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MAPEAMENTO GENÉTICO DA POPULAÇÃO TOROPI X IAC13 EM RESPOSTA
À FERRUGEM DA FOLHA E AO ALUMÍNIO TÓXICO

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Tese apresentada como um dos requisitos
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Dedico

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Minha querida mãe (*in memoriam*).
À Raphael Pinto
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"O cientista não é o homem que fornece as verdadeiras respostas

É quem faz as verdadeiras perguntas".

Claude Lévi-Strauss

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MAPEAMENTO GENÉTICO DA VARIEDADE DE TRIGO TOROPI EM RESPOSTA À FERRUGEM DA FOLHA E AO ALUMÍNIO TÓXICO[1]

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RESUMO

O trigo (*Triticum aestivum* L.) é uma cultura com distribuição mundial, razão pela qual está frequentemente exposto aos mais variados tipos de estresses de origem biótica e abiótica. Dentre as doenças, destaca-se a ferrugem da folha causada pelo fungo biotrófico *Puccinia triticina,* que tem a capacidade para ocasionar decréscimos de produtividade em torno de 50%. Nos estresses abióticos, destaca-se o proporcionado pelos solos ácidos, que está associado à toxicidade por alumínio (Al). Para lidar com estes problemas, a melhor estratégia é a utilização de cultivares resistentes a *P. triticina* e tolerantes ao Al. Sendo assim, os objetivos desse trabalho foram realizar uma revisão com o levantamento dos loci de resistência à ferrugem da folha publicados de 1971 a 2017, caracterizar fenotípica e genotipicamente uma população biparental de linhagens endogâmicas recombinantes (RILs), oriunda do cruzamento entre Toropi x IAC13, para a resistência à ferrugem da folha e tolerância ao Al. Foram realizadas três fenotipagens em casa de vegetação para ferrugem da folha e um conjunto de oito ensaios hidropônicos para o Al tóxico. O DNA de todas as RILs foi extraído para genotipagem utilizando marcadores DArT-Seq (*Diversity Array Technology*) e microssatélites. Foram revisados 188 QTLs (*Quantitative Trait Loci*) para ferrugem da folha derivados de populações de mapeamento biparental, 165 de estudos de GWA (*Genome-Wide Association*) e 35 Meta-QTLs. A partir dos ensaios realizados para ferrugem da folha foi possível identificar sete QTLs. Os loci derivados de Toropi foram nomeados de *QLr.ufrgs-1A, QLr.ufrgs-2A* e *QLr.ufrgs-3B.* Os associados ao IAC13 foram *QLr.ufrgs-2D*, *QLr.ufrgs-5A*, *QLr.ufrgs-6B* e *QLr.ufrgs-7D.* Este estudo gerou um mapa de ligação de 2.598,77 cM com uma distância média de 1,81 cM entre os marcadores. O mesmo mapa foi utilizado para a identificação de regiões genômicas de interesse nos ensaios de tolerância ao Al. Toropi mostrou-se como tolerante ao Al tóxico enquanto IAC13 foi moderadamente suscetível. Os genes *TaALMT1* e *TaMATE1B* foram confirmados no background de Toropi e nenhum loci de tolerância foi associado a cultivar IAC13. Adicionalmente, não foi possível encontrar nenhum loci novo associados à toxicidade por Al nesta população.

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GENETIC MAPPING OF TOROPI WHEAT VARIETY IN RESPONSE TO LEAF RUST AND ALUMINIUM TOXICITY[2]

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ABSTRACT

Wheat (*Triticum aestivum* L.) is a widespread crop, reason why it has frequently being exposed to the most diverse stress with biotic and abiotic origins. Among the diseases, it stands out the wheat leaf rust caused by the biotrophic fungus *Puccinia triticina*, which has the capacity to decrease yield around 50%. Among abiotic stresses, the one caused by acid soils, which is commonly associated to aluminium (Al) toxicity, is the most important. Therefore, the objectives of this work were to make a review of the data survey of the resistant loci to leaf rust published from 1971 to 2017 and to characterise phenotypic and genotypic a biparental population of recombinant inbreed lines (RILs) from the cross Toropi x IAC13 to leaf rust resistance and Al tolerance. It was conducted three phenotypic experiments to screen on greenhouse for leaf rust severity and infection type and eight hydroponic subset assays for Al toxicity. The DNA of all RIL was extracted for genotypic analyses using and DArT-Seq (*Diversity Array Technology*) and microsatellites markers. Altogether, it was reviewed 188 QTL (*Quantitative Trait Loci*) to leaf rust resistance coming from biparental mapping populations, 165 from GWA (*Genome-Wide Association*) studies and 35 Meta-QTL. From our leaf rust assays it was possible to identify seven QTL. The loci derived from Toropi were named as *QLr.ufrgs-1A*, *QLr.ufrgs-2A* and *QLr.ufrgs-3B*. Those associated to IAC13 were *QLr.ufrgs-2D*, *QLr.ufrgs-5A*, *QLr.ufrgs-6B* and *QLr.ufrgs-7D*. This study generates a linkage map of 2,598.77 cM with an average distance of 1.81 cM among markers. The same map was used to identify genomic regions of interest on the Al tolerance assays. Toropi showed to be tolerant to Al in toxic levels while IAC13 was moderately susceptible. The *TaALMT1* and *TaMATE1B* genes were confirmed on Toropi background and none tolerance loci was associated to cv. IAC13. Additionally, it was not possible to find any new loci to Al toxicity on this population.

SUMÁRIO

RELAÇÃO DAS TABELAS

RELAÇÃO DAS FIGURAS

Página

CAPÍTULO 5

1 INTRODUÇÃO

O trigo (*Triticum aestivum* L.) é a segunda cultura mais importante do mundo, ficando atrás somente do arroz e, por ser globalmente distribuída, é constantemente exposta a diversos estresses bióticos (Juliana *et al.*, 2017) e abióticos. A ferrugem da folha, causada por *Puccinia triticina,* é uma das principais doenças para a cultura, sendo responsável por causar reduções de produtividade, em média de 50%, dependendo do estádio de desenvolvimento da planta e da raça de *P. triticina* (Huerta-Espino *et al.*, 2011; Bolton *et al.*, 2008; Roelfs *et al.*, 1992). Da mesma forma, um dos principais estresses abióticos é o causado por solos ácidos, que é frequentemente acompanhado pela toxicidade ao alumínio (Al).

Para enfrentar os prejuízos ocasionados pela ferrugem e por solos ácidos a principal estratégia é o uso de variedades resistentes a *P. triticina* e tolerantes ao Al^{3+} (Juliana *et al.*, 2017). A resistência a doenças pode ser caracterizada de várias formas, sendo a resistência raça-específica baseada na interação gene a gene, porém fácil de ser superada por raças virulentas do patógeno, e a resistência de planta adulta (RPA) que na maioria das vezes é não-específica a raças, conhecida por sua longa vida útil a campo. Variedades de trigo contendo genes de RPA não impedem completamente a infecção pelo patógeno, mas atrasam o seu desenvolvimento. Atualmente existem 76 genes *Lr* (*Leaf rust*) que conferem resistência à ferrugem da folha (McIntosh *et al.*, 2017).

O mecanismo de tolerância ao Al^{3+} melhor caracterizado até o momento é o efluxo de ácidos orgânicos (AOs) pelas de raízes, através de transportadores localizados na membrana plasmática. Diversos AOs têm sido descritos sendo exsudados a partir de raízes de trigo, como malato, citrato, oxalato, succinato, ácido tartárico e fumarato (Garcia-Oliveira *et al.*, 2015). Os AOs mais importantes na detoxificação ao Al^{3+} em trigo são o malato, associado ao gene *TaALMT1* (*Aluminium-actived malate transporter 1*) e o citrato, associado a *TaMATE1B* (*Multi-drug and toxin extrusion 1*). Cultivares de trigo com alguma tolerância ao Al, desenvolvidas em diferentes países, geralmente possuem material brasileiro em seu *pedigree* (Garcia-Oliveira *et al.*, 2015).

Características agronômicas de importância, como resistência a doenças e tolerâncias a estresses, geralmente possuem herança poligênicas e/ou quantitativa. As regiões de um genoma que ancoram estas características são chamadas de *loci* de características quantitativas ou QTLs (*Quantitative Trait Loci*) (Lannou, 2012). Atualmente, com a criação de mapas genéticos é possível localizar e a avaliar o efeito das regiões genômicas associadas aos QTLs (Liu, 1988).

A cultivar de Toropi apresenta diversas características agronômicas de interesse, entre elas, resistência às ferrugens da folha (Casassola *et al.*, 2014), amarela (Rosa *et al.*, 2016) e do colmo (*dados não publicados*), além de tolerância ao Al^{3+} tóxico (Boff, 2006), aumento na absorção, distribuição e translocação de fósforo (Espindula *et al.*, 2009) e resistência a Fusarium (Kohli, 1989). Foram identificados até o momento dois genes recessivos e complementares de planta adulta, *Trp-1* e *Trp-2* (Barcellos *et al.*, 2000)*,* um gene de plântula *Trp-Se* e o gene *Trp-3*, que é de menor efeito, raça-específico e de planta adulta (Rosa, 2012). Mesmo assim, não se descarta a hipótese de que Toropi apresente outras regiões com resistência ainda não identificadas, posto que a identificação da resistência é fortemente influenciada pelas condições ambientais e, principalmente, pelo tamanho das populações em estudo (Kumar *et al.*, 2013). A natureza única da resistência observada em Toropi leva a crer que a mesma não é portadora de nenhum dos principais genes de resistência durável às ferrugens descritos até o momento (McIntosh *et al.*, 2017; Singla *et al.*, 2017; Rosa *et al.*, 2016).

