21, 3339–3349. doi:10.1105/tpc.109.070771.

Yamamoto, Y., Kobayashi, Y., Devi, S. R., Rikiishi, S., and Matsumoto, H. (2002). Aluminum Toxicity Is Associated with Mitochondrial Dysfunction and the Production of Reactive Oxygen Species in Plant Cells 1. *Plant Physiol.* 128, 63–72. doi:10.1104/pp.010417.1.

Yamamoto, Y., Kobayashi, Y., and Matsumoto, H. (2001). Lipid Peroxidation Is an Early Symptom Triggered by Aluminum, But Not the Primary Cause of Elongation Inhibition in Pea Roots. *Plant Physiol.* 125, 199–208. doi:10.1104/pp.125.1.199.

Yin, L., Mano, J., Wang, S., Tsuji, W., and Tanaka, K. (2010). The Involvement of Lipid Peroxide-Derived Aldehydes in Aluminum Toxicity of Tobacco Roots. *Plant Physiol.* 152, 1406–1417. doi:10.1104/pp.109.151449.

Yokosho, K., Yamaji, N., Fujii-Kashino, M., and Ma, J. F. (2016). Functional Analysis of a MATE Gene *OsFRDL2* Revealed its Involvement in Al-Induced Secretion of Citrate, but a Lower Contribution to Al Tolerance in Rice. *Plant Cell Physiol.* 57, 976–985. doi:10.1093/pcp/pcw026.

Yokosho, K., Yamaji, N., and Ma, J. F. (2010). Isolation and characterisation of two *MATE* genes in rye. *Funct. Plant Biol.* 37, 296–303. doi:10.1071/FP09265.

Yokosho, K., Yamaji, N., and Ma, J. F. (2011). An Al-inducible *MATE* gene is involved in external detoxification of Al in rice. *Plant J.* 68, 1061–1069. doi:10.1111/j.1365-313X.2011.04757.x.

Supplementary data

Table S1. Primers used for qPCR experiments with each candidate gene at study.

Primer	Sequence (5' → 3')	References
18S-F	TCAACGAGGAATGCCTAGTAAGC	Fontecha et al., (2007)
18S-R	ACAAAGGGCAGGGACGTAGTC	Fontecha et al. (2007)
ScALMT1-F	GCGGCTTTGTTGCAAGTGA	Fontecha et al. (2007)
ScALMT1-R	TCAACCAAGTCCGCGAGAAG	Fontecha et al. (2007)
ScMATE2-F	GCTGCATTCCAGATTTGCTTGC	Yokosho et al. (2010)
ScMATE2-R	GAGAAGCCCCAAGATCAATCC	Yokosho et al. (2010)
ScSTOP1-F	CTGGCTACCCCCCATTCTTT	Silva-Navas (2016)
ScSTOP1-R	CTGTGGAGGATTCGGTTCAAA	Silva-Navas (2016)
ScMDH1-F	GCGCCCTCGTGAAGGGGTTC	El-Moneim et al. (2014)
ScMDH1-R	GTGCAGAGGCCCTTGACGAT	El-Moneim et al. (2014)
ScMDH2 -F	CCTGCCCTGGTCAAGGGTTT	El-Moneim et al. (2014)
ScMDH2-R	CCGGCGTTAATGTTGAAGAG	El-Moneim et al. (2014)
ScCu/ZnSOD-F	TGCACATGACCAGCGGGGTTG	Sánchez-Parra et al. (2014)
ScCu/ZnSOD-R	GAGGGAGATGGCCCGACCACC	Sánchez-Parra et al. (2014)
ScMnSOD-F	ACCAAAATCCTCATCAATGGC	Sánchez-Parra et al. (2014)
ScMnSOD-R	AAGTTCAACGGCGGCGGTCAT	Sánchez-Parra et al. (2014)

References

El-Moneim, D. A., Contreras, R., Silva-Navas, J., Gallego, F. J., Figueiras, A. M., Benito, C. (2014). On the consequences of aluminium stress in rye: Repression of two mitochondrial malate dehydrogenase mRNAs. *Plant Biol.* 17, 123–133. https://doi.org/10.1111/plb.12219

Fontecha, G., Silva-Navas, J., Benito, C., Mestres, M. A., Espino, F. J., Hernández-Riquer, M. V., Gallego, F. J. (2007). Candidate gene identification of an aluminum-activated organic acid transporter gene at the *Alt4* locus for aluminum tolerance in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 114, 249–260. https://doi.org/10.1007/s00122-006-0427-7

Sánchez-Parra, B., Figueiras, A. M., El-Moneim, D. A., Contreras, R., Rouco, R., Gallego, F. J., Benito, C. (2014). The role of two superoxide dismutase mRNAs in rye aluminium tolerance. *Plant Biol.* 17, 694–702. https://doi.org/10.1111/plb.12281

Silva-Navas, J. (2016). Modulación del sistema radicular en condiciones de estrés. Tesis Doctoral en Ciencias Biologicas. Madrid: Universidad Complutense de Madrid, p 209.

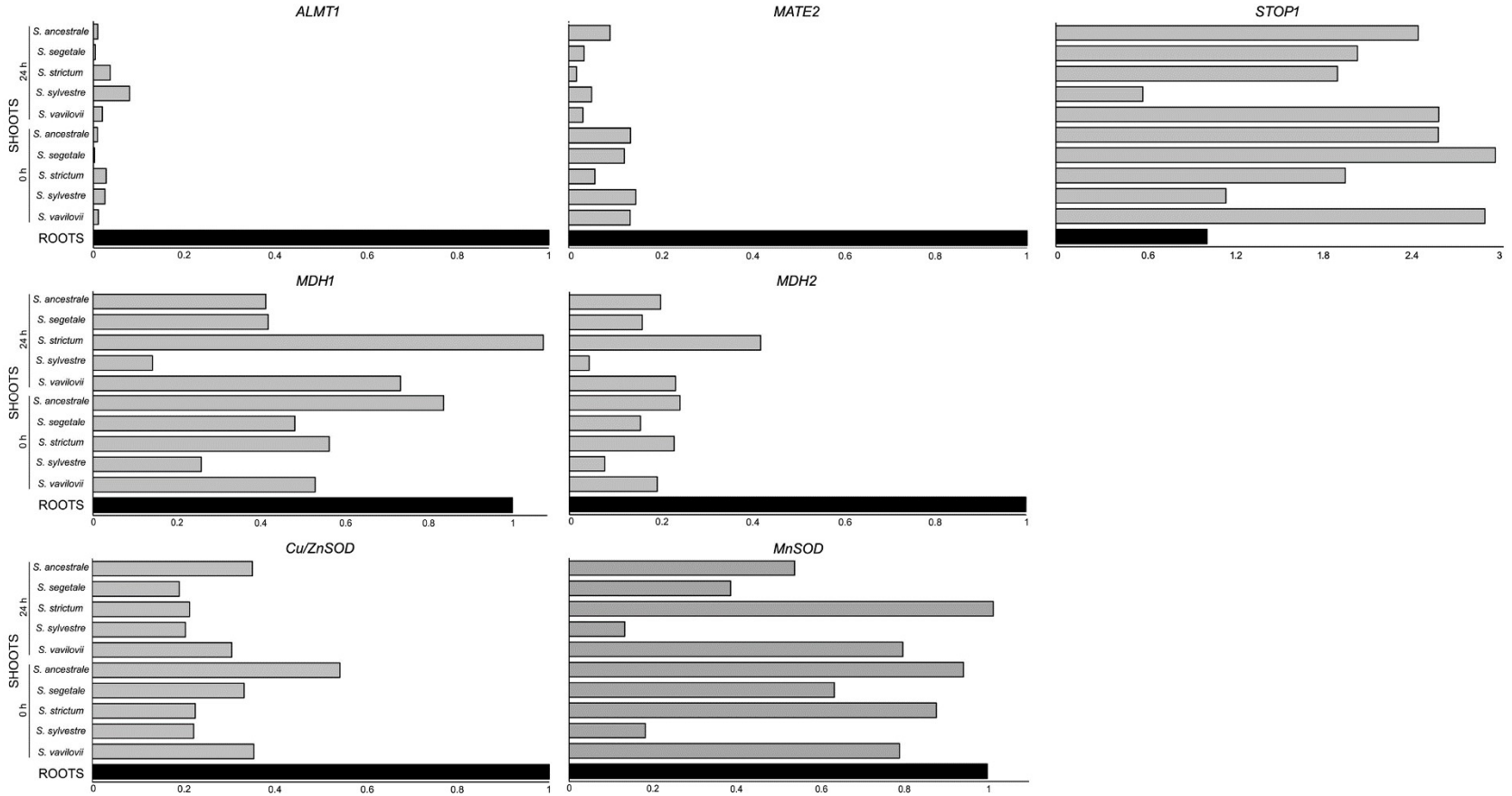


Figure S1. Comparison between the expression patterns (qRT-PCR) of root apices and shoot transcripts of several genes in five wild ryes treated with 300 µM $AlK(SO_4)_2$ (24 h) and without treatment (0 h). Change is expressed as relative difference in expression in roots compared with leaves at each time point (0 and 24 h). In all the cases, the data were normalized to *18S rRNA*; the columns represent mean of three replicates per treatment.

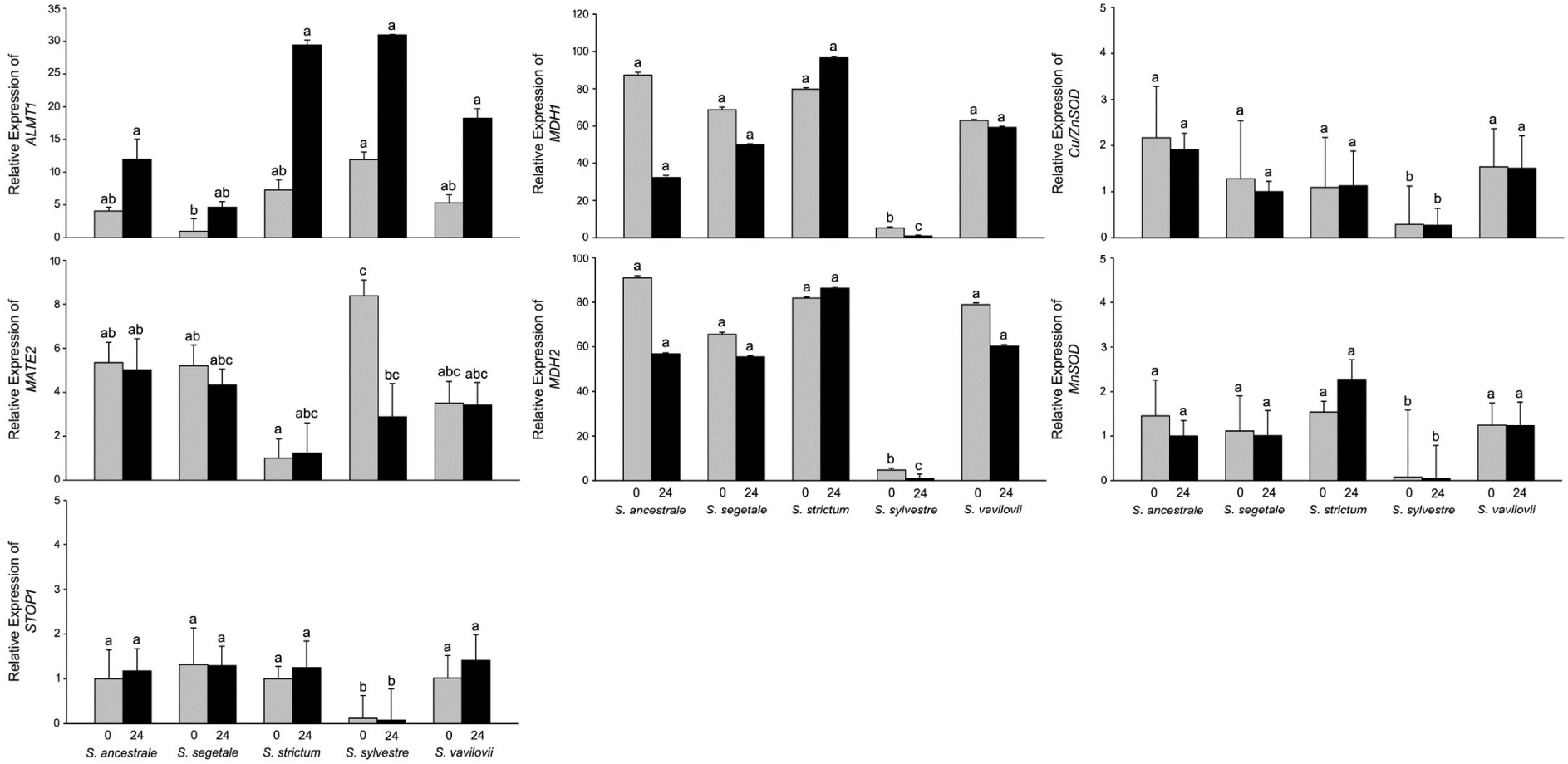


Figure S2. Quantitative RT-PCR showing expression patterns of shoot transcripts of several genes in five wild ryes treated with 300 µM $AlK(SO_4)_2$ (24 h) and without treatment (0 h). Data were normalized to *18S* gene. Change (fold difference) at each time point is expressed as the relative expression compared to the sample with lowest expression. Different lower case letters above bars represent differences between samples by Duncan ($P < 0.05$). The columns represent the mean of three replicates per time point and bars show ±SD.

SUBCHAPTER III-3

Isolation and characterization of a new *MATE* gene located in the same chromosome arm of the aluminum tolerance (*Alt1*) rye locus

E. Santos[1], M. Matos[1] and C. Benito[2]

[1]*Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, Quinta de Prados, 5001-801 Vila Real, Portugal; BioISI- Biosystems & Integrative Sciences Institute, University of Lisboa, Faculty of Sciences, Campo Grande, Lisboa, Portugal*

[2]*Departamento de Genética, Facultad de Biologia, Universidad Complutense, C/ José Antonio Novais, 12, 28040 Madrid, Spain*

Keywords: *Secale* spp.; locus *Alt1*; chromosome *6RS*; *ScMATE3;* Al resistance mechanisms.

*Submitted in *Theoretical and Applied Genetics* (TAAG-D-18-00347)

Abstract

Aluminum (Al) toxicity is the major constraint for crop productivity in acid soils. Wild rye species (*Secale* spp.) exhibited high Al-tolerance being a good source of genes related to this trait. The *Alt1* locus located on *6RS* chromosome arm is one of the four main loci controlling Al tolerance in rye and is known to harbor major genes but, so far, none has been found. We isolated and characterized the *ScMATE3* gene (a candidate *MATE* gene), in different *Secale* species. Three exclusive SNPs of the tolerant ryes were identified highlighting one non-synonymous that could change the expression of the encoded protein. The subcellular localization of *ScMATE3* seems to be the vacuolar membrane meaning that this could be a tonoplast MATE. The phylogenetic relationships performed with other *MATE* genes related to Al detoxification in Poaceae family suggest that *ScMATE3* could be involved in an internal tolerance mechanism. This work allow us to consider that beyond Al exclusion mechanism, already reported, an internal tolerance mechanism could be functional in rye. *ScMATE3* gene could be involved in Al resistance mechanisms in rye being a strong candidate to control the *Alt1* locus, however, future research will be needed to understand its role.

1. Introduction

Aluminum (Al) is a major component of the Earth's crust that solubilize in acidic soils with pH values below five, being converted in its phytotoxic form Al^{3+}. Two decades ago, Von Uexkull and Mutert (1995) estimated that 30-40 % of the arable land and over 50 % of the potentially arable land were acidic. Over time, these values have certainly been increasing due to the serious environmental problems we are facing and to the intensive farming practices (Samac and Tesfaye, 2003; Ma and Ryan, 2010). Al toxicity is one of the main constraints for crop production worldwide. The main consequent injury is the drastic inhibition of root growth which affect the uptake of water and nutrients, leading to crop yield reduction (Kochian et al., 2004). With a global growing population, it's crucial to overcome this problem in order to increase plant production around the world.

Internal and external Al tolerance mechanisms have been described in different species. Most Poaceae species share a common Al resistance mechanism: the exudation of organic acids from the roots in response to Al toxicity (Ma et al., 2014). *ALMT* and *MATE* are the most frequent genes implicated in the external mechanism, being related to the Al-activated release of malate and citrate, respectively.

MATE (Multidrug and toxic compound extrusion) transporters belong to one of the largest family in plants with a huge range of physiological functions such as Al detoxification. Several *MATE* genes with this function have been characterized in different plant species (For reviews see: Magalhaes, 2010; Takanashi et al., 2014). A remarkable number of members of this MATE family were found in many crops which emphasizes his importance in the plant kingdom: 49 *MATE* genes in maize (Zhu et al., 2016), 45 in rice (Wang et al., 2016), 56 in *Arabidopsis* (Wang et al., 2016), 117 in sorghum (Liu et al., 2016) and 33 in blueberry (Chen et al., 2015). Beyond its ubiquity, the MATE family is extremely flexible in function and could transports a broad range of substrates (For review see: Takanashi et al., 2014).

The response to Al presence varies between and within different plant species of the Poaceae family (Pinto-Carnide and Guedes-Pinto, 1999; Santos et al., 2018). Rye (*Secale cereale* L.) has been described as one of the most Al-tolerant species (Aniol and Gustafson, 1984; Gallego and Benito, 1997; Kim et al., 2001). Rye is the most productive cereal grain crop under any adverse conditions being important for breeding purposes and gene introgression in other related cereals more susceptible to this abiotic stress. Rye has a large genome size (~7.9 Gbp) which difficult genome analysis. The complete genome

sequence was not available until recently where new insights emerged with a whole-genome draft sequence of rye published by Bauer et al. (2017).

Four independent and dominant loci have been described that control Al tolerance in rye: *Alt1* on *6RS*, *Alt2* on *3RS*, *Alt3* on *4RL* and *Alt4* on *7RS* (Aniol and Gustafson, 1984; Gallego and Benito, 1997; Miftahudin et al., 2002; Matos et al., 2005; Benito et al., 2010). *ScALMT1* has been described as a candidate gene for the locus *Alt4*, moreover, the rye orthologue of *HvMATE1* (*ScMATE1*) was mapped to within 27.5 cM of this same locus (Fontecha et al., 2007; Collins et al., 2008; Benito et al., 2010). Al-tolerant genes located on the triticale chromosome arm *3RS* (*Alt2*) were linked with both malate and citrate release induced by Al (Ma et al., 2000). The high degree of Al tolerance in triticale is inherited from rye. Furthermore, several molecular markers were linked to *Alt3* and *Alt4* loci (Matos et al., 2005; Benito et al., 2010). The *Alt1* locus was linked to the aconitase-1 (*Aco1*), nicotinamide adenine dinucleotide dehydrogenase-2 (*Ndh2*), esterase-6 (*Est6*) and esterase-8 (*Est8*) loci, located on chromosome *6R*. Two SCARs ($ScR01_{600}$ and $ScB15_{790}$) were closely linked to the *Alt1* locus, $ScR01_{600}$ located 2.1 cM from *Alt1* and $ScB15_{790}$ located 5.5 cM from *Alt1*, on the *6RS* chromosome arm. Moreover, the SCAR $ScB15_{790}$ was linked to the *Lap1* locus (leucine aminopeptidase-1) (Gallego and Benito, 1997; Gallego et al., 1998). However, any candidate gene controlling *Alt1* tolerance was found up to date. The aim of this study was the identification, isolation and characterization of a candidate gene for the locus *Alt1* located on chromosome *6RS*, in different wild rye species.