Diante do exposto, é imprescindível que se dê continuidade aos estudos em Toropi a fim de desvendar inequivocamente os mecanismos e os genes de resistência e de tolerância operantes na cultivar, visto que inconsistências em relação a localização das regiões gênicas responsáveis pela resistência são evidentes em estudos publicados.

Objetivo geral

Investigar a base genética da resistência à ferrugem da folha do trigo e tolerância ao alumínio tóxico na cultivar Toropi, utilizando uma população biparental obtida do cruzamento de Toropi x IAC13 em F_{12} e F_{14}.

Objetivos específicos

1.1. Identificar e catalogar todos os QTLs de resistência a ferrugem da folha publicados de 1971 a 2017.

1.2. Caracterizar fenotípica e genotipicamente a população Toropi x IAC13 para resistência à ferrugem da folha e tolerância ao Al^{3+}.

1.3. Identificar regiões cromossômicas responsáveis pela expressão da resistência à ferrugem da folha e tolerância ao Al^{3+}.

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2 REVISÃO BIBLIOGRÁFICA

2.1 Trigo

2.1.1 Origem e domesticação

A domesticação do trigo (*Triticum aestivum* L.) hexaplóide ocorreu na região Sudeste da Ásia há mais 10.000 anos (Charmet, 2011), sendo originado a partir do cruzamento entre plantas nativas existentes no Crescente Fértil. Charmet (2011) afirma que cinco mil anos depois da domesticação, o trigo foi introduzido na Índia, na China e nos países da Europa.

O trigo é uma espécie alohexaplóide originada a partir da hibridação interespecífica de três diferentes espécies diplóides, que resultou em três sub-genomas, referidos como genomas A, B e D. O trigo hexaplóide domesticado não tem um genitor hexaplóide direto, visto que possui três conjuntos de cromossomos homólogos, sendo designado como A^uA^uBBDD. O expoente "u" no genoma A^u indica que ele é proveniente do genoma encontrado na espécie *Triticum urartu* Thumanian *ex* Gandilyan (Charmet, 2011). As primeiras evidências sugeriam que a espécie *Triticum monococcum* L. (2n = 14, AA) era progenitora doadora do sub-genoma A, para o trigo tetraplóide e hexaplóide (Gill & Kimber, 1974). No entanto, estudos realizados com uma espécie diploide similar, *T. urartu*, encontrada na mesma região geográfica que *T. monococcum*, forneceu uma forte evidência que estas duas espécies estejam fortemente relacionadas. Tal descoberta levou a crer que *T. monococcum* e *T. urartu* possam ser a mesma espécie, ou que *T. urartu* seja o único doador do sub-genoma A do trigo, esta última hipótese é a mais aceita. (Dvorak *et al.*, 1993; Kerby & Kuspira, 1987).

A identidade do progenitor do genoma B ainda não é clara, embora muitos estudos sugiram que *Triticum speltoides* L. (2n = 14, BB) seja o doador (Kimber & Rilley, 1963). Outras possibilidades, no entanto, têm sido propostas. Sears (1977) sugere que a espécie doadora do genoma B pode ainda não ter sido descoberta ou pode ter sido extinta e que tal genoma pode ser o resultado da introgressão de duas ou mais espécies parentais distintas. Nas décadas de 20 a 30 diversas espécies de *Aegilops* foram propostas como sendo possíveis progenitoras do genoma D (Kerby & Kuspira, 1987). Embora outras espécies tenham sido consideradas, muitas evidências suportam que *Aegilops tauschii*

Coss. (2n = 14, DD) seja um dos progenitores do trigo hexaplóide (Dubcovsky & Dvorak, 2007).

Atualmente são conhecidas seis espécies biológicas de trigo, que consistem em três níveis de ploidia: as diplóides *T. monococcum* ($A^m A^m$) e *T. urartu* (AA), as tetraplóides *T. turgidum* (AABB) e *T. Timopheevii* (AAGG), e as hexaplóides *T. aestivum* (AABBDD) e *T. zhukovskyi* ($AAGGA^m A^m$). As relações existentes entre os genomas citados mostram que *T. monococcum*, *T. timopheevii* e *T. zhukovskyi* correspondem a uma linhagem separada sem muita importância para o desenvolvimento da linhagem principal do trigo hexaplóide, que é formada por *T. urartu*, *T. turgidum* e *T. aestivum* (Charmet, 2011).

A convergência de duas ou mais espécies previamente adaptadas a diferentes ambientes, permitiu que espécies poliplóides domesticadas desenvolvessem vantagens adaptativas, as quais podem ser vistas em espécies poliplóides com habilidade de prosperar dentro de uma ampla gama de condições climáticas e ambientais (Dubcovsky & Dvorak, 2007). Algumas das importantes adaptações que permitiram ao trigo poliplóide prosperar como uma planta cultivada moderna, incluem: o desenvolvimento de uma ráquis não quebradiça, aumento do tamanho de grãos e número de afilhos (Dubcovsky & Dvorak, 2007). Inversamente, espécies poliplóides são limitadas em diversidade genética, visto que somente um pequeno número de plantas contribui para o seu *background* genético, o que é chamado de *bottleneck* (gargalo de garrafa) (Stebbins, 1950). O *bottleneck* mais severo é observado no genoma D de *T. aestivum*. Estudos indicam que os valores para diversidade são 15% menores do que aqueles observados no seu parente silvestre *Ae. tauschii* (Dvorak *et al.*, 1988). Devido a sua origem, a natureza hexaplóide do genoma do trigo faz com que os estudos genéticos sejam bem mais complexos do que os de outras espécies cultivadas com menores níveis de ploidia.

2.1.2 Importância econômica

O trigo é segundo alimento mais importante para humanidade, sendo considerado de primeira necessidade para mais de 50% da população mundial (Kiran *et al.*, 2016), principalmente na Europa, América do Norte e norte da Ásia. Além de ser o terceiro cereal mais cultivado, está amplamente distribuído ao redor do mundo, ocupando uma área de aproximadamente 220 milhões de hectares (Singh *et al.*, 2016). Segundo a Moore *et al.* (2015) o trigo sozinho é responsável por 1/5 de todas as calorias humanas consumidas no mundo, sendo a principal fonte de alimento para muitos países em desenvolvimento. Estima-se que a demanda para o consumo do grão aumente anualmente a uma taxa de 1,6% até 2050, devido principalmente, ao aumento populacional e da longevidade (Singh *et al.*, 2016). Como consequência, a média mundial de produtividade que é de 3 ton/ha, precisaria atingir um patamar de aproximadamente 5 ton/ha (Ray *et al.*, 2013).

No Brasil o trigo é tradicionalmente cultivado nos estados na região Sul. No entanto, sua atual produção é insuficiente para atender a demanda do consumo interno. Atualmente existem diversas cultivares de trigo desenvolvidas por entidades brasileiras que apresentam excelente qualidade de panificação, tornando o Brasil um pouco menos depende de outros países da América do Sul, como Argentina e Uruguai, que são os principais exportadores de trigo para o Brasil.

2.2 Entraves para o aumento da produtividade no Brasil

O trigo pode ser cultivado em uma grande diversidade de climas e solos pois é uma espécie amplamente adaptada. Algumas condições são mais favoráveis ao seu desenvolvimento e proporcionam maiores rendimentos, enquanto outras, causam enormes reduções de produtividade. Por ser amplamente distribuído, o trigo é frequentemente exposto a vários tipos de estresses de natureza biótica e abiótica, os quais impedem que cultivares de alto rendimento alcancem o seu máximo potencial genético, portanto limitam sua produtividade.

No Brasil o trigo é cultivado no período de outono-inverno, estendendo-se até a primavera, o que favorece a proliferação de diversas doenças, entre elas, as ferrugens dos cereais. Até o momento, três espécies de ferrugens são responsáveis por causarem reduções significativas em produtividade, sendo elas a ferrugem da folha (*Puccinia triticina* Eriks), ferrugem do colmo (*P. graminis* Pers. f. sp. *tritici*) e ferrugem amarela (*P. striiformis* Westend f. sp. *tritici*). A ferrugem da folha é a que apresenta maior ameaça, pois é a mais comum e amplamente distribuída, além de ser a mais prevalente das três na América do Sul. A ferrugem do colmo é considerada a mais destrutiva e a ferrugem amarela é uma importante doença em regiões de temperaturas baixas. A incidência das ferrugens é dependente das condições climáticas e do grau de resistência das cultivares de trigo predominantes em determinada região produtora (Kolmer *et al.*, 2008). Os danos ocasionados pela ferrugem da folha tendem a ser menos impactantes do que os causados pela ferrugem do colmo ou ferrugem amarela. Porém, devido a sua alta frequência e ocorrência generalizada ao redor do mundo, a ferrugem da folha resulta em maiores prejuízos anuais, quando comparada às demais (Huerta-Espino *et al.*, 2011).

A ferrugem da folha tem o ambiente como uma condição chave para a dispersão e germinação de esporos, o que influencia diretamente na severidade das epidemias de um ano para o outro e de local para local (McCallum *et al.*, 2007; Roelfs *et al.*, 1992). Esta doença provoca prejuízos que podem alcançar milhões de dólares, em parte devido ao aumento do custo de produção decorrente do uso de produtos químicos para o seu controle (Germán *et al.*, 2007). Causa ainda redução significava na área fotossinteticamente ativa e na qualidade e quantidade dos grãos produzidos, já que os danos são resultado do

decréscimo do número e do peso dos grãos por espiga (Herrera-Foessel *et al*., 2011; Germán *et al*., 2007). Segundo Bajwa *et al*. (1986), a diminuição no peso de grãos entre as cultivares de trigo, devido a infecção por ferrugem da folha, pode variar de 2 a 41% dependendo do nível de resistência apresentado pelas cultivares. A média de reduções, estimadas para Obregon/México, em cultivares suscetíveis, raça-específica e resistência parcial, pode ser de aproximadamente, 51%, 5% e 26%, respectivamente, quando a semeadura é realizada em época distintas e de, 71%, 11% e 44%, respectivamente, se realizada tardiamente (Herrera-Foessel *et al*., 2011).