2. Materials and Methods

2.1. Plant material and Growth conditions

In this work, three wild species/subspecies of the genus *Secale*, *S. strictum* (Persl) Persl ssp. *strictum* (R1211), *S. sylvestre* Host (R892) and *S. vavilovii* Grossh. (PI618682) were analyzed, as well as two subspecies of *Secale cereale* L.: *S. cereale* ssp. *ancestrale* Zhuk. (PI445975) and *S. cereale* ssp. *segetale* Zhuk. (PI326284). Ryes with accessions codes beginning with "PI" were kindly provided by The National Small Grains Collection (NSGC) of the United States Department of Agriculture - Agricultural Research Service (USDA-ARS) and those with "R" by Genebank Gatersleben, Institute of Plant Genetic and Crop Plant Research (IPK). In order to make it easier, the subspecies designation will be abbreviated in the text by *S. ancestrale, S. segetale* and *S. strictum*.

Moreover, for chromosomal location of the target gene we used the rye, *Secale cereale* L. cv. Imperial (I), a hexaploid wheat, *Triticum aestivum* cv. Chinese Spring (CS), their corresponding wheat–rye amphiploid (CS-I), the seven wheat–rye disomic addition lines (*1R* to *7R*) and two ditelosomic wheat–rye addition lines (*6RS* and *6RL*), kindly supplied by Dr. A. J. Lukaszewski, University of California (Riverside, CA, USA).

The Al-tolerance screening assays and the subsequent Al characterization of plants were described and carried out in Santos et al. (2018).

2.2. Bioinformatic analysis

Through comparative genomics, we checked the synteny shared by the major grasses and the region of the rye chromosome *6RS* where *Alt1* locus is located (Naranjo and Fernández-Rueda, 1991; Vogel et al., 2010; Martis et al., 2013; Herrero et al., 2016) (Fig. 1). *Brachypodium distachyon* is the model grass fully sequenced more closely related to rye (Draper et al., 2001), thus Al-related genes possibly orthologous to rye were scanned in his syntenic chromosome arm (*3S*) within PlantGDB database (http://www.plantgdb.org/BdGDB/). A BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was made in the selected coding sequences of *Brachypodium* to find homologies with other grasses and to confirm the orthology of the candidate genes found. Through the synteny among the main grass species, the most likely gene to be on the target *Alt1* locus in rye was elected and posteriorly the rye orthologous gene was isolated and characterized from wild ryes at study.

2.3. Candidate rye gene identification and chromosome location

Genomic DNA was extracted from young leaves of Imperial rye, Chinese Spring wheat and wheat–rye disomic and ditelosomic addition lines using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according the manufacturer's instructions. Three pairs of primers (Alt1ex1, Alt1ex7a9 and Alt1ex8a9 – R and F, Supplementary Table S1) were designed based on the sequence of the *Brachypodium* gene with the reference BRADI3G02170.2. All the primers of this work were designed with the support of Oligo 7.0 software (Rychlik, 2007). These primers were used for amplification in Imperial and Chinese Spring gDNA to obtain a first stretch of the target gene whose identity was confirmed using BLASTn search tool.

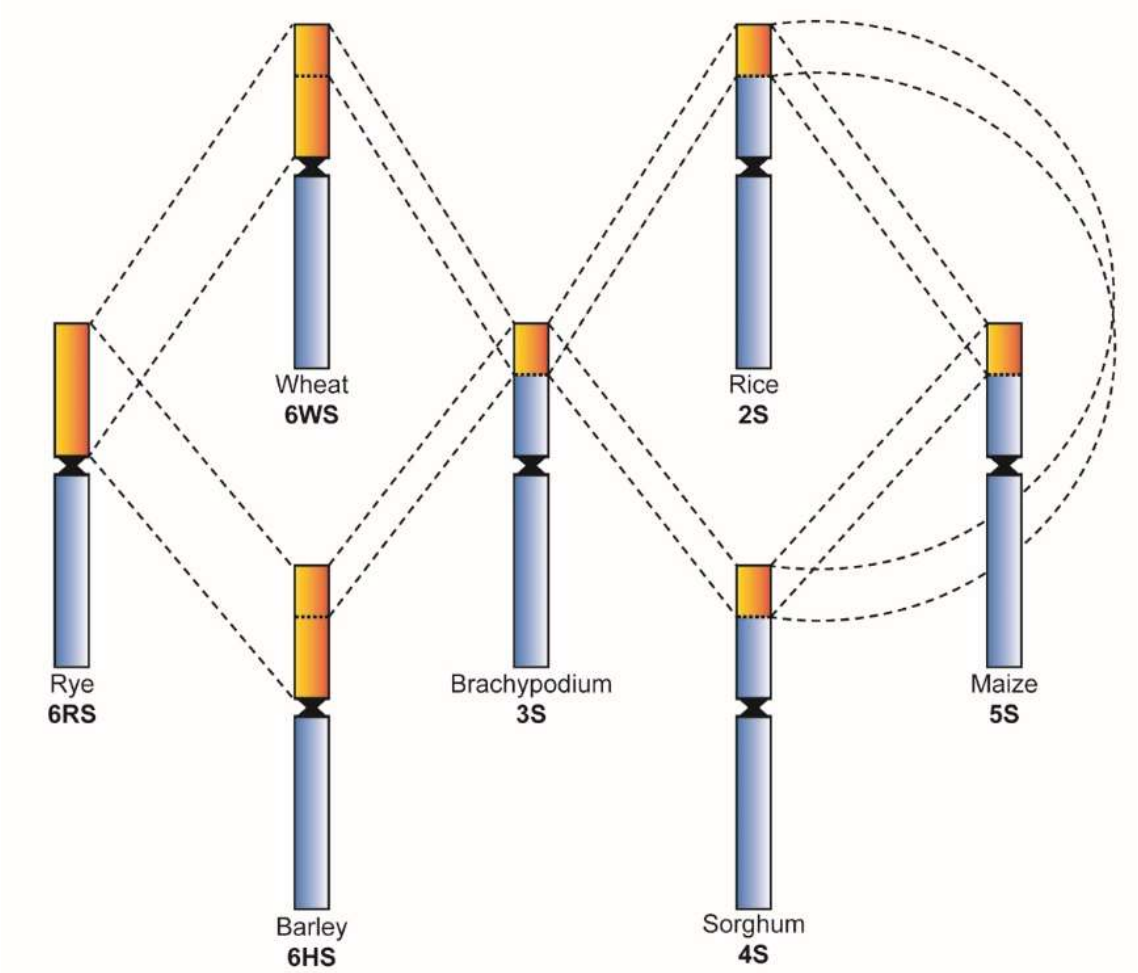


Figure 1. Syntenic regions between the major grasses and the rye chromosome *6RS* where *Alt1* locus is located obtained through comparative genomics (Naranjo and Fernández-Rueda, 1991; Vogel et al., 2010; Martis et al., 2013; Herrero et al., 2016).

In order to locate the target rye gene, two new pairs of primers (Alt1IMP1 – R and F and Alt1IMP2 – R and F, Supplementary Table S1) were designed in non-conserved regions of the sequences previously obtained in Imperial and Chinese Spring. These pairs of primers were designed to amplify only in Imperial rye. Besides the rye Imperial and the wheat Chinese Spring, these primers were also applied in the wheat-rye disomic and ditelosomic addition lines.

2.4. RNA extraction, cDNA synthesis and Sequencing of the candidate rye gene

7-day-old seedlings were exposed (24 h) or not (0 h) to 300 µM of Al nutrient solution. RNA extraction and cDNA synthesis were carried out from root apices (1 cm) and leaves with and without Al treatment following the procedures of Santos et al. (2018).

For *ScMATE3* gene sequencing only cDNAs obtained from roots of plants exposed to 24 h of Al were used. To isolate the complete CDS of *ScMATE3*, four pair of primers (cDNAMATE6RS – 1R, 2R, 3-4R and 1F, 2F, 3F, 4F, Supplementary Table S1) were designed based on conserved sequences among *Brachypodium distachyon* (reference BRADI3G02170.2) and barley (reference AK356145.1) which were used to amplify in rye cDNAs through PCR. The resultant PCR products were cloned into pGEM-T-Easy cloning Kit (Promega, Madison, USA) following the manufacturer's protocol.

2.5. Sequence analyses and phylogenetic trees assembly

Sequences were analyzed with Chromas Lite 1.0 (Technelysium, South Brisbane, Australia). Eleven putative MATE3 coding and protein sequences from rye related Poaceae species (*T. aestivum*, *Triticum urartu, Hordeum vulgare*, *Brachypodium distachyon*, *Brachypodium stacei, Sorghum bicolor*, *Zea mays*, *Oryza sativa, Setaria viridis, Setaria italica* and *Panicum virgatum*) were collected using BLASTn and BLASTp search, respectively. MATE1 and MATE2 proteins from different plant species were downloaded in the NCBI (http://www.ncbi.nlm.nih.gov/) and phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) databases using as queries the rye sequences ScMATE1 (KX632094; Santos et al., 2018) and ScMATE2 (BAJ61742; Yokosho et al., 2010). Alignments between different *MATE* sequences were made using the ClustalW algorithm (http://www.ebi.ac.uk/Tools/clustalw). DnaSP v5.1 (Librado and Rozas, 2009) was used to calculate sequence diversity parameters. Several software programs were used to predict the secondary structures and membrane topologies of the different ScMATE3 proteins (Supplementary Table S2). The Pfam (http://pfam.xfam.org) and InterPro (http://www.ebi.ac.uk/interpro/) software's were used to identify potential domains and to provide the *ScMATE3* gene ontology. Molecular weight (MW) and theoretical isoelectric point (pI) of rye MATE3 proteins were predicted by ExPASy Compute pI/Mw tool (http://web.expasy.org/compute_pi/). WOLF-PSORT (https://www.genscript.com/wolf-psort.html) was used to predict the intracellular localization of MATE3 proteins.

Phylogenetic relationships among different MATE proteins and cDNA sequences were analyzed using MEGA 6.06 (Tamura et al., 2013) using Neighbor-Joining method. Bootstraps with 1000 replicates were performed to test the robustness of the dendrograms.

The *MATE3* gene structure and evolution was studied with the support of PIECE 2.0 database (Wang et al., 2017b).

2.6. Determination of *ScMATE3* gene expression by semi-quantitative (sq) and quantitative (q) RT-PCR

For expression studies, cDNAs obtained from roots and leaves of plants (not exposed to Al and exposed for 24 h) of the five ryes at study were used. To determine the *ScMATE3* expression level, two pair of primers (qScMATE6RS and qScMATE3 – R and F, Supplementary Table S1) were designed. Primers for the housekeeping *18S* rye gene, described by Fontecha et al. (2007), were used as an internal control reference gene. SqRT-PCR was performed in a 10 μL reaction volume containing 2.5 μL of 1/10 diluted cDNA (RNA ~200 ng/μL), 0.5 μL of each gene-specific primer (10 μM) and 5 μL of Taq PCR MasterMix (Qiagen), using the following program: an initial step of 3 min at 94 °C, 30 cycles of 45 s at 94 °C, 45 s at 60 °C and 1 min at 72 °C, followed by a final extension step of 10 min at 72 °C. PCR products were visualized on 2% TBE agarose gels.

ScMATE3 transcripts of roots (0 and 24 h) were also quantified by qRT-PCR. Primers for *ScMATE3* and *18S* expression analyses were the same as employed in the sqPCR. PCR reactions were carried out using a Stratagene MX3005P QPCR systems (Agilent Technologies, Santa Clara, CA, USA) with the following program: one step at 95 °C for 10 min and 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 45 s at 72 °C, followed by a melting curve of 1 min at 95 °C, 30 s at 60 °C and 30 s at 95 °C. A 5-fold serial dilution of cDNA was performed to verify PCR efficiencies both for *ScMATE3* and for the control *18S*. Relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For each gene, expression values were normalized with the sample with the lowest transcript level. All PCR samples and controls were prepared in duplicate and all experiments were replicated at least three times. Analysis of variance (Two-way ANOVA) was performed using the SPSS statistical package for Windows (v. 23.0; IBM Corp., Armonk, NY, USA). Significant differences between means were determined using the Duncan test.

3. Results

3.1. Identification of the candidate gene and chromosome location

One *ALMT* and four *MATE* genes were found in the chromosome arm *3S* of *Brachypodium*. We elected the gene whose homologous are located in the short arm of the chromosome 6 in barley, 2 in rice, 4 in sorghum and 5 in maize. We obtained a DNA stretch in rye between the exon 7 and 9 of the candidate gene with the primers Alt1ex7a9 (R and F). The BLASTn search revealed that a possible candidate gene for controlling *Alt1* locus is a *MATE* gene. Since this *MATE* rye gene is a new discovery and only two of them are characterized to date (*ScMATE1* and *ScMATE2*; Yokosho et al., 2010; Silva-Navas et al., 2012; Santos et al., 2018) we decided hereafter to name it as *ScMATE3*.

We proceeded with the chromosome location of this candidate gene to ensure that it is in the same chromosome arm as the locus *Alt1*. The pair of primers Alt1IMP2 (R and F) amplified in the rye Imperial and not in the wheat Chinese Spring and were able to make the chromosomal localization of this *MATE* gene. The rye PCR-specific product obtained was present only in the *6R* and *6RS* addition lines (Fig. 2). Hence, the *ScMATE3* gene was located on the short arm of the chromosome *6R*, the same location of the *Alt1* locus.

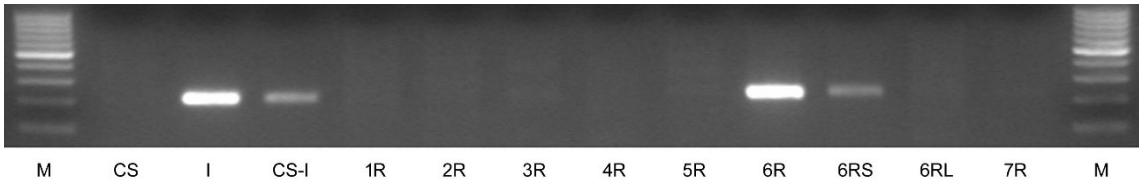


Figure 2. Chromosomal location of the *ScMATE3* gene. M: molecular weight marker (100 to 3,000 bp); CS: Chinese Spring (bread wheat); I: Imperial (rye); CS-I: wheat-rye amphiploid; *1R*, *2R*, *3R*, *4R*, *5R*, *6R* and *7R*: wheat-rye disomic addition lines (CS-I); *6RS* and *6RL*: ditelosomic addition lines with *6RS* and *6RL* chromosome arms.

3.2. Characterization of the cDNA sequences obtained

A total of 13 sequences of *ScMATE3* from different *Secale* species/subspecies were obtained: three from each *S. ancestrale*, *S. segetale* and *S. sylvestre,* as well as, two from each *S. strictum* and *S. vavilovii*. These sequences were deposited in Genbank (accessions numbers MF568517 to MF568528). The coding sequence of *Secale cereale* obtained, recently, by Bauer et al. (2017) was also analyzed and compared with the sequences obtained in this work. We found a 1278 bp coding region (including stop codon) for the

ScMATE3 gene in all the sequences analyzed. The sequence comparisons revealed both intraspecific and interspecific variability, although, two identical sequences were found in *S. segetale*. Moreover, this gene has a high sequence conservation in the *Secale* genus (C - 0.960) (Supplementary Table S3).

Alignment between these cDNA sequences and the gDNA and cDNA of, respectively, *Brachypodium* (BRADI3G02170.2) and barley (AK356145.1) orthologous genes, suggested that *ScMATE3* gene is composed of fourteen exons interrupted by thirteen introns (Fig. 3). The size of the intron sequences and the UTR (5' and 3') ends were estimated based on the gDNA sequence (FKKI011491151.1) of *S. cereale* obtained by Bauer et al. (2017). According to the diversity parameters analyzed, exons 1 and 14 are the most conserved whereas the exons 2, 7 and 13 are the most diverse (Supplementary Table S3).

Nine species/subspecies exclusive single nucleotide polymorphisms (SNPs) were found in the gene *ScMATE3*, each being shared with all the sequences obtained from the same species/subspecies: three from both *S. strictum* and *S. sylvestre*, two from *S. vavilovii* and one shared by *S. ancestrale*, *S. segetale* and *S. cereale*. Three SNPs were identified in the sensitive rye species (*S. sylvestre*) so, there is a common difference between the cDNAs of the tolerant ryes and the sensitive one. Two of these SNPs (exons 3 and 9) are synonymous changes where the amino acid does not change, thus, the protein is unaffected. However, one of them, located on the exon 6, is a non-synonymous or replacement substitution (Arg to Cys) which affect the amino acid and could change the expression of the protein coded. Moreover, SNPs with non-synonymous changes were also found in the exons 11 (Phe to Ile), 12 (Arg to Met) and 13 (Ser to Thr) of *S. strictum* and in the exon 9 (Val to Gly) of *S. vavilovii*. A total of 51 SNPs were found among the different rye species/subspecies in the *ScMATE3* gene, being 33 non-synonymous changes.

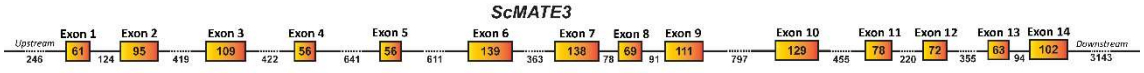


Figure 3. Schematic diagram of the predicted structure of the *ScMATE3* gene: fourteen exons (color boxes) and thirteen introns (black lines). Number are the size in base pairs (bp).

3.3. Characterization of the amino acid sequences

The deduced proteins of ScMATE3 consist of 425 amino acid residues and comprise the characteristic MatE domain of MATE family (Pfam: PF01554). The molecular weight of ScMATE3 range from 46.15 (*S. strictum* and *S. vavilovii*) to 46.22 (*S. ancestrale*) kDa and the isoelectric point value is 8.75, except for *S. vavilovii* (8.74). Subcellular prediction analysis indicated that ScMATE3 protein is localized in the vacuolar membrane (odds ratio - 0.77) and both TuMATE3 and BsMATE3 in the plasma membrane with 50% probability. Curiously, the other MATE3 proteins were located on the chloroplasts with high odds values. Depending on the protein structure prediction software used, six to eleven putative transmembrane helix regions (TMH1 to TMH11) were found, which six of them (TMH1, TMH2, TMH3, TMH5, TMH6 and TMH8) were predicted by all programs used (Supplementary Fig. S1).

3.4. Phylogenetic relationships

For the *ScMATE3* phylogenetic analysis only one coding or protein sequence from each species/subspecies, including *S. cereale*, was used (Fig. 4a). Moreover, the cDNAs and amino acid sequences from other Poaceae species such as *T. aestivum*, *T. urartu, H. vulgare*, *B. distachyon*, *S. bicolor*, *Z. mays*, *O. sativa, B. stacei, S. viridis, S. italica* and *P. virgatum* were added. The species *Spirodela polyrhiza* was used as outgroup. The dendrogram obtained showed very high bootstrap values. All *MATE3* sequences of the genus *Secale* were grouped in the same cluster, closer to the wild einkorn wheat and barley. *S. strictum* and *S. sylvestre* were clearly separate from the other ryes.

Phylogenetic studies were done among several potential orthologous of ScMATE3 protein and Poaceae orthologous of ScMATE1 and ScMATE2 (ScFRDL2) proteins (Fig 4b). The dendrogram obtained showed very high bootstrap values. Two main clusters were clearly defined (I and II), one containing all the MATE1 and MATE2 proteins and another with all the MATE3 proteins. Each group were divided in two subgroups: in cluster I, each subgroup was constituted by each of the MATE1 (I-1) and MATE2 (I-2) grouping whereas in cluster II, one subgroup inserted the MATE3 proteins of *Arabidopsis thaliana* and *Glycine max* (II-1) and the other contained all the Poaceae MATE3 proteins (II-2). ScMATE3 predict protein shared high identity with the homologous proteins of bread wheat (98.1%), barley (94%), einkorn wheat (93%), *Brachypodium* (91%), rice (88%), sorghum (86%) and maize (86%), in descending order.

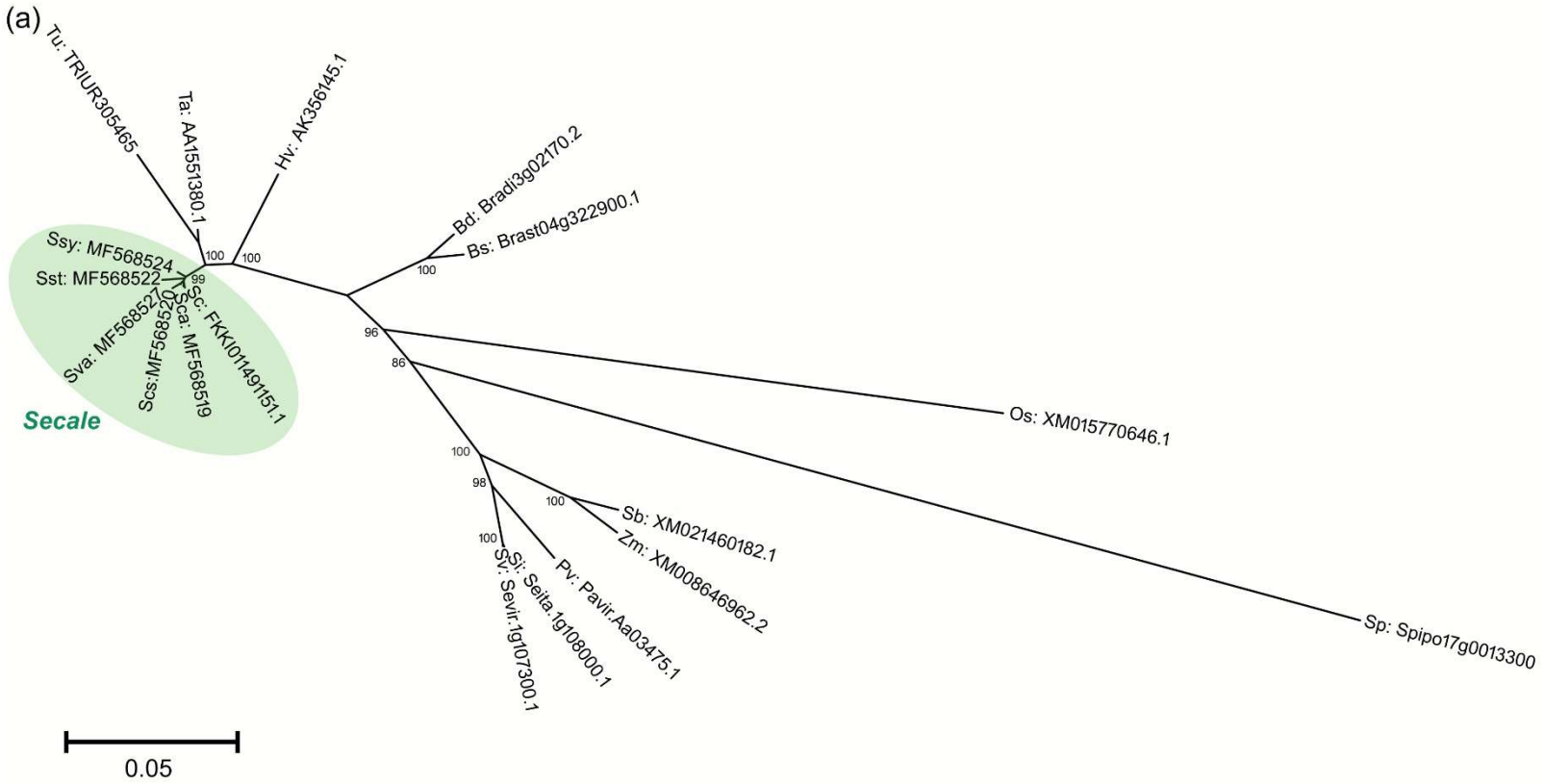
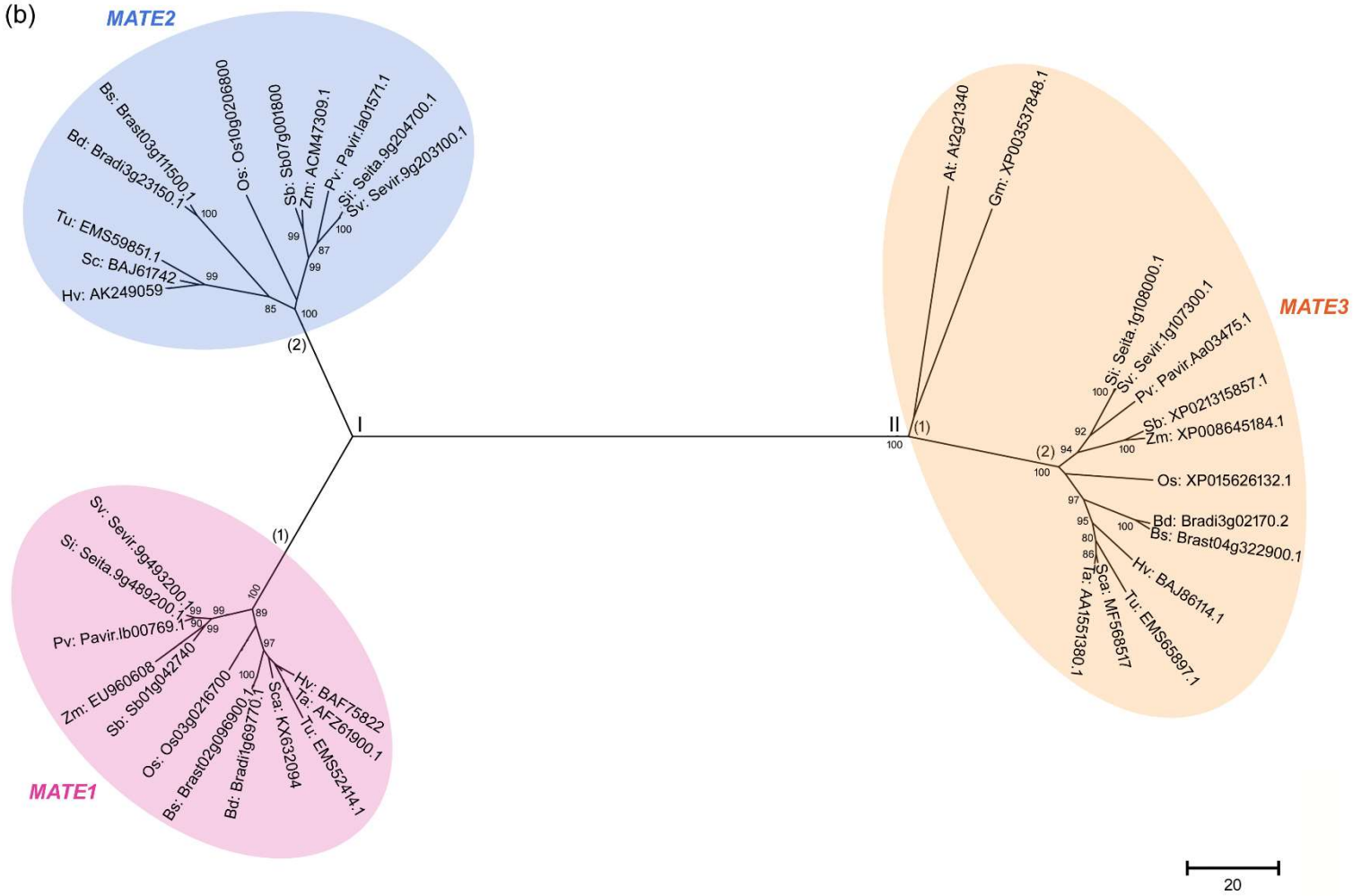


Figure 4. (a) Phylogenetic relationships obtained for coding region of the *MATE3* gene from different species/subspecies of the genus *Secale* compared with several orthologous Poaceae cDNA and (b) Phylogenetic relationships obtained with several potential orthologous of ScMATE3 protein and Poaceae orthologous of ScMATE1 and ScMATE2 (ScFRDL2) proteins. The species *Spirodela polyrhiza* (Sp) was used as outgroup. The cluster method used was neighbor-joining and the percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Abbreviations: At – *Arabidopsis thaliana*; Bd – *Brachypodium distachyon*; Bs – *Brachypodium stacei*; Gm – *Glycine max*; Hv – *Hordeum vulgare*; Os – *Oryza sativa*; Pv – *Panicum virgatum*; Sb – *Sorghum bicolor*; Sc – *Secale cereale*; Sca – *S. cereale* ssp. *ancestrale*; Scs – *S. cereale* ssp. *segetale*; Si – *Setaria italica*; Sst – *Secale strictum*; Ssy – *Secale sylvestre*; Sv – *Setaria viridis*; Sva – *Secale vavilovii*; Ta –*Triticum aestivum*; Tu – *Triticum urartu*; Zm – *Zea mays* (continues below).

(cont.)


(b)
MATE2
Bs: Brast03g111500.1
Bd: Bradi3g23150.1
Os: Os10g0206800
Sb: Sb07g001800
Zm: ACM47309.1
Pv: Pavir.Ia01571.1
Si: Seita.9g204700.1
Sv: Sevir.9g203100.1
Tu: EMS59851.1
Sc: BAJ61742
Hv: AK249059
(2)
I
II
(1)
At: At2g21340
Gm: XP003537848.1
MATE3
Si: Seita.1g108000.1
Sv: Sevir.1g107300.1
Pv: Pavir.Aa03475.1
Sb: XP021315857.1
Zm: XP008645184.1
Os: XP015626132.1
Bd: Bradi3g02170.2
Bs: Brast04g322900.1
Hv: BAJ86114.1
Tu: EMS65897.1
Sca: MF568517
Ta: AA1551380.1
(1)
Sv: Sevir.9g493200.1
Si: Seita.9g489200.1
Pv: Pavir.Ib00769.1
Zm: EU960608
Sb: Sb01g042740
Os: Os03g0216700
Bs: Brast02g096900.1
Bd: Bradi1g69770.1
Sca: KX632094
Ta: AFZ61900.1
Tu: EMS52414.1
Hv: BAF75822
MATE1
20

3.5. Expression analyses

The expression pattern of the gene *ScMATE3* was analyzed through sqRT-PCR in roots and shoots (Fig. 5) of the five *Secale* species/subspecies not treated (0 h) and exposed to Al for 24 h. Results were obtained comparing plants with and without Al exposure. In roots, a constitutive expression was observed in *S. ancestrale*, *S. segetale* and *S. vavilovii* while a repression seems to have occurred in *S. strictum* and *S. sylvestre* though more prominently in the last. At 24 h time point, *S. vavilovii* showed the highest amount of mRNA whereas *S. sylvestre* revealed the lowest. In shoots, all the ryes both with and without Al treatment exhibited a similar quantity of mRNA, except for *S. strictum* where a slight induction was detected. In general, no differences were observed in the transcript levels of *ScMATE3* after Al contact but *S. vavilovii* and *S. sylvestre* had more quantity in roots and shoots, respectively.

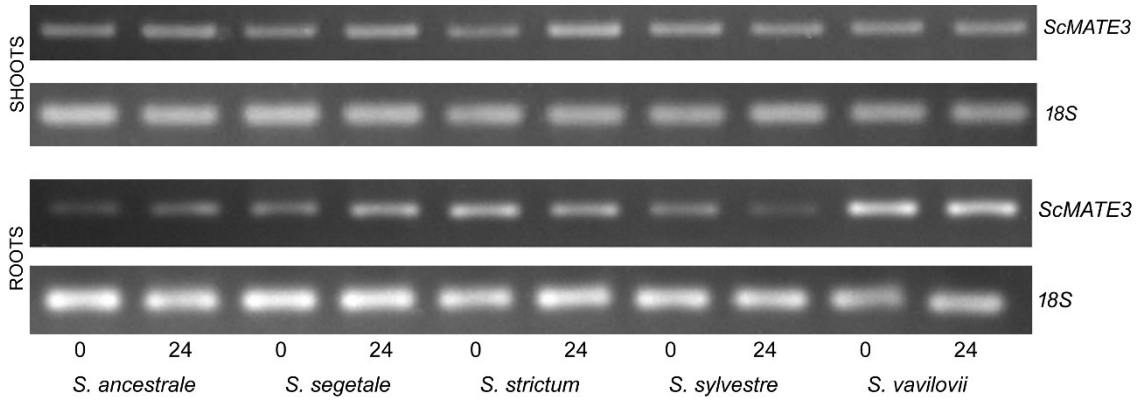


Figure 5. Semi-quantitative PCR results showing temporal expression patterns of shoots (above) and root tip (below) transcripts of *ScMATE3* gene from five wild ryes without Al (0 h) and with 300 µM $AlK(SO_4)_2$ for 24 h. The expression of the housekeeping gene *18S* was used as a reference in each case.

Furthermore, quantitative PCR analyses were carried on in the roots of the five ryes at study (Fig. 6) besides the exactly same results as the sqPCR were obtained. A constitutive expression pattern was found in *S. ancestrale* (1.12 fd – fold difference), *S. segetale* (1. 15 fd) and *S. vavilovii* (1. 10 fd), as well as, a downregulation in *S. strictum* (0.72 fd) and, especially, *S. sylvestre* (0. 29 fd).

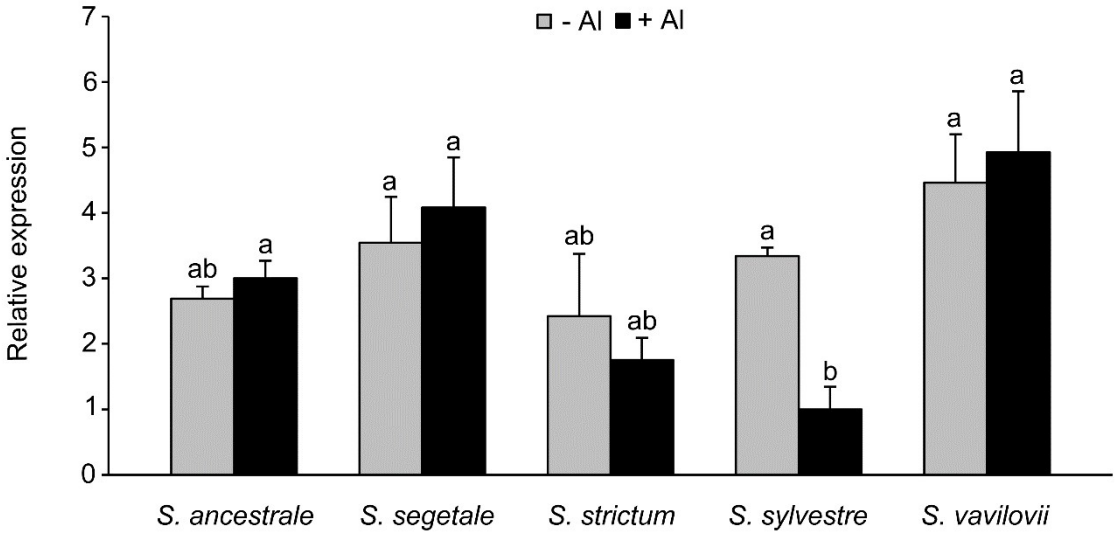


Figure 6. Quantitative RT-PCR showing expression patterns of root tip transcripts of *ScMATE3* gene in five wild ryes treated with 300 µM $AlK(SO_4)_2$ (24 h) and without treatment (0 h). Data were normalized to *18S* gene. Change (fold difference) at each time point is expressed as the relative expression compared to the sample with lowest expression. Different lower case letters above bars represent differences between samples by Duncan ($P < 0.05$). The columns represent the mean of three replicates per time point and bars show ±SD.

4. Discussion

Comparative genomics provides a powerful tool to identify genes that are conserved or common among species. Synteny-based comparisons with other cereal genomes allowed us to found a *MATE* candidate gene for the *Alt1* locus in rye located on the short arm of the chromosome 6 (*6RS*).

The exon-intron structure of this gene was obtained comparing our cDNA sequences with genomic sequence of rye obtained by Bauer et al. (2017). One orthologous gene was found in hexaploid wheat (*T. aestivum*) located on *6AS* chromosome arm with 14 exons and 13 introns, and another in the wild einkorn wheat (*T. urartu*), also with 14 exons and 13 introns, whose draft genome was already obtained (Ling et al., 2013).