Um dos principais estresses abióticos atuando na redução da produtividade do trigo é ocasionado por solos ácidos, atrelado à toxicidade por alumínio (Al). O sistema de plantio direto na palha, adotado com frequência pelos triticultores brasileiros, tem impossibilitado o revolvimento do solo para a incorporação de corretivos ao longo dos anos e a diminuição do pH nas camadas mais inferiores do solo. Em decorrência disso, os solos agrícolas começaram a apresentar toxidez por Al e/ou deficiência de cálcio (Ca) nas camadas mais profundas do solo, promovendo uma "barreira química" ao desenvolvimento normal de raízes, com a redução da capacidade das plantas em absorver água, o que afeta negativamente a produtividade das culturas (Lopes *et al*., 2004). Nas camadas mais ácidas do solo ocorrem significativas modificações, entre elas, a solubilização do Al na solução do solo, manganês (Mn) e ferro (Fe) atingindo níveis tóxicos, assim como a complexação de alguns nutrientes essenciais, sendo o fósforo (P) o mais limitado (Kochian, 1995).

A introdução do trigo no Brasil e, posteriormente no Rio Grande do Sul, forçou o desenvolvimento de novas cultivares mais produtivas, adaptadas às condições locais e tolerantes a estresses bióticos e abióticos. Atualmente há uma grande demanda de mercado por cultivares com atributos que vão além da alta produtividade, o que impulsiona as pesquisas relacionadas às características que mais causam reduções de produtividade.

2.3 Ferrugem da folha do trigo (*Puccinia triticina* Eriks.)
2.3.1 Ciclo de vida, hospedeiros alternativos e epidemiologia

Puccinia triticina de genoma estimado em 100-120 mb, é um fungo biotrófico obrigatório para trigo e algumas gramíneas (http://www.broadinstitute.org/annotation/genome/puccinia_group/Info.html). Pertence ao filo Basidiomycota, ordem Pucciniales, que juntamente com a ordem Ustilaginales, diferenciam-se da maioria dos outros Basidiomycotas por não terem seus basidiósporos localizados sobre ou dentro de corpos de frutificação. Ao invés disto, seus basidiósporos são localizados em tubos germinativos especializados, os basídios, que são produzidos pela germinação dos esporos de repouso ou teliósporos (Kolmer, 2013).

Este fungo é bastante complexo, visto que possui um ciclo de vida do tipo

heteroécio, com estágios sexual e assexual, que incluem cinco tipos diferentes de esporos (Bolton *et al.*, 2008). Além disso, possui dois hospedeiros alternativos, o *Thalictrum speciosissimum* L. e *Isopyrum fumaroides*, nos quais o fungo pode completar o seu ciclo sexual (Kolmer, 2013).

O hospedeiro primário de *P. triticina* é o trigo hexaplóide comum. Infecções nesse hospedeiro surgem quando os urediniósporos atingem a superfície foliar na presença de água livre. A partir da sua fixação e na presença de água livre, germinam e formam um tubo germinativo, que cresce em direção às aberturas naturais das folhas, os estômatos. Uma vez em contato com o estômato, o tubo germinativo se transforma em apressório dentro de um período de 3 h. A entrada na cavidade subestomatal é alcançada graças a pressão de turgor, a partir da qual é formada a vesícula subestomatal em aproximadamente 6 horas após a infecção (h.a.i.). Esta por sua vez, produz uma hifa infectiva que cresce intercelularmente até produzir uma célula mãe de haustório adjacente a uma célula do mesófilo da planta, em 12 h.a.i. (Bolton *et al.*, 2008). Sob condições favoráveis, dentro de um período de 24 h, ocorre a formação do haustório e a penetração real na parede celular e invaginação do plasmalema do hospedeiro, resultando no primeiro contato íntimo entre o fungo e a planta (Kolmer, 2013). Depois disto, uma interface haustorial, microscopicamente visível, é produzida ao redor de uma estrutura de alimentação madura, provavelmente composta por material de ambos, fungo e hospedeiro. Essa matriz extra-haustorial é fundamental no controle do tráfego de proteínas e metabólitos e no estabelecimento da nutrição fúngica (Bolton *et al.*, 2008). A partir da formação do haustório, a infecção tende a espalhar-se localmente através do tecido foliar sadio, o que resulta na formação de urédias infecciosas, as quais são hábeis para produzir continuamente um grande número de inóculo secundário, dando origem a diversos ciclos secundários de autoinfecção em um mesmo indivíduo.

P. triticina apresenta baixíssimos níveis de recombinação sexual na natureza. No Brasil esse tipo de reprodução ainda não foi relatada. Contudo, a sua habilidade em evoluir assexuadamente e superar a resistência do hospedeiro contribui de forma significante para o seu sucesso como uma doença amplamente distribuída (Bolton *et al.*, 2008; Dyck & Kerber, 1985). A população de *P. triticina* no Cone Sul é muito dinâmica, o que leva a uma vida curta da resistência em cultivares comerciais (Germán *et al.*, 2007), já que o fungo sobrevive no verão-outono parasitando plantas de trigo voluntárias, que correspondem a principal fonte de inóculo primário para a estação seguinte. A diversidade do patógeno é resultado de mutações ocorridas nas populações existentes ou devido a migração de outras áreas. Além disto, sabe-se que um grande número de raças está presente todos os anos. Germán *et al.* (2007) identificaram aproximadamente 100 diferentes combinações de virulência entre os anos de 2002 e 2004 somente da Região do Cone Sul. Esses relatos

evidenciam a dificuldade em controlar este fungo por métodos convencionais.

O uso de fungicidas e a utilização de cultivares resistentes são frequentemente empregados como métodos de controle para este patógeno. De acordo com Huerta-Espino *et al.* (2011), se nenhum fungicida for aplicado para o controle da ferrugem da folha, as reduções em produtividade podem alcançar cerca de 50%. Por isso, o melhoramento genético direcionado para a resistência de doenças oferece uma alternativa ambientalmente segura ao uso de produtos químicos. Por isso, é o método preferido no combate à ferrugem, uma vez que resulta no uso de cultivares que não exigem aplicação de fungicidas e, portanto, não onera custos aos produtores.

2.3.2 Terminologia da resistência

Embora na literatura exista uma variedade de interpretações sobre o termo resistência, nesta tese os termos seguem a definição adotada nos parágrafos abaixo.

Resistência é a capacidade que determinada planta apresenta em suprimir, retardar ou prevenir a entrada e/ou superar a subsequente infecção por um patógeno, ou seja, o seu crescimento e desenvolvimento nos tecidos vegetais (Parlevliet, 1997). A resistência pode ser classificada sob vários pontos de vista, como por exemplo, genético (monogênica, oligogênica ou poligênica), epidemiológico (específica à raça ou não específica à raça), entre outros.

A resistência específica à raça, completa ou qualitativa, é aquela onde a planta hospedeira proporciona resistência para uma única ou poucas raças do patógeno. De herança qualitativa, segue o modelo de reação gene-a-gene proposto por Flor (1956), onde cada gene de Resistência no hospedeiro somente reconhece o seu gene correspondente de Avirulência no patógeno, garantindo assim a incompatibilidade da reação da doença. Frequentemente encontra-se na literatura associada ao termo Resistência Vertical, como proposto por Van der Plank (1963). De um modo geral, as condições climáticas exercem menor influência sobre este tipo de resistência.

A resistência não específica à raça, parcial ou quantitativa é geralmente, mas não necessariamente, derivada da ação de muitos genes (herança poligênica) que apresentam efeito aditivo, o que promove uma resistência mais estável (ou durável) contra doenças. Sendo assim, a ação conjunta de alguns genes tem a capacidade para inibir simultaneamente o desenvolvimento de raças de um determinado patógeno sem especificidade. Neste tipo de resistência, o ambiente tem papel mais marcante por influenciar com maior ou menor grau a intensidade da doença. Resistência não específica a raça tem sido mais durável do que a específica à raça e é equivalente ao termo utilizado no passado por Van der Plank (1963) como resistência horizontal. Por ser quantitativa, permite a visualização de níveis de resistência intermediários, que podem variar desde

uma resistência muito alta, próxima da imunidade, até a níveis próximos da total suscetibilidade.

Por definição, resistência durável é aquela que se mantém efetiva quanto exposta a grandes áreas, por um longo período de tempo e em ambientes propícios ao desenvolvimento do patógeno (Johnson, 1984).

Existem ainda denominações que se referem à resistência que é expressa em diferentes estágios de desenvolvimento. Por exemplo, resistência de planta adulta, ou RPA, é aquela que somente se expressa a partir do momento em que a planta atinge o estágio reprodutivo ou adulto. Por outro lado, resistência de plântula é aquela condicionada desde a fase inicial de desenvolvimento e se mantém durante todo o ciclo de vida da cultura.

2.3.3 Resistência genética à *P. triticina*

A maneira mais eficiente e segura para se controlar a ferrugem da folha do trigo é através do uso de variedades resistentes à doença. Segundo Chaves *et al.* (2013), a resistência pode ser alcançada pela ação de muitos genes atuando de forma inespecífica às raças do patógeno ou pela ação de genes específicos atuando sobre algumas raças específicas.