In Santos et al. (2018) the characterization of the species/subspecies at study were based on their root regrowth after Al exposure in hydroponic experiments: *S. ancestrale*, *S. segetale* and *S. vavilovii* as Al-tolerant, *S. strictum* moderately Al-tolerant and *S. sylvestre* Al-susceptible. The existence of exclusive species/subspecies SNPs in the coding sequences of this gene can probably be related to Al tolerance. A favorable adaptation to this trait may have developed through this evolution leading to a new expression of the gene *ScMATE3*. SNPs can result in important phenotypic alterations, especially when they occur in coding regions (Ferreira et al., 2017) and, can be the result

of a strong selection pressure of *ScMATE3* gene. In a previous study (Santos et al., 2018), we characterized *ScMATE1* in the same rye species/subspecies of this current study and comparing data of the diversity indexes we observed that *ScMATE1* revealed higher values than *ScMATE3* meaning it is under a greater selection pressure. Fontecha et al. (2007) detected SNP polymorphisms for the gene *ScALMT1* among the parents of three F2 rye populations for the *Alt4* locus. In barley, SNPs were found in the ORF of *HvAACT1* gene in cultivars that differed in Al tolerance (Furukawa et al., 2007). Moreover, a non-synonymous SNP was identified in this gene and was correlated with larger root growth on acidic soil (Ferreira et al., 2017). In turn, Tovkach et al. (2013) found, in wheat, a SNP located in the *TaMATE1B* promoter which led to an increased expression gene level. All the variations observed in the different ScMATE3 proteins do not change his transmembrane structure, thus, the main function of the putative proteins is, probably, not affected.

Regarding the phylogenetic relationships of this gene, we could observe a monophyletic group formed by all the taxa of *Secale* at study. Moreover, the other members of the tribe Triticeae, *T. aestivum*, *T. urartu* and *H. vulgare*, were clearly closer to the genus *Secale* (Fig. 4a).

The phylogenetic relationships among the deduced MATE1, MATE2 and MATE3 proteins (Fig. 4b) originated two main groups (I and II), each enclosing two subgroups (I/II-1 and I/II-2). Cluster I includes the MATE1 and MATE2 proteins of several Poaceae species, each one forming a different subgroup (I-1 and I-2). On the other hand, cluster II includes all the MATE3 proteins. Our candidate gene (*ScMATE3*) is clearly different from the other two MATEs described in rye (*ScMATE1* and *ScMATE2*). Since they belong to the same cluster, the *MATE3* genes are probably orthologous which is supported by the syntenic relationships and the high level of similarity of the protein sequences. Moreover, the exon-intron structure of these genes is quite conserved (Fig. 7). All the orthologous genes have 14 exons and 13 introns, which means that gene functions of this group might be similar. This group was divided in two subgroups: in II-1 only the genes of *Arabidopsis thaliana* and *Glycine max* (soybean) were inserted and in II-2 all the eleven members belonging to Poaceae family were included. There is no much information about this group of genes that we named *MATE3* for being orthologues of the candidate gene in this study, *ScMATE3*.

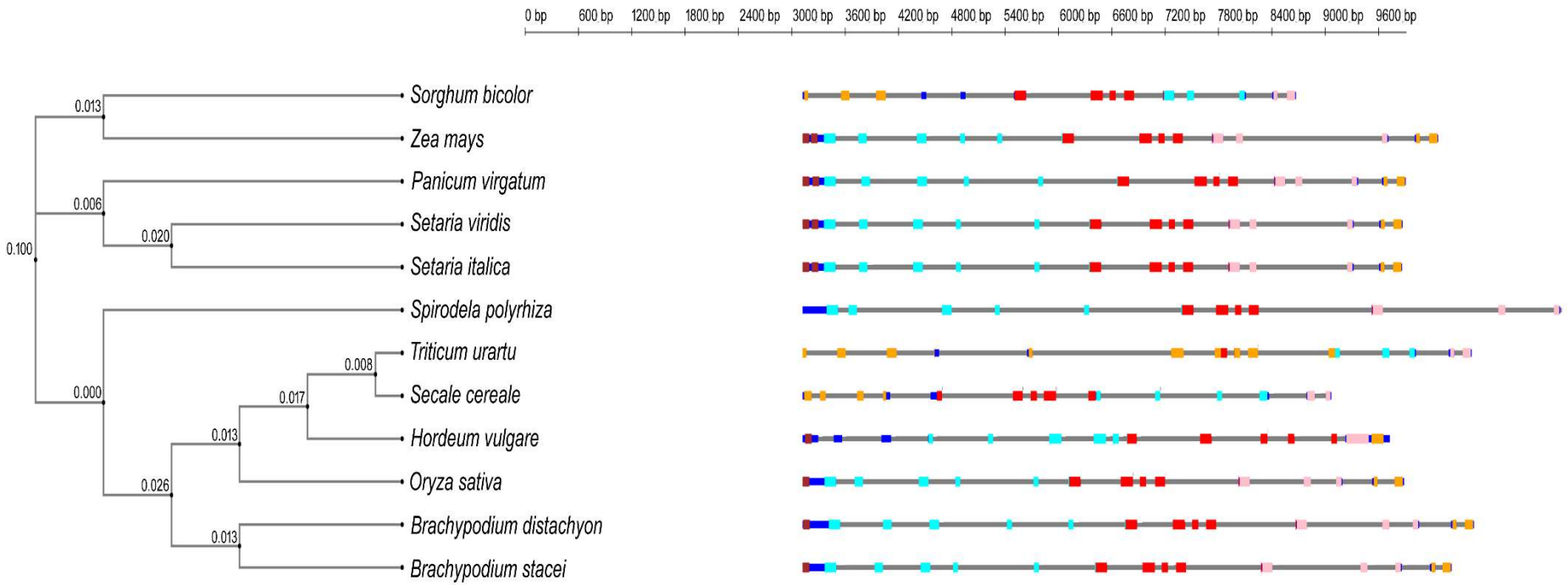


Figure 7. *MATE3* gene structure and evolution in Poaceae family obtained by PIECE 2.0 database. Colored boxes are representing exons and grey lines introns. Numbers next to the branches in the dendrogram are bootstrap values.

The gene At2g21340 (*A. thaliana*) was named EDS5H (Enhanced Disease Susceptibility5 Homologue) because of his homologue EDS5, already characterized as a salicylic acid (SA) transporter (Parinthawong et al., 2015). Both genes were localized at the envelope of the chloroplast and like EDS5, EDS5H may be an H^+/organic acid antiporter, however, they differ in the transport substrate since the latter was not involved in the SA accumulation and must be carrying related substances. The nature of the substrate could be the reason for the divergence of the MATE3 group, since most of the members of the other group are citrate-transporters and this one may transport phenolic compounds. It is known that transporter substrate specificity usually have an association with phylogeny (Chen et al., 2015; Wang et al., 2016), which means that ScMATE3 could transport phenolics instead of citrate. Tolrà et al. (2009) reported that phenolic compounds are strong ligands for Al^{3+} and form stable chelation complexes with it, possibly playing a role in Al tolerance by an internal mechanism.

Liu et al. (2016) identified 117 MATE transporters in soybean among which was found the *ScMATE3* orthologous gene which were denominated as GmMATE70. This gene was included in a primary clade with several MATE1 and MATE2 proteins of diverse crop species but as part of another subgroup with AtEDS5, described above. Interestingly, these three group of proteins seems to be closely related, thus, we could predict that functions of these MATE proteins have some correlation. It is recalled that the exon-intron structure of all the MATEs present in our phylogenetic study is very similar. Wang et al. (2016) considered AtEDS5, AtEDS5H, OsFRDL1 and OsFRDL2 members of the same subfamily, in a work containing 45 and 56 MATE genes from rice and *Arabidopsis*, respectively. Unfortunately, no data were found about Al tolerance of AtEDS5H and GmMATE70. However, the closeness between the two gene clusters (I and II) lead us to consider that it could be a relationship with Al tolerance and MATE3 proteins. In the grass subgroup (II-2), only the gene of maize was characterized and, thereafter, named ZmMATE32 in a study that comprises 49 MATE genes (Zhu et al., 2016). These authors also grouped in the same subfamily a member of MATE1 protein (ZmMATE1 – Maron et al., 2010) with the maize gene orthologous of *ScMATE3* (ZmMATE32). Quantitative RT-PCR was performed in roots of maize exposed to Al stress and the expression pattern of this gene was analyzed. As occurred with ZmMATE1, ZmMATE32 was evidently upregulated compared to controls (>2-fold) which means that this gene may be implicated in Al tolerance mechanisms. These data reinforce the possible implication of *ScMATE3* in this abiotic stress.

As we already seen, the subcellular localization is crucial to deduce the function of a transporter. In the MATE3 group, the unique gene whose location was confirmed by a study was the one of *Arabidopsis*, which was found in the chloroplasts. The subcellular localization of the remaining genes was predicted by WoLF PSORT also locating most of them in chloroplasts. Curiously, ScMATE3 was located at the vacuolar membrane and TuMATE3 at the plasma membrane with 76.9 and 50% of chances, respectively. It is noteworthy that almost 30% of the possibilities predicted, as well, the location of TuMATE3 in vacuoles. Previous works (Chen et al., 2015; Liu et al., 2016; Wang et al., 2016) shown that in the same grouping MATE, members shared the same or similar subcellular location as we verified in the current study. Indeed, chloroplasts and vacuoles are both cell organelles and the main function of these transporters may certainly be related. Several tonoplast MATE have been reported in plant species with diversified functions such as nicotine (NtMATE1 and NtMATE2 – Shoji et al., 2009), flavonoid (MtMATE2 – Zhao et al., 2011), proanthocyanidin (AtTT12 – Debeaujon et al., 2001; MdMATE1 and MdMATE2 – Frank et al., 2011; MtMATE1 – Zhao and Dixon, 2009) and anthocyanin (VvAM1 and VvAM3 – Gomez et al., 2009) accumulation. So far, no tonoplast MATE gene have been associated to Al tolerance mechanisms but, other vacuolar transporters like the half-size ABC AtALS1 (Larsen et al., 2007) and OsALS1 (Huang et al., 2012) as the aquaporin HmVALT (Negishi et al., 2012) have been implicated in the sequestration of Al into the vacuole, an internal mechanism of Al detoxification. Thus, considering all the functionally diversified existing tonoplast MATEs, it will not be surprising to find one involved in this internal Al tolerance mechanism, transporting any substrate such as citrate or phenolic compounds. Yokosho et al. (2016) localized the *MATE* gene *ScFRDL2* in vesicle-like organelles through GFP (Green Fluorescence Protein) technique and we localized it on the vacuolar membrane trough WoLF-PSORT. This could mean that we are facing a possible tonoplast MATE, involved in Al tolerance and citrate releasing. All this acquired knowledge allows us to speculate about the function of our candidate gene *ScMATE3*. Therefore, his localization requires further validation using more detailed and reliable techniques as immunolocalization.

To better understand if our candidate gene plays some role in rye Al tolerance mechanisms and can thus be the gene linked to *Alt1* locus, we proceeded with expression studies regarding this trait (Fig. 5 and 6). The semi-quantitative data revealed that the transcript level of *ScMATE3* gene after Al contact of shoots and roots varied according

the species/subspecies: no differences were observed in most of them whereas *S. vavilovii* and *S. sylvestre* had more transcript in roots and shoots, respectively (Fig. 5). Contreras et al. (2014) reported that BdMATE3, orthologous of ScMATE3, was more expressed in leaves compared to roots after Al exposure. In *Arabidopsis*, the activity of the EDS5H promoter was estimated by GUS staining and showed more expression in green tissues than in roots (Parinthawong et al., 2015). On the other hand, ZmMATE32 (Zhu et al., 2016) exhibited more expression in the primary root than other different tissues as leaves in several stages in a heatmap based on microarray data. A constitutive expression pattern was observed in the shoots both in the tolerant and sensitive ryes and no differences were detected in the transcript amount. *S. strictum*, the moderately Al-tolerant subspecies, was an exception and showed a slight induction in leaves. Recalling the existence of three non-synonymous SNPs in the *ScMATE3* coding sequence of this subspecies, we could speculate that Al tolerance in *S. strictum* might be operated by a different mechanism. Ma et al. (2016) found a different allele of *HvAACT1* in a moderately Al-tolerant barley variety and concluded that it might be controlled or regulated in a different way than the tolerant varieties. Expression data suggests that *ScMATE3* can be involved on an internal mechanism in *S. strictum* with Al being possibly translocated to the shoots and sequestered in the leaf vacuoles, a mechanism generally reported in Al-accumulator plant species such as buckwheat (Ma and Hiradate, 2000) and hydrangea (Ma et al., 1997).

We decided to carry on with quantitative studies only in roots (Fig. 6) since it is our point of interest for being the main target of Al toxicity and also, the results obtained in sqPCR caught our attention. Two facts lead us to believe that this gene may be involved in some Al resistance mechanism in roots: the constitutive expression in the tolerant ryes (*S. ancestrale*, *S. segetale* and *S. vavilovii*) and the downregulation in the sensitive one (*S. sylvestre*), as well as, the higher quantity of transcripts in the tolerant ryes compared to the sensitive. Not all the Al resistance genes are upregulated after Al exposure, which is the case of the genes *TaALMT1* (Sasaki et al., 2004) and *TaMATE1* (Ryan et al., 2009) as *BdALMT1* (Contreras et al., 2014) and *HvAACT1* (Furukawa et al., 2007), that contribute to Al tolerance in wheat, *Brachypodium* and barley, respectively. These genes exhibited a constitutively higher gene expression in the tolerant genotypes compared to the sensitives. It was reported that variations in the promoter could be implicated in the enhanced expression of these genes in Al-resistant plants. A series of cis mutations have generated duplicated and triplicated tandem repeat elements in the *TaALMT1* promoter increasing his expression level in wheat tolerant lines (Ryan et al., 2010a). In line,

Tovkach et al. (2013) found that the insertion of a large transposable element-like (11.1 kb) in the upstream region of *TaMATE1B* gene enhanced Al tolerance of the tolerant wheat cultivar. In the same way, Fujii et al. (2012) identified in tolerant barley cultivars, a 1-kb transposable element in the *HvAACT1* promoter which resulted in higher Al-induced release of citrate and, subsequently, Al tolerance. Likewise, an insertion in the upstream region of *BdALMT1* gene has also been detected in tolerant lines of *B. distachyon* and *B. hybridum* (Contreras et al., 2014). The detailed study of the ScMATE3 promoter may be the key to understanding how this gene confers Al tolerance, since his expression is not induced by Al.

Up to date, two different external Al resistance mechanisms were described in rye that relies on the release of malate (*ScALMT1* – Collins et al., 2008) and citrate (*ScMATE1* – Santos et al., 2018; *ScMATE2* – Yokosho et al., 2010) from the root apices. Therefore, in this study we are possibly facing an internal tolerance mechanism. For sure, multiple mechanisms are operating in tandem in rye species to circumvent the Al phytotoxicity, showing that Al tolerance in *Secale* is a very complex trait. The same occurs with other crop species such as wheat (Ryan et al., 2009), rice (Ma et al., 2014), buckwheat (Chen et al., 2017) and *Arabidopsis* (Liu et al., 2009). Very recently, it was discovered a coordinated function between Al exclusion and internal Al tolerance mechanisms linked by ALMT1-mediated root malate exudation and NIP1;2-mediated Al uptake from the root cell wall (Wang et al., 2017a).

In conclusion, clear evidences observed in the *ScMATE3* coding sequences (Al-related SNPs), phylogenetic relationships and expression data allowed us to prove that this gene have a role in Al tolerance, being a strong candidate to control the *Alt1* locus previously described. This work permit us to consider another mechanism operating in rye. Further investigations will be needed to ascertain the exact function of the *ScMATE3* gene.

Acknowledgments

This work was supported by research PhD grant from Fundação para a Ciência e Tecnologia de Portugal (SFRH/BD/65040/2009). We are grateful to the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) and to The National Small Grains Collection (NSGC) of the United States Department of Agriculture - Agricultural Research Service (USDA-ARS) for supplying us with seeds.

References

Aniol, A., and Gustafson, J. P. (1984). Chromosome location of genes controlling aluminium tolerance in wheat, rye and triticale. *Can. J. Genet. Cytol.* 26, 701–705.

Bauer, E., Schmutzer, T., Barilar, I., Mascher, M., Gundlach, H., Martis, M. M., et al. (2017). Towards a whole-genome sequence for rye (*Secale cereale* L.). *Plant J.* 89, 853–869. doi:10.1111/tpj.13436.

Benito, C., Silva-Navas, J., Fontecha, G., Hernández-Riquer, M. V., Eguren, M., Salvador, N., et al. (2010). From the rye *Alt3* and *Alt4* aluminum tolerance loci to orthologous genes in other cereals. *Plant Soil* 327, 107–120. doi:10.1007/s11104-009-0035-9.

Chen, L., Liu, Y., Liu, H., Kang, L., Geng, J., Gai, Y., et al. (2015). Identification and expression analysis of *MATE* genes involved in flavonoid transport in blueberry plants. *PLoS One* 10, 1–20. doi:10.1371/journal.pone.0118578.

Chen, W. W., Xu, J. M., Jin, J. F., Lou, H. Q., Fan, W., and Yang, J. L. (2017). Genome-wide transcriptome analysis reveals conserved and distinct molecular mechanisms of Al resistance in buckwheat (*Fagopyrum esculentum* moench) leaves. *Int. J. Mol. Sci.* 18, 1–17. doi:10.3390/ijms18091859.

Collins, N. C., Shirley, N. J., Saeed, M., Pallotta, M., and Gustafson, J. P. (2008). An *ALMT1* gene cluster controlling aluminum tolerance at the *Alt4* locus of rye (*Secale cereale* L.). *Genetics* 179, 669–682. doi:10.1534/genetics.107.083451.

Contreras, R., Figueiras, A. M., Gallego, F. J., and Benito, C. (2014). *Brachypodium distachyon*: A model species for aluminium tolerance in Poaceae. *Funct. Plant Biol.* 41, 1270–1283. doi:10.1071/FP13362.

Debeaujon, I., Peeters, A., and Leon-Kloosterziel, KM, Koornneef, M. (2001). The *TRANSPARENT TESTA12* gene of *Arabidopsis* encodes a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. *Plant Cell* 13, 853–871.

Draper, J., Mur, L. a. J., Jenkins, G., Ghosh-Biswas, G. C., Bablak, P., Hasterok, R., et al. (2001). *Brachypodium distachyon*. A New Model System for Functional Genomics in Grasses. *Plant Physiol.* 127, 1539–1555. doi:10.1104/pp.010196.1.

Ferreira, J. R., Faria, B. F., Junior, M. C., Delatorre, C. A., Minella, E., and Pereira, J. F. (2017). Is a non-synonymous SNP in the *HvAACT1* coding region associated with acidic soil tolerance in barley? *Genet. Mol. Biol.* 40, 480–490. doi:10.1590/1678-4685-GMB-2016-0225.