Os genes específicos à raça ou genes maiores, conferem resistência do estádio de plântula até a maturidade fisiológica. Essa resistência é baseada na ação de genes expressos de maneira qualitativa e que, frequentemente manifestam uma resposta hipersensitiva de morte celular, determinando uma reação de incompatibilidade entre a planta e o patógeno. Deste modo, limitam a expansão do patógeno no tecido vegetal sadio e, embora seja muito efetiva, continuamente mostra pouca durabilidade a campo (Parlevliet, 2002), pois é altamente vulnerável à evolução de novas raças virulentas. Em função disso, geralmente apresenta uma vida útil limitada (Juliana *et al.*, 2017; Lan *et al.*, 2017)

A resistência de planta adulta (RPA), por outro lado, não tem associação com respostas hipersensitivas e é frequentemente herdada de maneira quantitativa. A RPA caracteriza-se principalmente pelo lento desenvolvimento da doença, também conhecido como *slow-rusting*, sendo expresso apenas na fase adulta da planta. Esse tipo de resistência baseia-se em alguns poucos genes com efeitos parciais associados à QTLs (*Quantitative traits loci*) (St Clair, 2010). Diversos pesquisadores sugerem que a resistência quantitativa pode ser mais durável do que a qualitativa (Kumar *et al.*, 2013; St Clair, 2010; Parlevliet, 2002). Regiões do genoma contento QTLs ou genes de resistência ainda podem ser aditivas e seu acúmulo pode levar a altos níveis de RPA, muitas vezes aproximando-se da imunidade. Lan *et al.* (2017) considera que a RPA é um dos componentes chave

para a resistência durável, já que se expressa de maneira independe à raça do patógeno. Seu efeito no fenótipo da planta leva à redução da velocidade de crescimento da doença, mais do que a sua ausência total (Azzimonti *et al.*, 2013).

Atualmente existem cerca de 76 genes *Lr* (*Leaf rust*) que conferem resistência à ferrugem da folha do trigo (McIntosh *et al.*, 2017) e alguns que ainda possuem denominações temporárias, como é o caso de *Trp-1*, *Trp-2*, *Trp-3* e *Trp-Se*, todos estes oriundos de Toropi (Rosa *et al.*, 2016; Da Silva *et al.*, 2012; Barcellos *et al.*, 2000). A maioria dos genes *Lr* foram identificados no *background* do trigo hexaplóide. Contudo, muitos destes já não são mais efetivos a campo. O monocultivo de extensas áreas utilizando variedades de trigo possuindo um único gene de resistência, na maioria das vezes do tipo raça-específico, tem resultado na seleção de raças de *P. triticina* virulentas capazes de superar a resistência imposta pelas cultivares. Por isso, existe uma constante demanda para a identificação de genes que apresentem uma resistência mais durável a campo.

Diversos autores sugerem que a habilidade que o patógeno possui de superar múltiplos genes de resistência é teoricamente mais difícil do que para superar um único gene (Lan *et al.*, 2017; Singla *et al.*, 2017; Rosa *et al.*, 2016). Deste modo, a combinação de genes de resistência quantitativa apresenta excelente potencial para conferir uma resistência mais durável, além de aumentar a longevidade das cultivares. Atualmente existem cinco genes de resistência de planta adulta e parcial, sendo eles: *Lr34, Lr46, Lr67, Lr68* e *Lr75* (McIntosh *et al.*, 2017; Singla *et al.*, 2017). No entanto, devido à natureza parcial desses genes, é um desafio combiná-los em um único genótipo utilizando o melhoramento genético clássico (Singla *et al.*, 2017). Alguns desses genes foram mapeados em alta resolução ou clonados, tais como *Lr34*, *Lr67* e *Lr68* (Moore *et al.*, 2015; Krattinger *et al.*, 2009; Lagudah *et al.*, 2009).

O gene *Lr34* é o melhor caracterizado até o momento e localizado no cromossomo 7DS. Sabe-se que apresenta maior eficiência em baixas temperaturas e luminosidade, além de possuir um marcador fisiológico associado à necrose da ponta da folha ou *Ltn* (*Leaf tip necrosis*) (McIntosh *et al.*, 2017). Este gene tem origem nas cultivares de trigo Mentana e Ardito, ambas lançadas na Itália em meados de 1900 (Kolmer *et al.*, 2008). Nos anos 40, um cruzamento entre Mentana e Fronteira foi realizado no Brasil, por Iwar Beckman, dando origem à Frontana. Esta cultivar é até hoje utilizada por diversos programas de melhoramento genético de trigo ao redor do mundo (Krattinger *et al.*, 2013; Barcellos *et al.*, 2000). *Lr34* ainda está ligado pleiotropicamente a outros genes de interesse, sendo denominado como *Lr34/Yr18/Sr57/Pm38/Sb1/Ltn1* (Suenaga *et al.*, 2003; Lagudah *et al.*, 2009). Sua resistência tem se mostrado eficiente por mais de 60 anos e nenhum aumento de virulência tem sido observado (Herrera-Foessel *et al.*, 2014; Krattinger *et al.*, 2013; Krattinger *et al.*, 2009; Kolmer *et al.*, 2008). A partir da clonagem deste gene

confirmou-se que ele codifica um transportador cassete do tipo ATP-*binding*, porém o mecanismo de resistência conferido por esse transportador permanece desconhecido, assim como os substratos que ele transporta (Krattinger *et al.*, 2009).

Os genes *Lr46* e *Lr67* também apresentam características similares ao *Lr34*, como resistência a múltiplos patógenos e *Ltn*, sendo estes denominados como *Lr46/Yr29/Sr58/Pm39/Ltn2* e *Lr67/Yr46/Sr55/Pm46/Ltn3* (Herrera-Foessel *et al.*, 2014; 2011). *Lr46* tem sido mapeado em diversos trigos hexaplóides (Lan *et al.*, 2014; Rosewarne *et al.*, 2012) e mais recentemente no background de trigo duro (Lan *et al.*, 2017). Este gene tem sido usado extensamente no programa de melhoramento do CIMMYT com o objetivo de aumentar a resistência de amplo-espectro no avanço de linhagens de trigo (Singh *et al.*, 1998).

A clonagem do *Lr67* foi possível a partir de um BAC (*Bacterial Artificial Chromosome*) carregando o marcador *gwm165* estreitamente ligado ao gene no cromossomo 4DL (0,4 cM). Esse estudo permitiu caracterizar uma proteína de resistência de 514 aminoácidos contendo 12 hélices transmembrana que facilita o transporte de hexoses através da membrana. *Lr67* codifica um transportador de hexose (LR67res) que difere da sua forma suscetível (LR67sus) por dois SNPs (*Single Nucleotide Polymorphisms*) nos éxons 2 e 3. De acordo com Moore *et al.* (2015), a habilidade de reduzir o crescimento de múltiplos patógenos biotróficos pode ser explicada pela alteração no transporte de hexose em folhas infectadas, ou seja, o gene de resistência reduz significativamente a absorção de glicose, impedimento que o patógeno receba o suprimento de açúcares necessários ao seu desenvolvimento. Deste modo, ocorre um aumento de hexose/sacarose no apoplasto foliar, que por sua vez induz a uma sinalização de resposta mediada por açúcar que resulta em um ambiente mais hostil para o crescimento do patógeno (Moore *et al.*, 2015).

Lr68 e Lr75 foram os últimos genes APR mapeados e, portanto, quando comparados aos demais, apresentam poucas informações. *Lr68* foi mapeado no cromossomo 7BL, em uma área específica rica em genes, entre o *locus* associado à cor amarela do endosperma (*Psy1-1*) e o marcador *gwm146* (Herrera-Foessel *et al.*, 2012). Assim como o gene *Lr34*, o *Lr68* também tem origem na cultivar de trigo Frontana, a qual aparece com frequência no *background* de cultivares de trigo do CIMMYT. Em ensaios conduzidos na cidade de Obregon, México, de modo geral, o efeito de *Lr68* na resistência à ferrugem da folha foi menor do que *Lr34*, *Lr46* ou *Lr67*. No entanto, na safra 2010/2011 o *Lr68* apresentou efeito maior do que *Lr46*. Esta safra foi atipicamente mais fria, indicando que *Lr68* pode ter resistência condicionada por baixa temperaturas (Herrera-Foessel *et al.*, 2012).

O último gene RPA mapeado foi o *Lr75*, identificado no *background* da cultivar suíça

Forno, que foi lançada comercialmente em meados de 1986 (Singla *et al.*, 2017). Este fato claramente demonstra a falta de acurácia para a identificação de genes de resistência durável, visto que esta cultivar tem demonstrado respostas de quase-imunidade a todas as raças de *P. triticina* testadas até agora. *Lr75* foi mapeado no cromossomo 1BS entre os marcadores microssatélites *gwm604* (distal) e *swm271* (proximal) a uma distância de 1,6 e 2,7 cM respectivamente (Singla *et al.*, 2017). No estudo de Singla *et al.* (2017), ficou evidente que a associação de *Lr75* com *Lr34* foi positiva, já que as linhas contendo os dois genes mostraram-se mais resistentes do que quando usados de forma isolada.

2.4 Tolerância ao alumínio tóxico
2.4.1 Solos ácidos e danos associados ao Al^{+3}

Alumínio (Al) é o terceiro elemento mais abundante da crosta terrestre depois do oxigênio e do silício. Sob baixo pH, o Al dissolve-se nas formas iônicas, $[Al(H_2O)_6]^{3+}$, comumente conhecido como Al^{3+} e, $Al(OH)^{+2}$. Dentre essas formas, o Al^{3+} é a mais fitotóxica para a rizosfera de trigo, enquanto $Al(OH)^{+2}$ parece ser mais tóxicas para espécies dicotiledôneas (Kochian, 1995).

Em solos onde o pH é menor do que 5,0 a solubilidade do Al tende a aumentar causando limitação na absorção de nutrientes e de água pelo sistema radicular das plantas (Kochian *et al.*, 2015; Kochian, 1995). Isso ocorre principalmente devido ao acúmulo de íons tóxicos nas raízes, especialmente na forma do cátion trivalente Al^{3+}. Como consequência, ocorre a inibição do crescimento do sistema radicular e a produtividade é reduzida em decorrência da deficiência nutricional e do estresse hídrico. Plantas de trigo cultivadas em ambientes ácidos tendem a desenvolver raízes menores e com engrossamento característico. O Al também causa danos extensivos em outros processos celulares. Em cultivares sensíveis pode causar a inibição da síntese de DNA, alteração do potencial da membrana celular e redução do efluxo de H^+ pelo ápice radicular (Garcia-Oliveira *et al.*, 2015).

O estresse oxidativo produzido pelo Al tóxico causa incremento significativo na produção de espécies reativas de oxigênio (ROS), tais como os radicais de superóxido (O_2^-), radicais de hidroxila (OH^-) e peróxido de hidrogênio (H_2O_2) (Guo *et al.*, 2007). ROS podem afetar ácidos graxos insaturados, através da peroxidação da membrana de lipídios, o que por sua vez, leva a um severo dano celular. A morte celular programada ou apoptose também pode ocorrer em decorrência do acúmulo de Al (Inostrova-Blanchetau *et al.*, 2012), porque os íons de Al^{3+} têm alta afinidade por biomembranas, particularmente devido à carga negativa dos fosfolipídios que se ligam ao Al de forma irreversível.

Danos na parte aérea das plantas também sugerem que os efeitos fitotóxicos

podem afetar o metabolismo foliar através da redução de crescimento, conteúdo de clorofila, nutrientes minerais, taxa fotossintética e transpiração (Moustaka *et al.*, 2016). Al reduz ainda a acumulação de cátions divalentes, em especial Ca e Mg, causando prejuízos significativos no fotossistema II (PSII), que é o componente fotossintético mais sensível a estresses (Moustaka *et al.*, 2016; Li *et al.*, 2012).

Com o objetivo de diminuir a fitotoxicidade causada pelos solos ácidos, uma prática comum é a aplicação de calcário nessas áreas, o que aumenta o pH das camadas superiores do solo. No entanto, a utilização de tal prática nem sempre é economicamente, ou fisicamente viável, devido à enorme quantidade de calcário requerido nas aplicações, além de ser dependente do pH do solo, de sua textura e da acidez das camadas mais inferiores (Garcia-Oliveira *et al.*, 2015). O sistema de plantio direto, comumente adotado por produtores de trigo e soja, também dificulta a incorporação do calcário via revolvimento do solo, limitando a quantidade a ser aplicada. Neste caso, como consequência, ocorre a formação de um gradiente de pH no solo, o que por sua vez, limita o aprofundamento as raízes. Grandes aplicações de calcário podem ainda causar efeitos adversos nas plantas ou mesmo causar a deficiência de determinados nutrientes. Por isso, atualmente, muitos produtores têm recorrido ao uso de variedades de trigo tolerantes ao Al, pois além de ser uma alternativa eficiente é ambientalmente segura e não requer gastos expressivos para a correção do pH do solo.

Um dos primeiros desafios para a introdução do trigo no Brasil foi a presença de solos ácidos. Este é possivelmente o motivo pelo qual a tolerância ao Al^{3+} é uma característica relativamente comum nos genótipos brasileiros quando comparados aos genótipos oriundos de outros países (Pereira *et al.*, 2015). Em regiões tropicais e subtropicais, aproximadamente 60% do solo arável é acidificado (Figura 1) (Kochian *et al.*, 2015). No Brasil, os solos usados na triticultura são predominantemente ácidos (Echart & Cavali-Molina, 2001). Como consequência, boa parte dos genótipos de trigo desenvolvidos no Brasil são mundialmente reconhecidos como boa fonte de tolerância ao Al tóxico (Aguilhera *et al.*, 2016).

Historicamente, diversas cultivares de trigo tolerantes ao Al, tais como Fronteira, Surpresa, Minuano, Jesuíta, Guarani, BH1146, Carazinho e Toropi, foram desenvolvidas a partir dos genótipos brasileiros Alfredo Chaves 6-21 e Polyssu. Cultivares de trigo modernas com alguma tolerância ao Al, desenvolvidas em diferentes países, possuem material brasileiro em seu *pedigree* (Garcia-Oliveira *et al.*, 2015). Dentre as cultivares citadas destaque-se a Toropi, que além de apresentar altos níveis de resistência de planta adulta às ferrugens da folha e amarela (Rosa *et al.*, 2016; Casassola *et al.*, 2014), também é conhecida por seus altos níveis de tolerância ao Al tóxico em ensaios de campo e de hidroponia (Aguilera *et al.*, 2016; Ryan *et al.*, 2009; Boff, 2006).
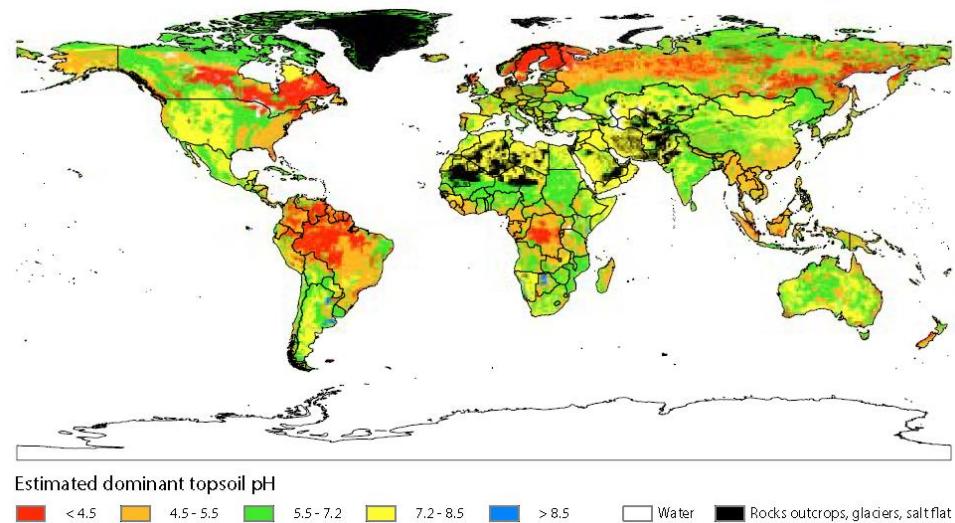
FIGURA 1. Estimativa do pH dominante do solo. Fonte: *World's Soil Resources*, 2015.

2.4.2 Mecanismos de tolerância ao Al^{3+}

As plantas podem lidar com o Al tóxico de diversas formas, mas atuam principalmente através da exclusão de Al^{3+} pelas raízes, prevenindo o acesso de Al^{3+} ao ápice radicular e/ou por mecanismos de tolerância, onde o Al^{3+} entra na planta e é destoxificado e sequestrado (Kochian *et al.*, 2015). Estes mecanismos visam principalmente evitar ou limitar o contato do Al^{3+} com a região meristemática das raízes, permitindo assim que as plantas continuem o seu desenvolvimento. Genes relacionados à tolerância podem estar envolvidos em múltiplos processos metabólicos, que incluem divisão e alongamento celular, formação da parede celular, estresse oxidativo, metabolismo de ferro, transdução de sinal, além de outros mecanismos celulares (Maron *et al.*, 2008). A proteína HvAACT1 (*Hordeum vulgare aluminium-activated citrate transporter*) presente em genótipos tolerantes de cevada é ativada por Al e tem ação principalmente na liberação de citrato pelo ápice radicular, protegendo o crescimento celular e permitindo que o alongamento continue (Ma *et al.*, 2016; Furukawa *et al.*, 2007).

Um dos mecanismos de tolerância mais estudados até o momento é o efluxo de ácidos orgânicos (AOs) pelo ápice de raízes de plantas tolerantes, através de transportadores transmembrana. O tipo de AO exsudado varia de acordo com a espécie vegetal e, embora a maioria secrete apenas um tipo, não é incomum encontrar espécies que liberem mais de um AO (Zheng *et al.*, 1988). Diversos AOs de baixo peso molecular foram encontrados sendo exsudados a partir de raízes de trigo, como malato, citrato, oxalato, succinato, ácido tartárico e fumarato (Garcia-Oliveira *et al.*, 2015). No entanto, em trigo os AOs mais importantes na detoxificação ao Al são o malato, associado ao gene *TaALMT1* (*Aluminium-actived malate transporter 1*) e o citrato, associado a *TaMATE1B*

(*Multi-drug and toxin extrusion 1*).

O malato tem a habilidade de quelar o Al^{3+} tóxico formando um complexo na proporção de 2:2 com os íons de Al^{3+}. Sendo assim, o malato reveste estes íons deixando-os em uma forma não-tóxica às plantas, protegendo o ápice radicular e reduzindo o contato de Al com as raízes. Este mecanismo previne que o Al se ligue aos sítios negativos da parede celular e da membrana plasmática (Garcia-Oliveira *et al.*, 2015), permitindo que genótipos tolerantes consigam ter raízes maiores e sejam mais produtivos do que os sensíveis. A indução dos sistemas de exsudação dos AOs, em resposta à toxicidade por Al, pode ser detectada de segundos a minutos após a exposição, enquanto outras respostas somente são discerníveis depois de horas ou dias (Kochian *et al.*, 2005). Magalhaes *et al.* (2007) observaram que o gene *SbMATE* de sorgo requer alguns dias para alcançar a liberação completa de citrato, enquanto que em *A. thaliana*, a liberação de malato alcança seu máximo em 6 h após a exposição ao Al (Magalhaes *et al.*, 2007).