Fontecha, G., Silva-Navas, J., Benito, C., Mestres, M. A., Espino, F. J., Hernández-Riquer, M. V., et al. (2007). Candidate gene identification of an aluminum-activated organic acid transporter gene at the *Alt4* locus for aluminum tolerance in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 114, 249–260. doi:10.1007/s00122-006-0427-7.

Frank, S., Keck, M., Sagasser, M., Niehaus, K., Weisshaar, B., and Stracke, R. (2011). Two differentially expressed *MATE* factor genes from apple complement the *Arabidopsis* transparent testa12 mutant. *Plant Biol* 13, 42–50.

Fujii, M., Yokosho, K., Yamaji, N., Saisho, D., Yamane, M., Takahashi, H., et al. (2012). Acquisition of aluminium tolerance by modification of a single gene in barley. *Nat. Commun.* 3, 713. doi:10.1038/ncomms1726.

Furukawa, J., Yamaji, N., Wang, H., Mitani, N., Murata, Y., Sato, K., et al. (2007). An

aluminum-activated citrate transporter in barley. *Plant Cell Physiol.* 48, 1081–1091.

Gallego, F. J., and Benito, C. (1997). Genetic control of aluminium tolerance in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 95, 393–399.

Gallego, F. J., Calles, B., and Benito, C. (1998) Molecular markers linked to the aluminium tolerance gene *Alt1* in rye (*Secale cereale* L.). *Theor. Appl. Genet*. 97, 1104–1109. doi: 10.1007/s001220050997.

Gomez, C., Terrier, N., Torregrosa, L, Vialet, S., Fournier-Level, A., Verries, C., et al. (2009). Grapevine MATE-type proteins act as vacuolar H^+-dependent acylated anthocyanin transporters. *Plant Physiol* 150, :402–415.

Herrero, J., Muffato, M., Beal, K., Fitzgerald, S., Gordon, L., Pignatelli, M., et al. (2016). Ensembl comparative genomics resources. *Database J. Biol. Databases Curation, 2016, bav096.* doi:10.1093/database/bav096.

Huang, C. F., Yamaji, N., Chen, Z., and Ma, J. F. (2012). A tonoplast-localized half-size ABC transporter is required for internal detoxification of aluminum in rice. *Plant J.* 69, 857–867. doi:10.1111/j.1365-313X.2011.04837.x.

Kim, B. Y., Baier, A. C., Somers, D. J., and Gustafson, J. P. (2001). Aluminium tolerance in triticale, wheat and rye. *Euphytica* 120, 329–337.

Kochian, L. V., Hoekenga, O. A., and Piñeros, M. A. (2004). How Do Crop Plants Tolerate Acid Soils? Mechanisms of Aluminum Tolerance and Phosphorous Efficiency. *Annu. Rev. Plant Biol.* 55, 459–493. doi:10.1146/annurev.arplant.55.031903.141655.

Larsen, P., Cancel, J., Rounds, M., and Ochoa, V. (2007). *Arabidopsis ALS1* encodes a root tip and stele localized half type ABC transporter required for root growth in an aluminum toxic environment. *Planta* 225, 1447–1458.

Librado, P., and Rozas, J. (2009). DnaSP v5.1: a software for comprehensive analysis of DNA polymorphism data. -. *Bioinformatics* 25, 1451–1452.

Ling, H.-Q., Zhao, S., Liu, D., Wang, J., Sun, H., Zhang, C., et al. (2013). Draft genome of the wheat A-genome progenitor Triticum urartu. *Nature* 496, 87–90. doi:10.1038/nature11997.

Liu, J., Li, Y., Wang, W., Gai, J., and Li, Y. (2016). Genome-wide analysis of MATE transporters and expression patterns of a subgroup of *MATE* genes in response to aluminum toxicity in soybean. *BMC Genomics* 17, 1–15. doi:10.1186/s12864-016-2559-8.

Liu, J., Magalhaes, J. V., Shaff, J., and Kochian, L. V. (2009). Aluminum-activated citrate and malate transporters from the *MATE* and *ALMT* families function independently to confer *Arabidopsis* aluminum tolerance. *Plant J.* 57, 389–399. doi:10.1111/j.1365-313X.2008.03696.x.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real- Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *METHODS* 25, 402–408. doi:10.1006.

Ma, J. . F., Taketa, S., and Yang, Z. M. (2000). Aluminum tolerance genes on the short arm of chromosome *3R* are linked to organic acid release in triticale. *Plant Physiol* 122, 687–694. doi:10.1104/pp.122.3.687.

Ma, J. F., Chen, Z. C., and Shen, R. F. (2014). Molecular mechanisms of Al tolerance in gramineous plants. *Plant Soil* 381, 1–12. doi:10.1007/s11104-014-2073-1.

Ma, J. F., and Hiradate, S. (2000). Form of aluminium for uptake and translocation in buckwheat (*Fagopyrum esculentum* Moench). *Planta* 211, 355–360.

Ma, J. F., Hiradate, S., Nomoto, K., Iwashita, T., and Matsumoto, H. (1997). Internal detoxification mechanism of Al in hydrangea: identification of Al form in the leaves. *Plant Physiol.* 113, 1033–1039.

Ma, J. F., and Ryan, P. R. (2010). Foreword: Understanding how plants cope with acid soils. *Funct. Plant Biol.* 37, 2008–2011. doi:10.1071/FPv37n4-FO.

Ma, Y., Li, C., Ryan, P. R., Shabala, S., You, J., Liu, J., et al. (2016). A new allele for aluminium tolerance gene in barley (*Hordeum vulgare* L.). *BMC Genomics* 17, 186. doi:10.1186/s12864-016-2551-3.

Magalhaes, J. V. (2010). How a microbial drug transporter became essential for crop cultivation on acid soils: Aluminium tolerance conferred by the multidrug and toxic compound extrusion (*MATE*) family. *Ann. Bot.* 106, 199–203. doi:10.1093/aob/mcq115.

Maron, L. G., Piñeros, M. A., Guimarães, C. T., Magalhaes, J. V., Pleiman, J. K., Mao, C., et al. (2010). Two functionally distinct members of the *MATE* (multi-drug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. *Plant J.* 61, 728–740. doi:10.1111/j.1365-313X.2009.04103.x.

Martis, M. M., Zhou, R., Haseneyer, G., Schmutzer, T., Vrana, J., Kubalakova, M., et al. (2013). Reticulate Evolution of the Rye Genome. *Plant Cell* 25, 3685–3698. doi:10.1105/tpc.113.114553.

Matos, M., Camacho, M. V., Pérez-Flores, V., Pernaute, B., Pinto-Carnide, O., and Benito, C. (2005). A new aluminum tolerance gene located on rye chromosome arm *7RS*. *Theor. Appl. Genet.* 111, 360–369. doi:10.1007/s00122-005-2029-1.

Miftahudin, T., Scoles, G., and Gustafson, J. (2002). AFLP markers tightly linked to the aluminum tolerance gene *Alt3* in rye. *Theor. Appl. Genet.* 104, 626–631. doi:10.1007/s00122-001-0782-3.

Naranjo, T., and Fernández-Rueda, P. (1991). Homoeology of rye chromosome arms to wheat. *Theor. Appl. Genet.* 82, 577–586. doi:10.1007/BF00226793.

Negishi, T., Oshima, K., Hattori, M., Kanai, M., Mano, S., Nishimura, M., et al. (2012). Tonoplast- and Plasma Membrane-Localized Aquaporin-Family Transporters in Blue Hydrangea Sepals of Aluminum Hyperaccumulating Plant. *PLoS One* 7. doi:10.1371/journal.pone.0043189.

Parinthawong, N., Cottier, S., Buchala, A., Nawrath, C., and Métraux, J.-P. (2015). Localization and expression of EDS5H a homologue of the SA transporter EDS5. *BMC Plant Biol.* 15, 135. doi:10.1186/s12870-015-0518-1.

Pinto-Carnide, O., and Guedes-Pinto, H. (1999). Aluminium tolerance variability in rye and wheat Portuguese germplasm. *Genet. Resour. Crop Ev.* 46, 81–85.

Ryan, P. R., Raman, H., Gupta, S., Horst, W. J., and Delhaize, E. (2009). A Second Mechanism for Aluminum Resistance in Wheat Relies on the Constitutive Efflux of Citrate from Roots. *Plant Physiol.* 149, 340–351. doi:10.1104/pp.108.129155.

Ryan, P. R., Raman, H., Gupta, S., Sasaki, T., Yamamoto, Y., and Delhaize, E. (2010a). The multiple origins of aluminium resistance in hexaploid wheat include *Aegilops tauschii* and more recent cis mutations to *TaALMT1*. *Plant J.* 64, 446–455. doi:10.1111/j.1365-313X.2010.04338.x.

Rychlik, W. (2007). OLIGO 7 primer analysis software. *Methods Mol. Biol.* 402, 35–59.

Samac, D. A., and Tesfaye, M. (2003). Plant improvement for tolerance to aluminum in acid soils – a review. *Plant Cell. Tissue Organ Cult.* 75, 189–207.

Santos, E., Benito, C., Silva-Navas, J., Gallego, F. J., Figueiras, A. M., Pinto-Carnide, O., et al. (2018). Characterization, genetic diversity, phylogenetic relationships, and expression of the aluminum tolerance *MATE1* gene in *Secale* species. *Biol. Plant.* 62, 109–120. doi:10.1007/s10535-017-0749-0.

Sasaki, T., Yamamoto, Y., Ezaki, B., Katsuhara, M., Ahn, S., Ryan, P., et al. (2004). A wheat gene encoding an aluminum-activated malate transporter. *Plant J* 37, 645–653.

Shoji, T., Inai, K., Yazaki, Y., Sato, Y., Takase, H., Shitan, N., et al. (2009). Multidrug and toxic compound extrusion-type transporters implicated in vacuolar sequestration of nicotine in tobacco roots. *Plant Physiol* 149, 708–718.

Silva-Navas, J., Benito, C., Téllez-Robledo, B., El-Moneim, D. A., and Gallego, F. J. (2012). The *ScAACT1* gene at the *Qalt5* locus as a candidate for increased aluminum tolerance in rye (*Secale cereale* L.). *Mol. Breed.* 30, 845–856. doi:10.1007/s11032-011-9668-5.

Takanashi, K., Shitan, N., and Yazaki, K. (2014). The multidrug and toxic compound extrusion (*MATE*) family in plants. *Plant Biotechnol.* 31, 417–430. doi:10.5511/plantbiotechnology.14.0904a.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol Biol Evol.* 30, 2725–2729.

Tolrà, R., Barceló, J., and Poschenrieder, C. (2009). Constitutive and aluminium-induced patterns of phenolic compounds in two maize varieties differing in aluminium tolerance. *J. Inorg. Biochem.* 103, 1486–1490. doi:10.1016/j.jinorgbio.2009.06.013.

Tovkach, A., Ryan, P. R., Richardson, A. E., Lewis, D. C., Rathjen, T. M., Ramesh, S., et al. (2013). Transposon-mediated alteration of *TaMATE1B* expression in wheat confers constitutive citrate efflux from root apices. *Plant Physiol.* 161, 880–92. doi:10.1104/pp.112.207142.

Vogel, J. P., Garvin, D. F., Mockler, T. C., Schmutz, J., Rokhsar, D., Bevan, M. W., et al. (2010). Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature* 463, 763–768. doi:10.1038/nature08747.

Von Uexkull, H. R., and Mutert, E. (1995). Global extent , development and economic impact of acid soils. *Plant Soil* 171, 1–15.

Wang, L., Bei, X., Gao, J., Li, Y., Yan, Y., and Hu, Y. (2016). The similar and different evolutionary trends of *MATE* family occurred between rice and *Arabidopsis thaliana*. *BMC Plant Biol.* 16, 207. doi:10.1186/s12870-016-0895-0.

Wang, Y., Li, R., Li, D., Jia, X., Zhou, D., Li, J., et al. (2017a). NIP1;2 is a plasma membrane-localized transporter mediating aluminum uptake, translocation, and tolerance in *Arabidopsis*. *Proc. Natl. Acad. Sci.* 114, 5047–5052. doi:10.1073/pnas.1618557114.

Wang, Y., Xu, L., Thilmony, R., You, F. M., Gu, Y. Q., and Coleman-Derr, D. (2017b). PIECE 2.0: An update for the plant gene structure comparison and evolution database. *Nucleic Acids Res.* 45, 1015–1020. doi:10.1093/nar/gkw935.

Yokosho, K., Yamaji, N., Fujii-Kashino, M., and Ma, J. F. (2016). Functional Analysis of a MATE Gene *OsFRDL2* Revealed its Involvement in Al-Induced Secretion of Citrate, but a Lower Contribution to Al Tolerance in Rice. *Plant Cell Physiol.* 57, 976–985. doi:10.1093/pcp/pcw026.

Yokosho, K., Yamaji, N., and Ma, J. F. (2010). Isolation and characterization of two *MATE* genes in rye. *Funct. Plant Biol.* 37, 296–303. doi:10.1071/FP09265.

Zhao, J., and Dixon, R. (2009). MATE transporters facilitate vacuolar uptake of epicatechin 3′-O-glucoside for proanthocyanidin biosynthesis in *Medicago truncatula* and *Arabidopsis*. *Plant Cell* 21, 2323–2340.

Zhao, J., Huhman, D., Shadle, G., He, X., Sumner, L., Tang, Y., et al. (2011). *MATE2* mediates vacuolar sequestration of flavonoid glycosides and glycoside malonates in *Medicago truncatula*. *Plant Cell* 23, 1536–1555.

Zhu, H., Wu, J., Jiang, Y., Jin, J., Zhou, W., Wang, Y., et al. (2016). Genome wide analysis of *MATE*-type gene family in maize reveals microsynteny and their expression patterns under aluminum treatment. *J. Genet.* 95, 691–704. doi:10.1007/s12041-016-0686-2.

Supplementary data

Table S1. Sequence of the primer pairs designed and employed in this work with the annealing temperature (Ta) and the purpose of each.

Primer	Sequence (5' → 3')	Ta	Purpose
Alt1ex1 – R	GGGCCGCAGATCCAGAG	62 ºC	Gene identification
Alt1ex1 – F	GCAGAGCTCGGCGTCA	62 ºC	Gene identification
Alt1ex7a9 – R	ATCAAATTGCGGTTAG	52 ºC	Gene identification
Alt1ex7a9 – F	ATTCGAAATCGCAGCTC	52 ºC	Gene identification
Alt1ex8a9 – R	CCGGCATAAAGGACTGT	52 ºC	Gene identification
Alt1ex8a9 – F	CGCGACTTCTATGGGAG	52 ºC	Gene identification
Alt1IMP1 – R	GACAATGACATCCAAGGTATCAT	52 ºC	Gene chromosome location
Alt1IMP1 – F	CCCAAACAGTGCACATGGATA	52 ºC	Gene chromosome location
Alt1IMP2 – R	TGGGTTATTAATGATCTGTGTCC	52 ºC	Gene chromosome location
Alt1IMP2 – F	CGAGATTAAAGTAAACAAAAGAT	52 ºC	Gene chromosome location
cDNAMATE6RS – 1R	CATCCTCCTATGTGGCGTTC	55 ºC	Full-lenght cDNA
cDNAMATE6RS – 1F	GCGTCATCCAGCCATGTCCG	55 ºC	Full-lenght cDNA
cDNAMATE6RS – 2R	TTGTAGAGCGCTTCCAAACC	58 ºC	Full-lenght cDNA
cDNAMATE6RS – 2F	GGCGTCATCCAGCCATGTCC	58 ºC	Full-lenght cDNA
cDNAMATE6RS – 3e4R	ACATGCTTGCGTAACTGC	54 ºC	Full-lenght cDNA
cDNAMATE6RS – 3F	CGGGACGTGTTGGTGTTC	54 ºC	Full-lenght cDNA
cDNAMATE6RS – 4F	CGGGCTCTGGATCTG	54 ºC	Full-lenght cDNA
qScMATE6RS – R	TGAAATCGCAGCTCCTGTTT	60 ºC	Quantitative RT-PCR
qScMATE6RS – F	CCTGATGGCCTGCAAGAG	60 ºC	Quantitative RT-PCR
qScMATE3 – R	GCGCTTCCAAACCGACTC	60 ºC	Quantitative and Semi-quantitative RT-PCR
qScMATE3 – F	GAACATTGCTGGCTGGAAGG	60 ºC	Quantitative and Semi-quantitative RT-PCR

Table S2. Software programs used to deduce the structure of ScMATE3 proteins.

Program	URL address	Reference
TopPred2	http://www.sbc.su.se/~erikw/toppred2/	Claros and von Heijne (1994)
SOSUI	http://harrier.nagahama-i-bio.ac.jp/sosui/	Hirokawa et al. (1998)
PSIpred	http://bioinf.cs.ucl.ac.uk/psipred/	McGuffin et al. (2000)
TMHMM	http://www.cbs.dtu.dk/services/TMHMM/	Krogh et al. (2001)
HMMTOP	http://www.enzim.hu/hmmtop	Tusnády and Simon (2001)
DAS	http://www.enzim.hu/DAS/DAS.html	Cserzo et al. (2002)

References

Claros, M. G., and von Heijne, G. (1994). TopPred II: an improved software for membrane protein structure redictions. *CABIOS* 10, 685–686.

Cserzo, M., Eisenhaber, F., Eisenhaber, B., and Simon, I. (2002). On filtering false positive transmembrane protein predictions. *Protein Eng* 15, 745–752.

Hirokawa, T., Boon-Chieng, S., and Mitaku, S. (1998). SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* 14, 378–379.

Krogh, A., Larsson, B., von Hiejne, G., and Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 305, 567–580.

McGuffin, L. J., Bryson, K., and Jones, D. T. (2000). The PSIPRED protein structure prediction server. *Bioinformatics* 16, 404–405.

Tusnády, G. E., and Simon, J. (2001). The HMMTOP transmembrane topology prediction server. *Bioinformatics* 17, 849–850.

Table S3. Diversity parameters obtained comparing fourteen different sequences of cDNA from different species/subspecies of *Secale* using the DnaSP v. 5.0 software.

***Secale* genus**	**Ex1**	**Ex2**	**Ex3**	**Ex4**	**Ex5**	**Ex6**	**Ex7**	**Ex8**	**Ex9**	**Ex10**	**Ex11**	**Ex12**	**Ex13**	**Ex14**	**cDNA**
Exon size (bp)	61	95	109	56	56	139	138	69	111	129	78	72	63	102	1278
IMS	60	91	104	53	54	133	132	63	107	126	75	70	58	101	1227
VPS	1	4	5	3	2	6	6	6	4	3	3	2	5	1	51
SVS	1	1	4	3	2	5	5	6	2	3	2	0	2	1	37
PIS	0	3	1	0	0	1	1	0	2	0	1	2	3	0	14
TNSC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16
TNRC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	35
h	2	5	5	2	3	6	6	3	5	4	4	3	6	2	13
Hd	0,143	0,791	0,593	0,143	0,275	0,681	0,736	0,275	0,67	0,396	0,396	0,648	0,813	0,143	0,989
ND (Pi)	0,00234	0,01735	0,00857	0,00765	0,0051	0,00775	0,00876	0,01242	0,00822	0,00332	0,00704	0,01114	0,02128	0,0014	0,00842
k	0,143	1,648	0,934	0,429	0,286	1,077	1,209	0,857	0,912	0,429	0,549	0,802	1,341	0,143	10,758
C	0,984	0,958	0,954	0,946	0,964	0,957	0,957	0,913	0,964	0,977	0,962	0,972	0,921	0,99	0,96

Ex – exon; IMS – invariable (monomorphic) sites; VPS – variable (polymorphic) sites; SVS – singleton variable sites; PIS – parsimony informative sites; TNSC – total number of synonymous changes; TNRC – total number of replacement changes; h – number of haplotypes; Hd – Haplotype (gene) diversity; ND (Pi) – nucleotide diversity; k – average number of nucleotide differences; C – sequence conservation.

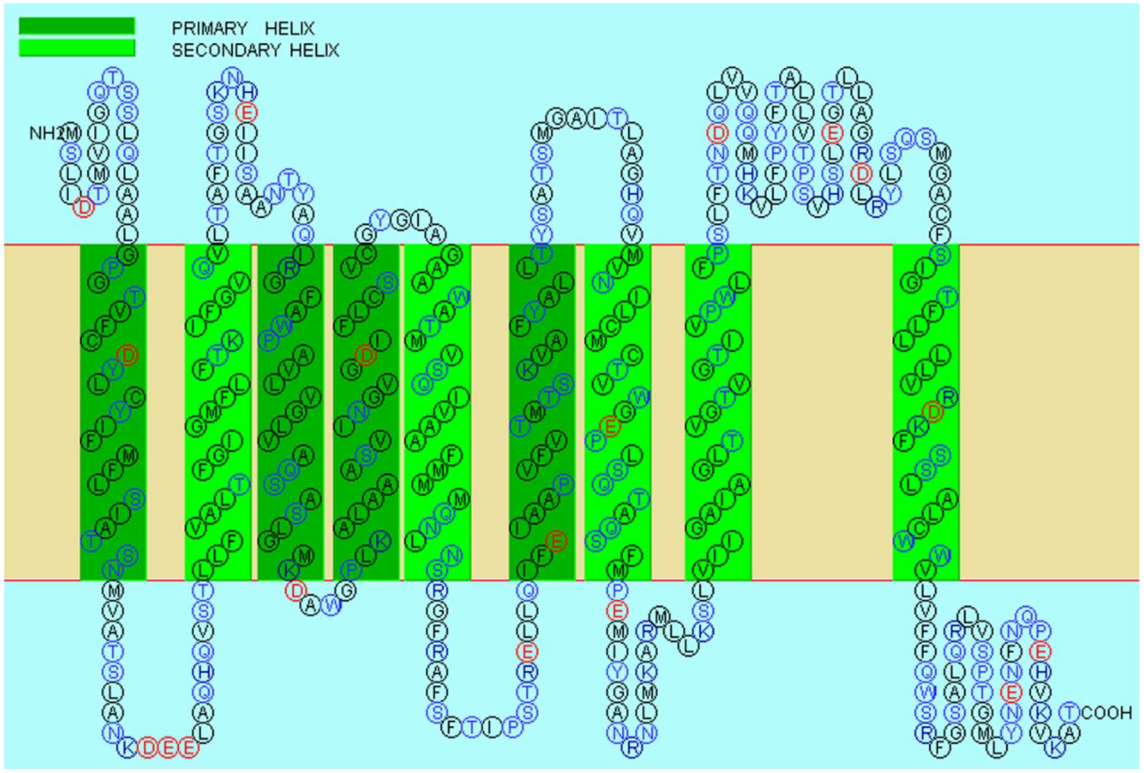


Figure S1. Transmembrane domains of the ScMATE3 protein of *S. segetale* predicted by SOSUI software. This software predicted nine transmembrane domains for all ScMATE3 proteins analyzed except for *S. strictum*, with a prediction of ten transmembrane domains. The synonymous changes (blue circles), the conserved amino acids (yellow circles) and the non-synonymous changes (white circles) detected are indicated.

CHAPTER IV

General Discussion and Concluding Remarks

IV. General Discussion and Concluding Remarks

With the current rate of human population growth, an additional demand for food will be required around the world. Cereals sustain the bulk of mankind's basic nutritional needs with more than 50% of our food coming from the three major cereals wheat, maize and rice. Also barley, sorghum, millet, oats and rye make important contributions for global agriculture and diet (Morris and Bryce, 2000). Even with the impressive growth in food production in the recent decades, cereal production alone will have to rise about 20% of recent production levels to satisfy the increasing food demand (FAO, 2018). Therefore, major soil constraints currently limiting crop productivity such as acidic soils where Al toxicity affects directly the grain quality and the plant yield (Kochian et al., 2005) need to be overcome. A better understanding of fundamental plant science and its application in plant breeding is the key for the enhancement of food production since much of the increase in both quantity and quality of the harvested grains can be attributed to improvements in cereal crops.

Genetic diversity is an important prerequisite for breeding programs since different loci and alleles of loci are crucial for crop improvement. In the last two decades, DNA-based markers have revolutionized plant biotechnology, and became the marker of choice to study crop genetic diversity partly because of its great advantages (Amom and Nongdam, 2017). For a better characterization of the rye genome for breeding purposes, the first aim of this thesis was the assessment of the genetic diversity among different cultivated and wild ryes through molecular markers (Chapter II-1) (Santos et al., 2016). To an overall variability study of the different rye species/subspecies and cultivars, bulks were effectively used applying ISSR and RAPD markers with a dominant inheritance. Bulk method includes several distinct genotypes belonging to a particular taxon, which allow a better interspecific and intercultivar genetic characterization. Several authors used bulks in their studies and confirmed their effectiveness and usefulness (Loarce et al., 1996; Matos et al., 2001; Fernández et al., 2002; Tanyolac, 2003). High polymorphism values were found in *Secale* genus with both markers evidencing a high variability, essential for the reduction of biotic and abiotic stresses vulnerability. Genetic diversity studies in *Secale* genus have been successfully performed using different molecular markers (Matos et al., 2001; Shang et al., 2006; Ren et al., 2011; Chikmawati et al., 2012; Bolibok-Brągoszewska et al., 2014; Hagenblad et al., 2016; Targońska et al., 2016;

Petrovičová et al., 2017), however, this was the first published work with wild ryes applying both ISSR and RAPD markers.

The genetic variability of Al-related genes within *Secale* genus was also studied (Chapters II-1, III-1 and III-3). The diversity parameters of two genes belonging to *MATE* family, *ScMATE1* (Chapters II-1 and III-1) and *ScMATE3* (Chapters III-3), were analyzed and a great genetic variability was found within *Secale* genus in both genes. At least two copies of the *ScMATE1* gene were found in the species *S. sylvestre* and *S. vavilovii*, with one and two 3 bp insertion, respectively, occurring on the exon 1 of sensitive genotypes (Santos et al., 2016, 2018). Silva-Navas et al. (2012) also found in Riodeva (Al-sensitive) an additional cDNA with the same two INDELs of *S. vavilovii*. These INDELs have a potential relation with Al sensitivity/tolerance indicating a probable evolution for the Al tolerance trait in *Secale*. Collins et al. (2008) and Maron et al. (2013) also found several copies of the genes *ScALMT1* and *ZmMATE1* with a correlation to Al tolerance in rye and in maize, respectively. In turn, exclusive species/subspecies SNPs probably related to Al tolerance were found in the coding sequences of *ScMATE3* gene, which is also a signal of this trait evolution. Several authors reported SNPs as being related to the Al tolerance trait in different cereals (Fontecha et al., 2007; Furukawa et al., 2007; Tovkach et al., 2013; Ferreira et al., 2017). A wide variability was also found in the deduced proteins from both genes, however, changes detected in the different proteins do not alter their transmembrane structure being the main function, probably, not affected. Taken together, these data suggests that rye and wild rye can easily adapt to any adverse environmental conditions being more likely to evolve.

To better understand the evolution of rye genome, genetic relationships among cultivated and wild ryes were assessed through molecular markers (Chapter II-1) and in specific Al-related genes (Chapters III-1 and III-3). Firstly, both ISSR and RAPD markers were used for a more reliable study and a greater genome coverage allowing a better rye characterization (Santos et al., 2016). Secondly, rye phylogenies were inferred in the genes *ScMATE1* and *ScMATE3* to understand the evolution of these genes and the possible relation with the Al tolerance trait in rye (Santos et al., 2018). It is consistent that *S. strictum* and *S. sylvestre* are the oldest ryes but which of them is the common ancestor is still a subject under discussion although most of the studies point to *S. sylvestre* (Reddy et al., 1990; De Bustos and Jouve, 2002; Chikmawati et al., 2005; Shang et al., 2006; Ren et al., 2011; Bolibok-Brągoszewska et al., 2014; Al-Beyroutiová et al., 2016) as occurred in *ScMATE3* gene analysis. In turn, in *ScMATE1* gene analysis both species/subspecies

share similar coding and protein sequences and own equal ancestry degree. Within the cultivated subspecies (*S. cereale* ssp. *cereale*), we could verify the landraces to be grouped by geographical location and to be apart from the rye varieties, possibly because of a common origin or as an environment adaptation. Using different molecular markers, some authors clustered ryes by geographical location (Ma et al., 2004; Hagenblad et al., 2016) while others didn't found clear geographic patterns (Chikmawati et al., 2012; Bolibok-Brągoszewska et al., 2014), which may be due to the distinct features of each DNA-based marker. *S. ancestrale* and *S. segetale* also considered as *S. cereale* subspecies, remained always close to the cultivated representative making clear their relationship. Finally, *S. vavilovii* seems to be closely related to the *S. cereale* subspecies and shows no obvious molecular differences to be an individual rye species. In the Chapter II-1, the six *ScMATE1* gene exons analyzed (about half) made *S. vavilovii* closer to *S. strictum* for being quite conserved among them. The evidences described above lead us to recognize only three species in *Secale* (*S. cereale*, *S. strictum* and *S. sylvestre*) which is in accordance with this genus last taxonomic revision of Frederiksen and Petersen (1998). Several molecular studies achieved in the last decade also agrees with this taxonomic classification (Chikmawati et al., 2005; Shang et al., 2006; Ren et al., 2011; Bolibok-Brągoszewska et al., 2014; Al-Beyroutiová et al., 2016; Hagenblad et al., 2016). Since the most ancient ryes have less Al tolerance, it´s possible to say that Al-tolerance is an evolutive trait where crops adapted to acidic environments and progressed to overtake this abiotic adversity.

Phylogenetic relationships were also performed to infer about the function of the deduced ScMATE1 (Chapter III-1) and ScMATE3 (Chapter III-3) proteins, being the last discussed in more detail further on. *ScMATE1* gene is orthologous of genes involved in the Al stress response of barley (*HvMATE1*, Furukawa et al., 2007; Wang et al., 2007), wheat (*TaMATE1*, Ryan et al., 2009; Garcia-Oliveira et al., 2014), maize (*ZmMATE1*, Maron et al., 2010) and *Brachypodium distachyon* (*BdMATE1*, Contreras et al., 2014). So, a role in Al tolerance trait was inferred for the *ScMATE1* gene which was reinforced by synteny associations (Naranjo et al., 1987; Gale and Devos, 1998; Collins et al., 2008; Silva-Navas et al., 2012). However, *ScMATE1* is not orthologous of the genes involved in the Al tolerance of sorghum (*SbMATE*, Magalhaes et al., 2007) but it is orthologous of *OsFRDL1* gene in rice which is a citrate transporter in charge for Fe translocation with no Al tolerance correlation (Yokosho et al., 2009, 2016b). Expression studies of *ScMATE1* gene were then carried out, which will be discussed later. On the other hand,

ScMATE2 gene which was related to Al-tolerance in rye (Yokosho et al., 2010) grouped with other putative orthologous genes with a similar function such as *OsFRDL2* (rice, Yokosho et al., 2016), *ZmMATE2* (maize, Maron et al., 2010) and *BdMATE2* (*B. distachyon*, Contreras et al., 2014). Since orthologous genes share common ancestor, it is most likely to preserve a similar function in the course of evolution being a good way to predict about new gene function.

Al tolerance of cultivated and wild ryes was evaluated through hydroponic assays which have several advantages (Samac and Tesfaye, 2003), using both root staining and root regrowth measurement (Chapter II-2 and III-1). Such techniques are appropriate tools for Al tolerance screening since the first and main morphological symptom of Al phytotoxicity is the inhibition of the root elongation, as we also observed in this work. Plants with an unstained root segment (root regrowth) after an Al exposed heavily stained root, had the ability to recover easily after Al contact and were considered Al-tolerant. On the other hand, plants with intensely stained root apices (no root regrowth) had the root system damaged and had difficulties to recover and thus, were considered Al-sensitive. The greater plant ability to recover, the greater their Al tolerance degree. We were able to classify all ryes at study concerning Al tolerance at 5 ppm (185 μM) (Chapter II-2) and 150 μM (Chapter III-3) Al concentration. Gallego and Benito (1997) concluded that this latter Al concentration was the most discriminating among different rye cultivars. Although wild rye accessions used in Chapter II and III were different, the same Al tolerance classification was obtained in each species/subspecies, thus they possibly share a common genetic background. The high Al tolerance found in the *Secale* genus, even at huge Al concentrations, clearly put rye as one of the most Al-tolerant cereal which, together with the vast genetic diversity found for this trait both within and between different rye species/subspecies/cultivars, becomes a valuable source of germplasm for crop improvement. It is important to emphasize that even the Al-sensitive rye genotypes are more Al-tolerant than the most tolerant genotypes of wheat (Garcia-Oliveira et al., 2016), barley (Echart et al., 2002) and *Brachypodium* spp. (Contreras et al., 2014). In this work, both Riodeva and *S. sylvestre* classified as sensitive presented tiny root regrowths in some genotypes at 150 μM which is a toxic and fatal Al concentration for these plant species just cited. The high Al tolerance, genetically diversified in rye cultivars was also witnessed by other authors (Bona et al., 1993; Pinto-Carnide and Guedes-Pinto, 1999; Kim et al., 2001) but, the first Al tolerance screening data of wild ryes was published in this work (Santos et al., 2018). According to Santos et al. (2018), cultivated ryes (except

Riodeva) are more Al tolerant than wild ryes whose are classified by descending order of Al tolerance as *S. segetale* (tolerant) < *S. ancestrale* (tolerant) < *S. vavilovii* (tolerant) < *S. strictum* (moderately tolerant) < *S. sylvestre* (sensitive).

A better understanding of the Al resistance mechanisms as well as the physiological and genetic processes related is imperative for successful breeding strategies. Crop species have evolved mechanisms to survive in acid soils with Al toxicity which consists in Al tolerance (internal) and Al exclusion (external). In the former, Al is sequestered and detoxified or/and translocated after it enters the root apex whereas the latter is based on exudation of Al-chelating organic compounds into the rhizosphere, preventing Al from entering the root tip (Kochian et al., 2015). Al toxicity have multiple target sites within root cells evidencing the complexity of Al resistance mechanisms (Aggarwal et al., 2015). In this thesis, four Al-related root cellular disturbances were highlighted in cultivated and wild ryes (Chapter III-2). In all cases, Al content in root tips was directly proportional to ROS and lipid peroxidation production as well as death cell. All these physiological symptoms showed a positive correlation with the inhibition of root elongation and were suitable to identify tolerant and sensitive rye genotypes and thus, for Al tolerance characterization. This correlation was corroborated in other crop species (Yamamoto et al., 2001; Contreras et al., 2014; Awasthi et al., 2017). Recently, phytohormones were implicated in the negative regulation of Al-induced root growth inhibition (An et al., 2017; Zhang et al., 2018). Changes in root morphology were observed mainly in sensitive genotypes and the most Al-sensitive site was in the root meristem (DTZ) instead of root cap as occurred with sorghum (Sivaguru et al., 2013). Tolerant genotypes did not accumulate Al in root apices being able to exclude it, thus an Al-exclusion mechanism is operating in the Al-tolerant ryes of this thesis to avoid Al toxicity. In this type of mechanism is the most recognized and highlighted Al resistance mechanism namely the organic acids (OA) exudation from roots. Therefore, citric (Chapter III-1) and malic (Chapter III-2) acid release were quantified in the root ryes analyzed in this work. Both malate and citrate exudation were Al-induced in all wild rye species/subspecies unless *S. strictum* which was constitutive exudation but also can be involved in Al tolerance because of the great amount released. Within *S. cereale* variations were found: inbred lines showed the same behavior than this last subspecies but only with citrate, all the rest was Al-induced. Inducible pattern in both sensitive and tolerant genotypes was reported in earlier works (Ma et al., 2000; El-Moneim et al., 2014). Based on the timing of OA release two different Al-inducible patterns were found within *Secale*: OA efflux

immediately after Al exposure (I) or with a lag phase (II) (Ma, 2000). Up to date, only pattern II was reported in rye cultivars (Li et al., 2000; Yokosho et al., 2010) and there is still no data about wild ryes, being this work the first record (Santos et al., 2018).

The highlight of Al-related gene expression and function clarify the Al-resistance mechanisms implicated enabling the development of new and better Al-tolerant crops. Therefore, expression studies of nine candidate genes (*ScALMT1*, *ScMATE1*, *ScMATE2*, *ScMATE3*, *ScSTOP1*, *ScMDH1*, *ScMDH2*, *ScCu/ZnSOD* and *ScMnSOD*) were performed on rye shoots and root tips (Chapter III). Two main evidences were found in almost all the candidate genes for a role in Al resistance mechanisms in wild ryes. Firstly, most of the genes especially *ScALMT1* gene expressed more in roots which is the critical site for Al toxicity and, consequently, the most likely region for Al tolerance gene expression. Contrarily, *ScSTOP1* expressed more in shoots but only in the tolerant ryes suggesting a mechanism acting in shoots. On the other hand, mRNA quantity of *ScMATE3* gene after Al contact of shoots and roots varied according the species/subspecies: no differences were observed in most of them whereas *S. vavilovii* and *S. sylvestre* had more transcript in roots and shoots, respectively. A slight induction was observed in the leaves of *S. strictum* which together with the SNPs found in the coding sequence of the gene of this rye suggests a different mechanism such as an internal Al detoxification. However, a constitutive expression pattern was observed in the shoots of the remaining ryes (tolerant and sensitive). Also Ma et al. (2016) reported a different way to resist Al stress for a moderately Al-tolerant barley variety comparing to the tolerant ones. Moreover, a clear repression was detected in the roots of the sensitive rye (*S. sylvestre*) whereas the other ryes (tolerant) presented a constitutive expression pattern. Taken together, we concluded about a role of *ScMATE3* gene in the Al resistance mechanisms of the roots of the tolerant species/subspecies and of the shoots of *S. strictum*.