Segundo Ma *et al.* (2001), o padrão de secreção dos AOs pode ser identificado com base no momento da sua liberação, podendo ser divido em dois padrões: Padrão I, onde a secreção ocorre quase imediatamente após o contato com o Al, sugerindo que o Al ativa um canal de ânions na membrana plasmática pré-existente e a indução de genes não é requerida e, Padrão II, que ocorre horas após a exposição ao Al, sugerindo que a indução de genes é requerida para a ativação de respostas. O tempo de resposta está diretamente vinculado ao grau de tolerância intrínseco da espécie testada. Usando linhagens de trigo tolerantes, He *et al.* (2015) encontraram que estas respondem rapidamente ao estresse por Al e liberam os AOs, em média, 10 min após o contato, através da ativação dos canais de ânions presentes na membrana plasmática. Sendo assim, a maior parte dos estudos acerca dos mecanismos de tolerância em trigo estão focados nas respostas que ocorrem rapidamente após a exposição por Al tóxico.

2.4.3 Os principais genes envolvidos na tolerância ao Al tóxico
2.4.3.1 *TaALMT1*

O primeiro membro identificado da família dos *ALMT* (*Aluminium-actived malate transporter*) foi o *TaALMT1*, descoberto nas pontas das raízes de trigo expressando uma proteína transmembrana com expressão constitutiva (Sasaki *et al.*, 2004). Os genes desta família possuem um alto grau de similaridade e, basicamente, consistem de uma região N-terminal altamente conservada com seis domínios transmembrana, seguida por uma longa, variável e hidrofílica região C-terminal, que pode conter um ou mais domínios transmembrana (Kochian *et al.*, 2015).

TaALMT1 está localizado no cromossomo 4DL de trigo e é responsável por codificar uma proteína transportadora de malato, que apesar de ser funcional na ausência de Al^{3+}

extracelular, possui atividade basal aumentada na presença de Al^{3+} (Kobayashi *et al.*, 2007; Sasaki *et al.*, 2004). Já o seu nível de expressão é fortemente correlacionado com as repetições em bloco promotores, os quais são nomeados de acordo com o número de cópias, que variam de I a VII (Figura 2) (Aguilhera *et al.*, 2016; Pereira *et al.*, 2015). Promotores contendo grande número de repetições, ou seja, de V a VII, apresentam altos níveis de expressão gênica, sendo, portanto, mais tolerantes ao Al^{3+}, em contraste aos promotores com baixo número de repetições, de I a II, que apresentam baixíssimas taxas de expressão gênica e consequentemente maior sensibilidade ao Al^{3+} (Aguilhera *et al.*, 2016). Evidências farmacológicas ainda apontam que uma fosforilação direta da proteína TaALMT1 por uma proteína quinase C (PKC) é um dos pré-requisitos para a ativação da proteína (Ligaba *et al.*, 2009).
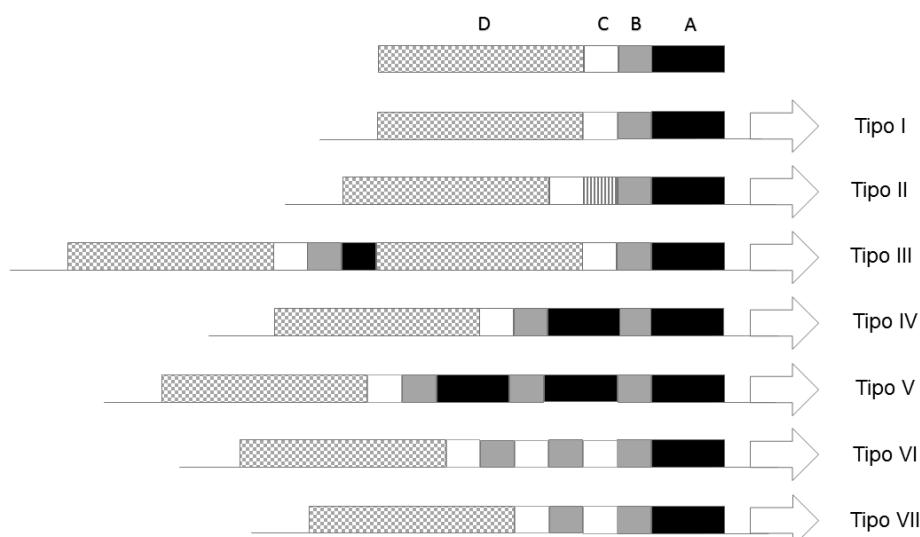
FIGURA 2. Representação esquemática dos alelos promotores do gene *TaALMT1*. O bloco A está representado em preto (172 pb), o bloco B em cinza (108 pb), o bloco C em branco (97 pb) e o bloco D em preto e branco pontilhado (528 pb). Uma repetição (31 pb) dentro do bloco B no promotor Tipo II está representado por linhas verticais. A segunda repetição do bloco A no promotor Tipo III é a menor (70 pb). As linhas representam sequencias não identificadas como repetições por Sasaki et al. (2006) 210 pb à direita e 75 pb à esquerda). As setas indicam a região codificante. [Adaptado de Pereira *et al.*, 2015].

Ainda não é claro a origem das repetições em bloco no promotor de *TaALMT1*. No entanto, estas repetições frequentemente aparecem em *T. aestivum* dentro dos últimos 10.000 anos e, alelos com diferentes padrões de repetições, em sua maioria, têm origem independente (Ryan *et al.*, 2010). Desde a descoberta de *TaALMT1* diversos ortólogos deste gene foram identificados nas mais distintas espécies (Tabela 1), indicando que o mecanismo de liberação de malato é compartilhado por uma ampla gama de espécies.

TABELA 1. Genes ortólogos de *TaALMT1* encontrados em diversas espécies vegetais.

Gene	Espécie	Referência
BnALMT1	Canola (*Brassica napus*)	Ligaba et al., 2006
AtALMT1	Arabidopsis (*Arabidopsis thaliana*)	Kobayashi et al., 2007
GmALMT1	Soja (*Glycine max*)	Fontecha et al., 2007
ScALMT1	Centeio (*Secale cereale*)	Collins et al., 2008
HvALMT1	Cevada (*Hordeum vulgare*)	Gruber et al., 2010
ZmALMT1	Milho (*Zea mays*)	Piñeros et al., 2008
MsALMT1	Alfafa (*Medicago sativa*)	Chen et al., 2013
HlALMT1	Erva-lanar (*Holcus lanatus*)	Chen et al., 2013

Diferentemente de *TaALMT1*, o *AtALMT1* não é expresso constitutivamente em *A. thaliana*. *AtALMT1* tem expressão induzida por Al (Kobayashi *et al.*, 2007), sendo mediada pelos fatores de transcrição STOP1, STOP2 e WRKY46 (Ding *et al.*, 2013), que também são responsáveis por regular outros genes críticos para a tolerância a solos ácidos. Para checar a especificidade do *ALMT1* ao Al, raízes de *A. thaliana* foram expostas a cádmio (Cd), cobre (Cu), érbio (Er), lântano (La), sódio (Na) e baixo pH. Como resposta, somente o Al foi capaz de induzir a liberação de malato acima do nível observado nas plantas controle (Magalhaes *et al.*, 2007), indicando que o mecanismo desempenhado por este gene tem expressão gênica aumentada somente na presença de Al e não sofre influência significativa de outros elementos. No entanto, estudos posteriores encontraram outros 13 membros da família *ALMT* no genoma de *A. thaliana*, sugerindo que eles podem estar envolvidos nos mais diversos processos e não só na tolerância ao Al^{3+} (Meyer *et al.*, 2011).

Diferenças significativas de funções podem ser encontradas entre genes ortólogos com sequências altamente similares (Palmer *et al.*, 2016). *ZmALMT1* foi o primeiro membro da família *ALMT* descrito que não possui atividade na tolerância ao Al. Este gene localiza-se na membrana plasmática de células de milho, porém é menos permeável aos AOs e, provavelmente, está envolvido na homeostase de ânions inorgânicos e nutrição mineral (Piñeros *et al.*, 2008). O gene *HvALMT1* de cevada é um outro exemplo bem característico, pois codifica um canal de malato expresso em células guardas, na zona de alongamento da raiz, em tecidos florais e em sementes (Gruber *et al.*, 2010). Embora tenha alta similaridade à sequência de *TaALMT1* e se localize na membrana plasmática, curiosamente *HvALMT1* não está envolvido na tolerância ao Al. Estudos mais recentes têm demostrado que o canal codificado por *HvALMT1* tem papel primordial no desenvolvimento dos grãos e, durante a germinação, atua na acidificação do amido no endosperma (Xu *et al.*, 2015).

2.4.3.2 *TaMATE1B*

As proteínas MATE (*Multi-drug and toxin extrusion*) utilizam-se do gradiente

eletroquímico da troca de Na$^+$/prótons para exportar uma ampla variedade de substratos, incluindo metabólitos secundários e xenobióticos (He *et al.*, 2015). Essas proteínas estão presentes em diversos organismos, como bactérias, fungos, plantas e mamíferos (Omote *et al.*, 2006). A família dos genes *MATE* foi identificada inicialmente em resposta ao Al tóxico em sorgo (*Alt*$_{SB}$) e cevada. Por isso, os estudos pioneiros nessa família são provenientes destas duas espécies. Sabe-se, no entanto, que existem 58 ortólogos de *MATE* no genoma de *Arabidopsis* (Yazaki, 2005) e pelo menos outros 40 genes em arroz (Yokosho *et al.*, 2009), mas somente poucos membros desta família foram funcionalmente caracterizados até o momento. A cevada é o cereal mais sensível ao Al tóxico (Furukawa *et al.*, 2007), mas ainda assim apresenta significativa variação entre genótipos (Ma *et al.*, 2016; Bian *et al.*, 2015).