Secondly, all genes unless *ScMATE1* exhibited more transcript amount in the Al stressed roots of tolerant ryes than of the sensitive *S. sylvestre*. The same occurred in the shoots of *ScSTOP1*, reinforcing a possible mechanism operating in shoots. Expression studies with *ScMATE1* were extended to cultivated ryes whose were Al-upregulated and displayed a larger expression level than the wild ones (Chapter III-1). An environmental adaptation to acidic soils may have generated more Al-tolerant genotypes. The contribution of the *ScMATE1* gene in rye is complex: the sensitive ryes (Riodeva and *S. sylvestre*) displayed more transcripts than the tolerant ones and few ryes induced after Al stress including the sensitive ryes. As we seen earlier, sensitive ryes are tolerant compared

to other plant species and may thus have alleles conferring Al tolerance. Apparently, not all *ScMATE1* alleles contribute for Al tolerance. Some works found contributing alleles (Silva-Navas et al., 2012; Santos et al., 2018) while others didn't (Collins et al., 2008; Yokosho et al., 2010).

All candidate genes seems to have an active contribution on Al resistance mechanisms of, at least, one wild rye. There are many factors acting simultaneously that can influence Al tolerance of each rye species/subspecies, however, we inferred about a hypothetical contribution of the candidate genes in each of them according to the OA release and gene expression level (Fig. 1).

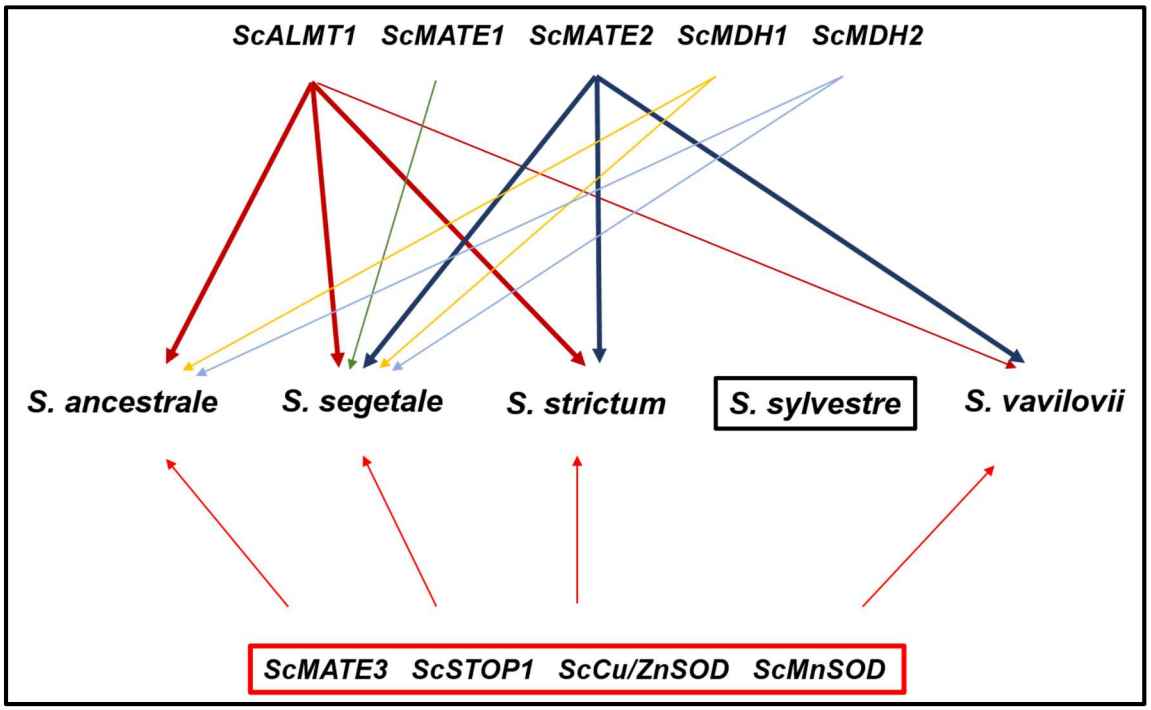


Figure 1. Scheme showing a hypothetical Al tolerance gene contribution in each rye species/subspecies based on expression data of roots and shoots as the OA exudation. Thick lines mean a greater role.

S. ancestrale exhibited a pattern II of OA release with both citrate and malate but only *ScALMT1* expression was Al-induced. The gene encoding the citrate transporter involved in the citrate secretion of this subspecies can be the constitutively expressed *ScMATE3* or another gene not studied in this thesis. On the other hand, *S. segetale* showed a pattern I with both OA exudation and was Al-upregulated in *ScMATE2*, *ScALMT1* and *ScMATE1* by descending order. This rye exhibited a highly Al-triggered OA efflux which can be the reason for his great Al tolerance. It has been reported that this pattern do not require genes, however, in this case genes seems to be involved in the Al resistance mechanisms of this subspecies. In turn, *S. vavilovii* varied between pattern I for malic acid and pattern II for

citric acid being the expression of *ScMATE2* and *ScALMT1* highly and slightly Al-upregulated, respectively. The induction of genes encoding malate transporters cannot be required for Al-stimulated malate efflux in this rye species. The moderately Al-tolerant *S. strictum* had a constitutive pattern of OA secretion but in high quantities and both *ScMATE2* and *ScALMT1* expression was greatly Al-induced. As we seen above, OA exudation of sensitive genotypes can also be Al-induced as occurred in this work. Usually, sensitive genotypes exude less OA quantity than the tolerant ones, however, wild ryes of this thesis can't be compared because each of them belongs to different species/subspecies and the amount of OA exudates may vary among them. For comparison purposes, OA release from both sensitive and tolerant ryes of *S. cereale* was quantified and we verified that Imperial (tolerant) had an higher Al-induced malate efflux than Riodeva (sensitive) whereas in citrate efflux Riodeva showed a constitutive pattern contrasting with the Al-induced of Imperial and the other inbred line P105 (tolerant) also was constitutively exuded but in higher quantity than Riodeva. The Al-triggered OA secretion of sensitive ryes seems to be regular and is made in smallest quantities than the tolerant. Finally, as we already seen *ScMATE3* also can contribute in the Al tolerance of each rye and both *ScMDH1* and *ScMDH2* seems to be involved in the malate metabolism of *S. ancestrale* and *S. segetale* because of their constitutive expression and the clear repression in *S. sylvestre*. Indeed, *ScCu/ZnSOD*, *ScMnSOD* and *ScSTOP1* genes were constitutively expressed being also involved in the Al resistance mechanisms of each rye. It is recalled that all these genes with a constitutive expression exhibited more transcripts in the tolerant ryes comparing to the sensitive. The constitutively higher gene expression was reported to be related to the Al-enhanced tolerance of rye relative cereals (Sasaki et al., 2004; Furukawa et al., 2007; Ryan et al., 2009; Tovkach et al., 2013; Contreras et al., 2014). The involvement of these constitutively expressed genes (*ScMATE3*, *ScMDH1*, *ScMDH2*, *ScCu/ZnSOD*, *ScMnSOD* and *ScSTOP1*) in Al tolerance must involve posttranslational processes (Kochian et al., 2015), though, the detailed study of their promoter may be the key to understanding how these genes confer Al tolerance. Both antioxidant genes (*ScCu/ZnSOD* and *ScMnSOD*) expressed inversely to oxidative stress production certainly taking also part on internal Al-detoxification. Apparently, each rye species/subspecies adopt different strategies to circumvent Al phytotoxicity where *ScALMT1* and *ScMATE2* have clearly a key role in their improved Al-tolerance because of the great Al-upregulation.

ScMATE3 is a new *MATE* gene isolated and characterized for the first time in this thesis (Chapter III-3). It is a candidate gene for controlling the *Alt1* locus in rye located on the short arm of the chromosome 6 (*6RS*) (Gallego et al., 1998a). To infer about his function, phylogenetic relationships were assessed among two other MATE proteins (*ScMATE1* and *ScMATE2*) already studied in rye along with their putative orthologous genes. Both *ScMATE1* and *ScMATE2* genes are much related with very similar exon-intron structure and grouped with citrate-transporting members which have been reported in Al detoxification and/or Fe translocation. Expression data revealed a complex contribution to Al-tolerance for *ScMATE1* and evidenced a clear implication of *ScMATE2* to this abiotic trait. Phylogenies indicated that *ScMATE3* is clearly different from these two *MATE* genes, however, its close relationships and similar gene structure suggest functions with some correlation. *ScMATE3* expression studies confirmed an involvement in Al resistance mechanisms as we seen earlier. Moreover, other evidences supporting this function were found, such as an orthologous gene in maize involved in this abiotic trait (Zhu et al., 2016) and the SNPs probably related to Al tolerance, reported above in *ScMATE3* cDNA sequences. The difference of MATE3 proteins seems to be the nature of the substrate, this one may transport phenolic compounds instead of citrate and according to his putative subcellular localization (vacuolar membrane) they could be involved in an internal Al tolerance mechanism. These conjectures were made based on the phylogenetic relationships obtained. An association with phylogeny and both transporter substrate specificity and subcellular location was reported by some authors (Chen et al., 2015; Liu et al., 2016; Wang et al., 2016). *ScMATE3* is a strong candidate to control the *Alt1* locus and we can be facing with another mechanism operating in rye, however, the exact function of this gene needs to be clarify.

Another goal of this thesis was the identification of Al tolerance linked markers with two marker systems (ISSRs and RAPDs) characterized above as highly polymorphic (Chapter II-2). The detection of 34 DNA markers (10 RAPDs and 24 ISSRs) able to distinguish between sensitive and tolerant rye genotypes, is the first step to complement conventional breeding strategies through marker assisted selection (MAS) for Al tolerance crop improvement. To simplify this task, these Al-tolerance linked DNA fragments can in future be converted into SCAR markers. The high number of putative Al-linked markers found is an indication of this abiotic trait complexity. Several DNA-based Al-linked markers have been found in rye through different molecular techniques (Gallego et al., 1998a, 1998b; Miftahudin et al., 2002; Camacho et al., 2005; Matos et al.,

2005; Fontecha et al., 2007; Benito et al., 2010), useful for developing rye chromosome physical maps. Furthermore, Contreras et al. (2017) were able to distinguish between Al tolerant and sensitive lines of *B. distachyon* and *B. hybridum* using SSR and ISSR markers.

The effectiveness of the molecular markers was verified in this work in different approaches (Chapter II). Both RAPD and ISSR markers revealed high polymorphism values in rye being good tools for genetic relationships and variability studies and for the identification of DNA fragments associated to Al tolerance. These two dominant markers were efficient both in bulk and individual genotypes analyses. In all cases, ISSRs were the most informative and effective molecular marker. However, both marker systems provide important resources for applied research such as MAS which has revolutionized breeding programs. With the advent of next generation sequencing (NGS) technology ultimate MAS tool will be provided to accelerate plant improvement research (Amom and Nongdam, 2017). Furthermore, RNA-sequencing (RNA-seq) is a relatively new method for both quantifying and mapping transcriptomes (Wang et al., 2009) and has been widely used lately in Al tolerance purposes (Yokosho et al., 2014; Guo et al., 2017; Jiang et al., 2018).

Al tolerance in *Secale* genus seems to be a genetically complex trait where different resistance mechanisms coexist giving it better Al toxicity circumventing and, consequently, greater Al tolerance. In this work, we found evidences for an exclusion mechanism but also for an internal mechanism of Al-detoxification which possibly operate in parallel. A coordinated operation between Al exclusion and Al internal tolerance mechanisms linked by ALMT1-mediated root malate exudation and NIP1;2-mediated Al uptake from the root cell wall was found in *Arabidopsis* (Wang et al., 2017). Moreover, an internal Al-detoxification based on Al-malate complexes was reported recently in wheat (Kopittke et al., 2017), demonstrating that the OA release is not the only mechanism operating in this cereal.

In short, rye species/subspecies have a valuable genetic background which can be used in breeding programs to improve related crops economically important and more susceptible to this abiotic stress. Moreover, the study of wild relatives provides new sources of genes that can be exploited. Taking together all the data of this thesis, we can confirm the potential of rye as a model species for the study of Al tolerance purposes. Rye has already been used as source of resistance to biotic constraints in wheat (Crespo-Herrera et al., 2017).

In summary:

- Rye is clearly one of the most Al-tolerant cereal with a vast genetic diversity concerning this trait which makes it crucial for crop improvement programs.
- ISSR and RAPD markers were absolutely able to assess genetic diversity and relationships as to identify putative Al tolerance linked DNA fragments in *Secale* genus, being ISSRs the most informative and effective.
- *Secale* proved to own a valuable genetic background because of the high genetic variability found.
- Phylogenetic relationships led us to recognize only three species in *Secale* (*S. cereale*, *S. strictum* and *S. sylvestre*) being in agreement with this genus last taxonomic revision.
- Landraces grouped by geographical location possibly as a common origin or an environment adaptation.
- Phylogeny studies indicated that the ryes with less Al tolerance, *S. strictum* and *S. sylvestre*, are the oldest ryes.
- 34 putative Al-linked DNA markers that can be used in the forthcoming traditional strategies combined with MAS were identified.
- Al tolerance in *Secale* genus seems to be a genetically complex trait where different resistance mechanisms coexist and where each rye species/subspecies adopt different strategies.
- Evidences indicated that Al-tolerant ryes strategies are based on Al-exclusion and also internal Al tolerance mechanisms.
- Al accumulation was directly proportional to ROS and lipid peroxidation production as well as death cell, allowing the distinction of tolerant and sensitive rye genotypes which can be used to classify ryes since a positive correlation was found with root regrowth measurement.
- Organic acid exudation was Al-induced in most of the rye species/subspecies being implicated in Al resistance mechanisms in some of them.
- All candidate genes seem to have an active contribution on enhanced Al-tolerance of, at least, one wild rye where *ScALMT1* and *ScMATE2* have a key role.

- INDELs and SNPs possibly related to Al tolerance were found in coding sequences of *MATE* genes indicating an evolution of the Al tolerance trait.
- Cultivated ryes exhibited higher expression level than wild ryes possibly due to an environmental adaptation to acidic soils.
- A *MATE* candidate gene to control the rye *Alt1* locus located at 6RS was isolated and characterized, that we named *ScMATE3*.
- *ScMATE3* can differ in the nature of substrate and be involved in a distinct internal Al-tolerance mechanism not reported so far in rye.
- Phylogenies showed that *ScMATE1* and *ScMATE2* are much related and quite close to *ScMATE3* with some correlate function to Al tolerance.
- *MATE* genes function inferred by phylogenetic relationships were in agreement with most of the expression data.
- Further research will be needed to accurate the exact function of *ScMATE3* and how it is operating in rye Al tolerance.
- The search of new candidate genes and the study of the regulatory processes implicated are crucial for a better understanding of how Al tolerance is conferred in *Secale*.

References

Aggarwal, A., Ezaki, B., Munjal, A., and Tripathi, B. N. (2015). "Physiology and Biochemistry of Aluminum Toxicity and Tolerance in Crops," in *Stress Responses in Plants - Mechanisms of Toxicity and Tolerance*, eds. B. N. Tripathi and M. Muller (Springer International Publishing Switzerland), 35–58. doi:10.1007/978-3-319-13368-3.

Al-Beyroutiová, M., Sabo, M., Sleziak, P., Dušinský, R., Birčák, E., Hauptvogel, P., et al. (2016). Evolutionary relationships in the genus *Secale* revealed by DArTseq DNA polymorphism. *Plant Syst. Evol.* 302, 1083–1091. doi:10.1007/s00606-016-1318-2.

Amom, T., and Nongdam, P. (2017). The Use of Molecular Marker Methods in Plants : A Review. *Int J Cur Res Rev* 9, 1–7. doi:10.7324/IJCRR.2017.9171.

An, J. P., Wang, X. N., Yao, J. F., Ren, Y. R., You, C. X., Wang, X. F., et al. (2017). Apple *MdMYC2* reduces aluminum stress tolerance by directly regulating *MdERF3* gene. *Plant Soil*, 1–12. doi:10.1007/s11104-017-3297-7.

Awasthi, J. P., Saha, B., Regon, P., Sahoo, S., Chowra, U., Pradhan, A., et al. (2017). Morpho-physiological analysis of tolerance to aluminum toxicity in rice varieties of North East India. *PLoS One* 12, 1–23. doi:10.1371/journal.pone.0176357.

Benito, C., Silva-Navas, J., Fontecha, G., Hernández-Riquer, M. V., Eguren, M.,

Salvador, N., et al. (2010). From the rye *Alt3* and *Alt4* aluminum tolerance loci to orthologous genes in other cereals. *Plant Soil* 327, 107–120. doi:10.1007/s11104-009-0035-9.

Bolibok-Brągoszewska, H., Targońska, M., Bolibok, L., Kilian, A., and Rakoczy-Trojanowska, M. (2014). Genome-wide characterization of genetic diversity and population structure in *Secale*. *BMC Plant Biol.* 14, 184. doi:10.1186/1471-2229-14-184.

Bona, L., Wright, R. J., Baligar, V. C., and Matuz, J. (1993). Screening wheat and other small grains for acid soil tolerance. *Landsc. Urban Plan.* 27, 175–178. doi:10.1016/0169-2046(93)90046-G.

Camacho, M. V., Matos, M., González, C., Pérez-Flores, V., Pernaute, B., Pinto-Carnide, O., et al. (2005). *Secale cereale* inter-microsatellites (SCIMs): Chromosomal location and genetic inheritance. *Genetica* 123, 303–311. doi:10.1007/s10709-004-5553-z.

Chen, L., Liu, Y., Liu, H., Kang, L., Geng, J., Gai, Y., et al. (2015). Identification and expression analysis of *MATE* genes involved in flavonoid transport in blueberry plants. *PLoS One* 10, 1–20. doi:10.1371/journal.pone.0118578.

Chikmawati, T., Miftahudin, M., Skovmand, B., and Gustafson, J. P. (2012). Amplified fragment length polymorphism-based genetic diversity among cultivated and weedy rye (*Secale cereale* L.) accessions. *Genet. Resour. Crop Evol.* 59, 1743–1752. doi:10.1007/s10722-012-9796-8.

Chikmawati, T., Skovmand, B., and Gustafson, J. P. (2005). Phylogenetic relationships among *Secale* species revealed by amplified fragment length polymorphisms. *Genome* 48, 792–801. doi:10.1139/g05-043.

Collins, N. C., Shirley, N. J., Saeed, M., Pallotta, M., and Gustafson, J. P. (2008). An *ALMT1* gene cluster controlling aluminum tolerance at the *Alt4* locus of rye (*Secale cereale* L.). *Genetics* 179, 669–682. doi:10.1534/genetics.107.083451.

Contreras, R., Figueiras, A. M., Gallego, F. J., Benavente, E., Manzaneda, A. J., and Benito, C. (2017). Neutral molecular markers support common origin of aluminium tolerance in three congeneric grass species growing in acidic soils. *AoB Plants* 9, 1–16. doi:10.1093/aobpla/plx060.

Contreras, R., Figueiras, A. M., Gallego, F. J., and Benito, C. (2014). *Brachypodium distachyon*: A model species for aluminium tolerance in Poaceae. *Funct. Plant Biol.* 41, 1270–1283. doi:10.1071/FP13362.

Crespo-Herrera, L. A., Garkava-Gustavsson, L., and Åhman, I. (2017). A systematic review of rye (*Secale cereale* L.) as a source of resistance to pathogens and pests in wheat (*Triticum aestivum* L.). *Hereditas* 154, 14. doi:10.1186/s41065-017-0033-5.

De Bustos, A., and Jouve, N. (2002). Phylogenetic relationships of the genus *Secale* based on the characterisation of rDNA ITS sequences. *Plant Syst. Evol.* 235, 147–154. doi:10.1007/s00606-002-0215-z.

Echart, C. L., Fernandes Barbosa-Neto, J., Garvin, D. F., and Cavalli-Molina, S. (2002). Aluminum tolerance in barley: Methods for screening and genetic analysis. *Euphytica* 126, 309–313. doi:10.1023/A:1019964410057.

El-Moneim, D. A., Contreras, R., Silva-Navas, J., Gallego, F. J., Figueiras, A. M., and Benito, C. (2014). Pectin methylesterase gene and aluminum tolerance in *Secale cereale*. *Environ. Exp. Bot.* 107, 125–133. doi:10.1016/j.envexpbot.2014.06.006.

FAO: Food and Agriculture Organization (2018). World Food Situation. Available at: http://www.fao.org/worldfoodsituation/csdb/en/.

Fernández, M. E., Figueiras, A. M., and Benito, C. (2002). The use of ISSR and RAPD markers for detecting DNA polymorphism, genotype identification and genetic diversity among barley cultivars with known origin. *Theor. Appl. Genet.* 104, 845–851. doi:10.1007/s00122-001-0848-2.

Ferreira, J. R., Faria, B. F., Junior, M. C., Delatorre, C. A., Minella, E., and Pereira, J. F. (2017). Is a non-synonymous SNP in the *HvAACT1* coding region associated with acidic soil tolerance in barley? *Genet. Mol. Biol.* 40, 480–490. doi:10.1590/1678-4685-GMB-2016-0225.

Fontecha, G., Silva-Navas, J., Benito, C., Mestres, M. A., Espino, F. J., Hernández-Riquer, M. V., et al. (2007). Candidate gene identification of an aluminum-activated organic acid transporter gene at the *Alt4* locus for aluminum tolerance in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 114, 249–260. doi:10.1007/s00122-006-0427-7.

Frederiksen, S., and Petersen, G. (1998). A taxonomic revision of the genus *Secale* (Triticeae; Poaceae). *Nord. J. Bot.* 18, 399–420.

Furukawa, J., Yamaji, N., Wang, H., Mitani, N., Murata, Y., Sato, K., et al. (2007). An aluminum-activated citrate transporter in barley. *Plant Cell Physiol.* 48, 1081–1091.

Gale, M. D., and Devos, K. M. (1998). Comparative genetics in the grasses. *Proc. nat. Acad. Sci. USA* 95, 1971–1974.

Gallego, F. J., and Benito, C. (1997). Genetic control of aluminium tolerance in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 95, 393–399.

Gallego, F. J., Calles, B., and Benito, C. (1998a). Molecular markers linked to the aluminium tolerance gene *Alt1* in rye (*Secale cereale* L.). *Theor. Appl. Genet.* 97, 1104–1109. doi:10.1007/s001220050997.

Gallego, F. J., López-Solanilla, E., Figueiras, A. M., and Benito, C. (1998b). Chromosomal location of pcr fragments as a source of DNA markers linked to aluminium tolerance genes in rye. *Theor. Appl. Genet.* 96, 426–434. doi:10.1007/s001220050759.

Garcia-Oliveira, A. L., Martins-Lopes, P., Tolrà, R., Poschenrieder, C., Guedes-Pinto, H., and Benito, C. (2016). Differential physiological responses of portuguese bread wheat (*Triticum aestivum* L.) genotypes under aluminium stress. *Diversity* 8, 1–12. doi:10.3390/d8040026.

Garcia-Oliveira, A. L., Martins-Lopes, P., Tolrá, R., Poschenrieder, C., Tarquis, M., Guedes-Pinto, H., et al. (2014). Molecular characterization of the citrate transporter gene *TaMATE1* and expression analysis of upstream genes involved in organic acid transport under Al stress in bread wheat (*Triticum aestivum*). *Physiol. Plant.* 152, 441–452. doi:10.1111/ppl.12179.

Guo, P., Qi, Y.-P., Yang, L.-T., Lai, N.-W., Ye, X., Yang, Y., et al. (2017). Root adaptive responses to aluminum-treatment revealed by RNA-Seq in two citrus species with different aluminum-tolerance. *Front. Plant Sci.* 8, 1–18. doi:10.3389/fpls.2017.00330.

Hagenblad, J., Oliveira, H. R., Forsberg, N. E. G., and Leino, M. W. (2016). Geographical distribution of genetic diversity in *Secale* landrace and wild accessions. *BMC Plant Biol.* 16, 1–20. doi:10.1186/s12870-016-0710-y.

Jiang, C., Liu, L., Li, X., Han, R., Wei, Y., and Yu, Y. (2018). Insights into aluminum-tolerance pathways in *Stylosanthes* as revealed by RNA-Seq analysis. *Sci. Rep.* 8, 6072. doi:10.1038/s41598-018-24536-3.

Kim, B. Y., Baier, A. C., Somers, D. J., and Gustafson, J. P. (2001). Aluminium tolerance

in triticale, wheat and rye. *Euphytica* 120, 329–337.

Kochian, L. V., Piñeros, M. A., and Hoekenga, O. A. (2005). The physiology, genetics and molecular biology of plant aluminum resistance and toxicity. *Plant Soil* 274, 175–195. doi:10.1007/s11104-004-1158-7.

Kochian, L. V., Piñeros, M. A., Liu, J., and Magalhaes, J. V. (2015). Plant Adaptation to Acid Soils: The Molecular Basis for Crop Aluminum Resistance. *Annu. Rev. Plant Biol.* 66, 571–598. doi:10.1146/annurev-arplant-043014-114822.

Kopittke, P. M., McKenna, B. A., Karunakaran, C., Dynes, J. J., Arthur, Z., Gianoncelli, A., et al. (2017). Aluminum Complexation with Malate within the Root Apoplast Differs between Aluminum Resistant and Sensitive Wheat Lines. *Front. Plant Sci.* 8, 1–11. doi:10.3389/fpls.2017.01377.

Li, X. F., Ma, J. F., and Matsumoto, H. (2000). Pattern of aluminum-induced secretion of organic acids differs between rye and wheat. *Plant Physiol.* 123, 1537–1544. doi:10.1104/pp.123.4.1537.

Liu, J., Li, Y., Wang, W., Gai, J., and Li, Y. (2016). Genome-wide analysis of MATE transporters and expression patterns of a subgroup of *MATE* genes in response to aluminum toxicity in soybean. *BMC Genomics* 17, 1–15. doi:10.1186/s12864-016-2559-8.

Loarce, Y., Gallego, R., and Ferrer, E. (1996). A comparative analysis of the genetic relationships between rye cultivars using RFLP and RAPD markers. *Euphytica* 88, 107–115.

Ma, J. . F., Taketa, S., and Yang, Z. M. (2000). Aluminum tolerance genes on the short arm of chromosome *3R* are linked to organic acid release in triticale. *Plant Physiol* 122, 687–694. doi:10.1104/pp.122.3.687.

Ma, J. F. (2000). Role of organic acids in detoxification of aluminum in higher plants. *Plant Cell Physiol.* 41, 383–390. doi:10.1093/pcp/41.4.383.

Ma, R., Yli-Mattila, T., and Pulli, S. (2004). Phylogenetic relationships among genotypes of worldwide collection of spring and winter ryes (*Secale cereale* L.) determined by RAPD-PCR markers. *Hereditas* 140, 210–221. doi:10.1111/j.1601-5223.2004.01844.x.

Ma, Y., Li, C., Ryan, P. R., Shabala, S., You, J., Liu, J., et al. (2016). A new allele for aluminium tolerance gene in barley (*Hordeum vulgare* L.). *BMC Genomics* 17, 186. doi:10.1186/s12864-016-2551-3.

Magalhaes, J. V, Liu, J., Guimarães, C. T., Lana, U. G. P., Alves, V. M. C., Wang, Y.-H., et al. (2007). A gene in the multidrug and toxic compound extrusion (*MATE*) family confers aluminum tolerance in sorghum. *Nat. Genet.* 39, 1156–1161. doi:10.1038/ng2074.

Maron, L. G., Guimaraes, C. T., Kirst, M., Albert, P. S., Birchler, J. A., Bradbury, P. J., et al. (2013). Aluminum tolerance in maize is associated with higher *MATE1* gene copy number. *Proc. Natl. Acad. Sci.* 110, 5241–5246. doi:10.1073/pnas.1220766110.

Maron, L. G., Piñeros, M. A., Guimarães, C. T., Magalhaes, J. V., Pleiman, J. K., Mao, C., et al. (2010). Two functionally distinct members of the *MATE* (multi-drug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. *Plant J.* 61, 728–740. doi:10.1111/j.1365-313X.2009.04103.x.

Matos, M., Camacho, M. V., Pérez-Flores, V., Pernaute, B., Pinto-Carnide, O., and Benito, C. (2005). A new aluminum tolerance gene located on rye chromosome arm

7RS. *Theor. Appl. Genet.* 111, 360–369. doi:10.1007/s00122-005-2029-1.

Matos, M., Pinto-Carnide, O., and Benito, C. (2001). Phylogenetic relationships among Portuguese rye based on isozyme, RAPD and ISSR markers. *Hereditas* 134, 229–236. doi:10.1111/j.1601-5223.2001.00229.x.

Miftahudin, Chikmawati, T., Ross, K., Scoles, G. J., and Gustafson, J. P. (2005). Targeting the aluminum tolerance gene *Alt3* region in rye, using rice/rye micro-colinearity. *Theor. Appl. Genet.* 110, 906–913. doi:10.1007/s00122-004-1909-0.

Miftahudin, T., Scoles, G., and Gustafson, J. (2002). AFLP markers tightly linked to the aluminum tolerance gene *Alt3* in rye. *Theor. Appl. Genet.* 104, 626–631. doi:10.1007/s00122-001-0782-3.

Morris, P. C., and Bryce, J. H. (2000). "Introduction," in *Cereal Biotechnology*, eds. P. C. Morris and J. H. Bryce (Cambridge: Woodhead Publishing Limited).

Naranjo, T., Roca, A., Goicoechea, P. G., and Giráldez, R. (1987). Arm homoeology of wheat and rye chromosomes. *Genome* 29, 873–882.

Petrovičová, L., Balážová, Ž., Vivodík, M., and Gálová, Z. (2017). Detection genetic variability of *Secale cereale* L. by SCoT markers. *Potravin. Slovak J. Food Sci.* 11, 197–202.

Pinto-Carnide, O., and Guedes-Pinto, H. (1999). Aluminium tolerance variability in rye and wheat Portuguese germplasm. *Genet. Resour. Crop Ev.* 46, 81–85.

Reddy, P., Appels, R., and Baum, B. R. (1990). Ribosomal DNA spacer-length variation in *Secale* spp. (Poaceae). *Plant Syst. Evol.* 171, 205–220. doi:10.1007/BF00940606.

Ren, T. H., Chen, F., Zou, Y. T., Jia, Y. H., Zhang, H. Q., Yan, B. J., et al. (2011). Evolutionary trends of microsatellites during the speciation process and phylogenetic relationships within the genus *Secale*. *Genome* 54, 316–326. doi:10.1139/g10-121.

Ryan, P. R., Raman, H., Gupta, S., Horst, W. J., and Delhaize, E. (2009). A Second Mechanism for Aluminum Resistance in Wheat Relies on the Constitutive Efflux of Citrate from Roots. *Plant Physiol.* 149, 340–351. doi:10.1104/pp.108.129155.

Samac, D. A., and Tesfaye, M. (2003). Plant improvement for tolerance to aluminum in acid soils – a review. *Plant Cell. Tissue Organ Cult.* 75, 189–207.

Santos, E., Benito, C., Silva-Navas, J., Gallego, F. J., Figueiras, A. M., Pinto-Carnide, O., et al. (2018). Characterization, genetic diversity, phylogenetic relationships, and expression of the aluminum tolerance *MATE1* gene in *Secale* species. *Biol. Plant.* 62, 109–120. doi:10.1007/s10535-017-0749-0.

Santos, E., Matos, M., Silva, P., Figueiras, A. M., Benito, C., and Pinto-Carnide, O. (2016). Molecular diversity and genetic relationships in *Secale*. *J. Genet.* 95, 273–281. doi:10.1007/s12041-016-0632-3.

Sasaki, T., Yamamoto, Y., Ezaki, B., Katsuhara, M., Ahn, S., Ryan, P., et al. (2004). A wheat gene encoding an aluminum-activated malate transporter. *Plant J* 37, 645–653.

Shang, H. Y., Wei, Y. M., Wang, X. R., and Zheng, Y. L. (2006). Genetic diversity and phylogenetic relationships in the rye genus *Secale* L. (rye) based on *Secale cereale* microsatellite markers. *Genet. Mol. Biol.* 29, 685–691. doi:10.1590/S1415-47572006000400018.

Silva-Navas, J., Benito, C., Téllez-Robledo, B., El-Moneim, D. A., and Gallego, F. J. (2012). The *ScAACT1* gene at the *Qalt5* locus as a candidate for increased aluminum tolerance in rye (*Secale cereale* L.). *Mol. Breed.* 30, 845–856. doi:10.1007/s11032-011-9668-5.

Sivaguru, M., Liu, J., and Kochian, L. V. (2013). Targeted expression of *SbMATE* in the

root distal transition zone is responsible for sorghum aluminum resistance. *Plant J.* 76, 297–307. doi:10.1111/tpj.12290.

Tanyolac, B. (2003). Inter simple sequence repeat (ISSR) and RAPD variation among wild barley (*Hordeum vulgare* ssp. *spontaneum*) populations from west Turkey. *Genet. Resour. Crop Evol.* 50, 611–614. doi:10.1023/A:1024412814757.

Targońska, M., Bolibok-Brągoszewska, H., and Rakoczy-Trojanowska, M. (2016). Assessment of Genetic Diversity in *Secale cereale* Based on SSR Markers. *Plant Mol. Biol. Report.* 34, 37–51. doi:10.1007/s11105-015-0896-4.

Tovkach, A., Ryan, P. R., Richardson, A. E., Lewis, D. C., Rathjen, T. M., Ramesh, S., et al. (2013). Transposon-mediated alteration of *TaMATE1B* expression in wheat confers constitutive citrate efflux from root apices. *Plant Physiol.* 161, 880–92. doi:10.1104/pp.112.207142.

Wang, J., Raman, H., Zhou, M., Ryan, P. R., Delhaize, E., Hebb, D. M., et al. (2007). High-resolution mapping of the *Alp* locus and identification of a candidate gene *HvMATE* controlling aluminium tolerance in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.* 115, 265–276. doi:10.1007/s00122-007-0562-9.

Wang, L., Bei, X., Gao, J., Li, Y., Yan, Y., and Hu, Y. (2016). The similar and different evolutionary trends of *MATE* family occurred between rice and *Arabidopsis thaliana*. *BMC Plant Biol.* 16, 207. doi:10.1186/s12870-016-0895-0.

Wang, Y., Li, R., Li, D., Jia, X., Zhou, D., Li, J., et al. (2017). NIP1;2 is a plasma membrane-localized transporter mediating aluminum uptake, translocation, and tolerance in *Arabidopsis*. *Proc. Natl. Acad. Sci.* 114, 5047–5052. doi:10.1073/pnas.1618557114.

Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 10, 57–63.

Yamamoto, Y., Kobayashi, Y., and Matsumoto, H. (2001). Lipid Peroxidation Is an Early Symptom Triggered by Aluminum, But Not the Primary Cause of Elongation Inhibition in Pea Roots. *Plant Physiol.* 125, 199–208. doi:10.1104/pp.125.1.199.

Yokosho, K., Yamaji, N., Fujii-Kashino, M., and Ma, J. F. (2016a). Functional Analysis of a *MATE* Gene *OsFRDL2* Revealed its Involvement in Al-Induced Secretion of Citrate, but a Lower Contribution to Al Tolerance in Rice. *Plant Cell Physiol.* 57, 976–985. doi:10.1093/pcp/pcw026.

Yokosho, K., Yamaji, N., and Ma, J. F. (2010). Isolation and characterisation of two *MATE* genes in rye. *Funct. Plant Biol.* 37, 296–303. doi:10.1071/FP09265.

Yokosho, K., Yamaji, N., and Ma, J. F. (2014). Global transcriptome analysis of al-induced genes in an al-accumulating species, common buckwheat (*Fagopyrum esculentum* Moench). *Plant Cell Physiol.* 55, 2077–2091. doi:10.1093/pcp/pcu135.

Yokosho, K., Yamaji, N., and Ma, J. F. (2016b). *OsFRDL1* expressed in nodes is required for distribution of iron to grains in rice. *J. Exp. Bot.* 67, 5485–5494. doi:10.1093/jxb/erw314.

Yokosho, K., Yamaji, N., Ueno, D., Mitani, N., and Ma, J. F. (2009). *OsFRDL1* Is a Citrate Transporter Required for Efficient Translocation of Iron in Rice. *Plant Physiol.* 149, 297–305. doi:10.1104/pp.108.128132.

Zhang, M., Lu, X., Li, C.-L., Zhang, B., Zhang, C., Zhang, X. S., et al. (2018). Auxin Efflux Carrier ZmPGP1 Mediates Root Growth Inhibition under Aluminum Stress. *Plant Physiol.* doi:10.1104/pp.17.01379.

Zhu, H., Wu, J., Jiang, Y., Jin, J., Zhou, W., Wang, Y., et al. (2016). Genomewide analysis

of *MATE*-type gene family in maize reveals microsynteny and their expression patterns under aluminum treatment. *J. Genet.* 95, 691–704. doi:10.1007/s12041-016-0686-2.

"My watch is ended"