Sabe-se que *TaMATE1B* está localizado no cromossomo 4BL de trigo, codificando uma proteína transmembrana transportadora de citrato. Ryan *et al.* (2009) identificaram o *locus Xce$_c$* na cultivar brasileira Carazinho como sendo o responsável por mais de 50% da variação fenotípica total do efluxo de citrato. Devido à importância da família *MATE* nas mais diversas espécies, a tabela 2 apresenta alguns de seus ortólogos descritos nos últimos anos.

TABELA 2. Genes ortólogos de *TaMATE1B* encontrados em diversas espécies vegetais.

Genes	Espécie	Referência
ZmMATE1	Milho (*Zea mays*)	Maron et al., 2010
AtMATE1	Arabidopsis (*A. thaliana*)	Liu et al., 2009
VuMATE1	Feijão (*Vigna umbellata*)	Yang et al., 2011
OsFRDL1	Arroz (*Oryza sativa*)	Yokosho et al., 2011
SbMATE	Sorgo (*Sorgum bicolor*)	Magalhaes et al., 2007
HvMATE (*HvAACT1*)	Cevada (*Hordeum vulgare*)	Furukawa et al., 2007; Bian et al., 2015
ScAACT1	Centeio (*Secale cereale*)	Silva-Navas et al., 2011
GmMATE	Soja (*Glycine max*)	Yokosho et al., 2009
MtMATE	Luzerna-cortada (*Medicago truncatula*)	Chandran et al., 2008
LaMATE	Tremoço (*Lupinus albus*)	Uhde-Stone et al., 2005

Alguns membros da família *MATE* apresentam mecanismos de tolerância ao Al^{3+} singulares. Os genes *OsFRDL1* e *GmMATE* codificam proteínas envolvidas na translocação de ferro a partir das raízes em direção para a parte aérea de arroz e soja, respectivamente. Essas proteínas estão localizadas nas células do periciclo e medeiam a liberação de citrato para o xilema (Yokosho *et al.*, 2009). Ao entrar em contato com o ferro, o citrato forma um complexo que é translocado em direção à parte aérea das plantas.

2.4.3.3 Relação entre *TaALMT1* e *TaMATE1B*

Repetições em bloco no promotor de *TaALMT1,* bem como a inserção de um *transposon* na região promotora de *TaMATE1B*, têm sido relacionadas aos níveis de expressão gênica, efluxo de AOs e tolerância ao Al^{3+} em diversas cultivares brasileiras de trigo (Pereira *et al.*, 2015). Dentre 300 cultivares brasileiras de trigo testadas, o maior crescimento relativo da raiz em resposta ao Al foi observado nos genótipos que apresentavam os promotores de *TaALMT1* do tipo V e VI e a inserção do promotor de *TaMATE1B* (Pereira *et al.*, 2015). Zheng *et al.* (1998) observaram que a liberação de malato por *TaALMT1* tem maior impacto do que a liberação de citrato por *TaMATE1B,* mesmo sendo o citrato oito vezes mais eficiente que o malato em quelar Al^{3+}. Uma das razões para isso está na quantidade de malato liberado, sendo 10 vezes maior do que a de citrato em raízes de trigo tolerantes (Tovkach *et al.*, 2013; Ryan *et al.*, 2009).

Estudos relacionados aos genes *TaALMT1* de trigo (Sasaki *et al.*, 2004), *SbMATE* de sorgo (Magalhaes *et al.*, 2007) e *HvMATE* e *HvAACT1* de cevada (Bian *et al.*, 2015; Furukawa *et al.*, 2007) sugerem que os *loci* de tolerância ao Al^{3+} apresentam maior efeito e herança monogênica. No entanto, há diversos casos onde uma aparente segregação transgressiva pode ser observada (Caniato *et al.*, 2011; Boff, 2006). O que levanta a hipótese de que este tipo de herança pode ser bem mais complexa e que outros genes importantes ainda podem estar não-identificados nessas espécies (Garcia-Oliveira *et al.*, 2015; Kochian *et al.*, 2015).

2.4.4 Outras regiões de interesse no genoma de trigo

Nos últimos anos a tecnologia direcionada para estudos em biotecnologia vegetal tem evoluído de forma exponencial. A disponibilidade e a facilidade de obtenção de diferentes populações de mapeamento associadas a marcadores moleculares mais robustos não só facilitam a identificação de vários *loci* genéticos envolvidos em características complexas como também ajudam a entender como os genes atuam em determinada característica. O mapeamento de QTLs tem ajudado muitos geneticistas a identificar regiões de interesse dentro do genoma de diversas espécies vegetais. Numerosos estudos foram realizados utilizando diferentes tipos de populações de mapeamento, as quais são derivadas de diversos parentais (Ma *et al.*, 2016; Nava *et al.*, 2016; Bian *et al.*, 2015; Yokosho *et al.*, 2010; Ryan *et al.*, 2009). Tais estudos demonstram que há de um a três QTLs envolvidos na tolerância ao Al tóxico em trigo, nas populações estudadas.

Recentemente, a identificação de QTLs sem a necessidade de populações de mapeamento tornou-se possível devido ao uso do *Genome-wide association mapping* (GWAS), que representa uma abordagem complementar aos estudos de mapas de ligação (Gao *et al.*, 2016). GWAS é baseado no desequilíbrio de ligação em germoplasmas

geneticamente diferentes. Utilizando essa tecnologia, Raman *et al.* (2010) identificaram pelo menos 16 *loci* genéticos para tolerância ao Al tóxico em trigo, nos cromossomos 1A, 1B, 2A, 2B, 2D, 3A, 3B, 4A, 4B, 4D, 5B, 6A, 6B, 7A e 7B.

Navakode *et al.* (2014) identificaram um novo *locus* para tolerância a Al no cromossomo 1DL e o atribuíram ao *bin* 1DL2-0,41-1,00 onde o gene candidato *wali5* (*wheat aluminium induced*) responsivo a Al havia sido previamente identificado. Já o gene *TaSTOP1* foi localizado do braço longo do grupo cromossomal 3 [3AL (*TaSTOP1-A*), 3BL (*TaSTOP1-B*) e 3DL (*TaSTOP1-D*)]. Tais informações evidenciam a diversidade genética entre genótipos em relação à tolerância ao alumínio tóxico e a existência de outros genes além de *TaALMT1* e *TaMATE1B* agindo no genoma de trigo.

2.4.5 Fenotipagem utilizando hidroponia

Ensaios de fenotipagens são pré-requisitos básicos para qualquer programa de melhoramento mundo afora. Esses experimentos devem ser, além de confiáveis, simples de executar. Diferentes metodologias podem ser utilizadas para a avaliação da tolerância ao Al^{3+} tóxico em trigo. Nos ensaios conduzidos a campo é muito difícil isolar os fatores nutricionais e os mecanismos de absorção de nutrientes do solo devido à heterogeneidade espacial presente naturalmente no solo, além das propriedades químicas e físicas que simultaneamente afetam o desenvolvimento das plantas. A dificuldade de remoção das plantas do solo sem causar danos às raízes também faz desse tipo de metodologia um desafio (Nava *et al.*, 2016). Por isso, o uso de hidroponia para fenotipagem tem se mostrado uma alternativa bastante segura, permitindo a análise de diversas características. Este método é vantajoso frente aos ensaios de campo, pois é rápido, não-destrutivo e permite um melhor controle sobre as condições ambientais (Shaurukov *et al.*, 2012).

A hidroponia ainda permite um fácil acesso ao sistema radicular das plantas, sem causar danos. Garante uma alta acurácia nas concentrações de Al testadas, permitindo também um controle na disponibilidade de nutrientes e pH. Correlação significativa tem sido reportada entre ensaios de campo e avaliações realizadas em hidroponia em aveia (Nava *et al.*, 2016), trigo (Pereira *et al.*, 2015) e cevada (Bian *et al.*, 2016). O que permite realizar fenotipagens em grandes populações de mapeamento, utilizando pequenos espaços e em curtos períodos de tempo.

Os ensaios utilizando hidroponia só são válidos quando conduzidos em condições controladas. A quantidade de Al^{3+} aplicada em cada tratamento deve ser precisa, de modo a evitar erros básicos de fenotipagens, como o uso de diferentes fontes de Al em um mesmo ensaio ou o desbalanceamento de soluções padrões. O uso de variedades tolerantes e sensíveis deve ser padrão em todos os ensaios, a fim de se ter um controle sob todas as condições testadas. A hidroponia deve ser usada como um aliado nas

fenotipagens de tolerância à toxicidade, porém deve obedecer a preceitos básicos acima mencionados para que seus resultados sejam validados.

2.5 Mapeamento de QTLs utilizando marcadores DArT
2.5.1 Mapeamento de QTLs

Boa parte das características agronômicas de importância são poligênicas ou de herança quantitativa, isto é, são resultado da ação de vários *loci* que podem exibir efeitos variáveis. As regiões cromossômicas que contém estes *loci* são denominadas como QTLs (Lannou, 2012). Para Liu (1998), os QTLs são supostos genes descobertos a partir de inferências baseadas em análises estatísticas e cujos efeitos no mapeamento podem ter um significado biológico. De acordo com Khan (2015), um QTL é uma região do genoma ou um *locus* do gene que está associado a uma característica quantitativa. Um determinado QTL pode ser um único gene ou pode ser um grupo de genes ligados controlando uma determinada característica. As análises de mapa de ligação em populações segregantes experimentais são comumente usadas para dissecar a arquitetura genética de características complexas (Bazakos *et al.*, 2017).

A identificação e a localização de *loci* específicos mediando caracteres quantitativos são abordagens de grande importância no melhoramento de plantas. Para identifica-los é necessário que se faça um mapeamento genético, o qual consiste na distribuição de marcadores moleculares ao longo de um genoma de referência, associados à determinação da distância genética entre eles. Estes marcadores são arranjados nos grupos de ligação, os quais correspondem ao número de cromossomos da espécie em estudo. Quando os marcadores ligados à característica de interesse são identificados através de ferramentas computacionais, é possível selecionar os indivíduos com base no genótipo, técnica conhecida como MAS (*Marker-Assisted Selection*). Esse tipo de ferramenta é importante nos casos em que a característica está presente em espécies anuais exigentes em condições climáticas específicas, como é o caso do trigo (Lannou, 2012).

O mapeamento genético é uma ferramenta que exige a utilização de uma grande quantidade de marcadores a fim de se obter uma ampla cobertura do genoma (St Clair, 2010). De acordo com Liu (1988) mapas genéticos permitem a localização e a avaliação da magnitude do efeito das regiões genômicas associadas a caracteres de importância agronômica. Mapear um QTL significa fazer deduções em todo o genoma sobre as relações existentes entre o genótipo e o fenótipo dos caracteres quantitativos. Estas deduções compreendem informações a respeito do número e da posição dos *loci* no genoma em estudo, além dos efeitos destes *loci* sobre a característica de interesse, modo de ação gênica e a possibilidade de analisar a interação genótipo x ambiente em cada QTL

(Lannou, 2012). No entanto, o mapeamento é altamente dependente da acurácia da fenotipagem em múltiplos ambientes e entre diferentes escalas espaciais e temporais, o que ainda é limitado pela sistemática da genética quantitativa (Bazakos *et al.*, 2017). A reprodutibilidade dos ensaios de fenotipagens também é um fator limitante. Por isso, variações não-genéticas causadas por perturbações ambientais devem ser evitadas ou minimizadas.

Os recentes avanços em tecnologia de sequenciamento genético agora permitem uma genotipagem mais rápida e economicamente viável em populações F_2, além de uma investigação complexa de processos fundamentais na genética vegetal, tais como a recombinação meiótica. No entanto, a necessidade de genotipar os indivíduos em cada experimento e a impossibilidade de usar o mesmo conjunto de linhagens em diferentes condições têm forçado os geneticistas a produzirem populações de linhagens endogâmicas recombinantes (RILs), a partir de linhas F_2 por sucessivas autofecundações (Bazakos *et al.*, 2017). Cada RIL de uma determinada população é caracterizada por apresentar alta taxa de homozigose e, por isso, apresenta um mosaico único dos dois genomas parentais. A principal vantagem de se usar populações RILs é que estas podem ser fenotipadas em diferentes condições e para diferentes características de interesse e, no entanto, serem genotipadas somente uma única vez devido à alta homozigose.

2.5.2 Marcadores DArT (*Diversity arrays technology*)

Atualmente existe uma boa disponibilidade de marcadores moleculares passíveis de serem usados para o mapeamento genético, como os RFLP (*Restriction Fragment Length Polymorphism*), RAPD (*Random Amplified Polymorphic DNA*), AFLP (*Amplified Fragment Length Polymorphism*) e SSR (*Simple Sequence Repeat*) ou microssatélites. No entanto, a maioria gera um número relativamente baixo de polimorfismos ao longo do genoma, o que dificulta a construção de mapas de ligação, bem como a detecção de *loci* de interesse. Com o surgimento dos marcadores DArT (*Diversity Arrays Technology*) essa limitação tem sido contornada, visto que esta tecnologia permite a identificação de polimorfismo no DNA, oferecendo um sistema rápido, robusto, que requer mínimas quantidades de DNA e elimina completamente a subjetividade dos géis de eletroforese (Kilian *et al.*, 2009). Os marcadores DArT foram originalmente desenvolvidos para a cultura do arroz (Kilian *et al.*, 2009), embora sejam utilizados com sucesso para a construção de mapas genéticos, estudos de caracterização de germoplasma e de diversidade genética de várias espécies, como cevada (Wenzl *et al.*, 2004), trigo (Lan *et al.*, 2015), aveia (Cover, 2010) e cana-de-açúcar (Heller-Uszynska *et al.*, 2011).

Os DArT objetivam detectar um grande número de variações genéticas dentro de um genoma, através da utilização de plataformas de microarranjos ou de sequenciamento

(DArT-Seq) para a análise dos polimorfismos no DNA (Jaccoud *et al.*, 2001). Marcadores DArT baseados em microarranjos são obtidos através da redução da complexidade do genoma por meio da digestão do DNA com enzimas de restrição (Kilian *et al.*, 2009). Os fragmentos de DNA resultantes da clivagem são utilizados para a construção de uma biblioteca que irão dar origem ao microarranjo. O DNA a ser analisado é então marcado por fluorescência e sua leitura é feita com base na detecção de presença e ausência dos fragmentos hibridizados no microarranjo.

Devido ao avanço tecnológico de genotipagem por sequenciamento de nova geração ou NGS (*Next Generation Sequencing*), surgiu uma variante dos marcadores DArT, os então conhecidos como DArT-Seq, os quais diferem pela genotipagem via sequenciamento (Kilian *et al.*, 2009). No DArT-Seq as sequências resultantes da clivagem pelas enzimas de restrição são sequenciadas e alinhadas com o genoma de referência e detectados os polimorfismos a partir da comparação destas sequências (Sansaloni *et al.*, 2011). Neste caso, a presença ou ausência são identificadas nos fragmentos em comum das amostras sob análise, além de permitir a detecção de variações, ou seja, os SNPs. Sendo assim, os DArT-Seq fornecem duas classes de marcadores, os dominantes baseados na presença/ausência de fragmentos e, os codominantes ou SNPs.

A utilização das informações obtidas com o mapeamento genético tradicional e com o mapeamento físico do genoma devem facilitar a caracterização de genes e de regiões do genoma relacionados com a expressão fenotípica. Essas informações oferecem subsídios para a clonagem de genes desejáveis, tais como genes de resistência às doenças e de tolerância a estresses abióticos.

2.6 Toropi

A cultivar de trigo Toropi com pedigree Frontana 1971.37/Quaderna//Petiblanco 8 (Figura 3), foi lançada comercialmente pela Estação Experimental Federal de Júlio de Castilhos/RS em 1965 e possui resistência parcial de planta adulta à ferrugem da folha, sendo também não específica às raças do patógeno (Figura 4.A). Esta cultivar foi produzida em escala comercial durante aproximadamente 15 anos, sendo gradativamente substituída por outras variedades de trigo mais produtivas. No entanto, a sua resistência tem demonstrado eficácia por mais de 50 anos, mesmo sob alta pressão de inóculo, quando submetida em ensaios conduzidos a campo ou inoculada em casa de vegetação, em ambas as condições sob uma ampla gama de raças de *P. triticina*. Além da resistência à ferrugem da folha, Toropi ainda apresenta outras características agronômicas de interesse, entre elas, o aumento da absorção, distribuição e translocação de fósforo, tolerância ao Al^{3+} tóxico (Figura 4.B) (Boff, 2006), resistência a Fusarium (Kohli, 1989), resistência à ferrugem amarela (Rosa *et al.*, 2016) e à ferrugem do colmo (*dados não publicados*).
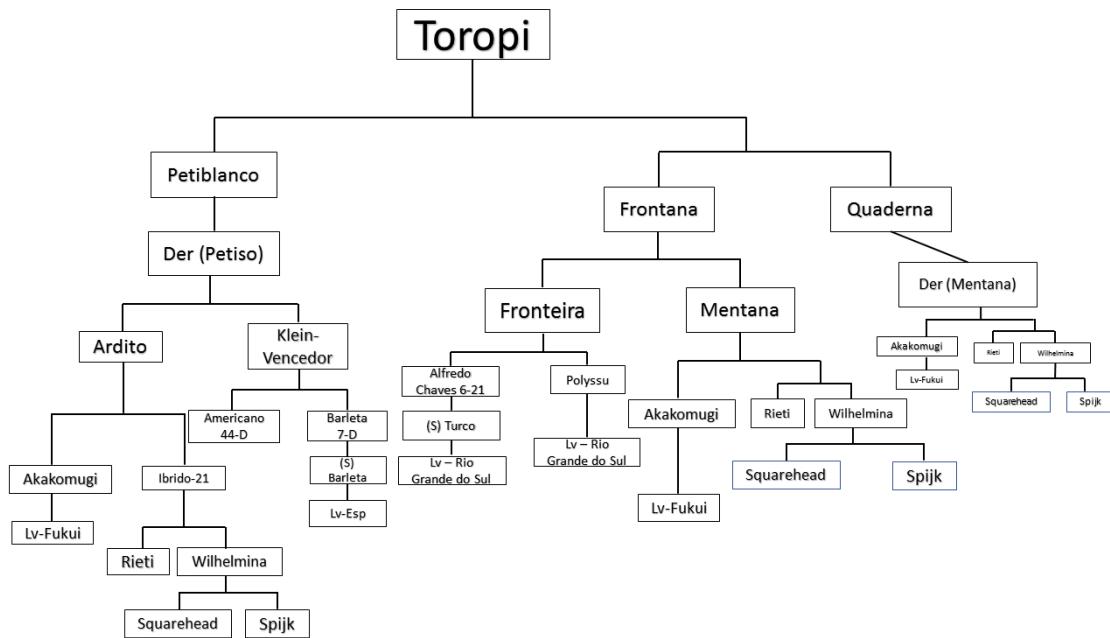
FIGURA 3. Genealogia da cultivar Toropi. [Adaptado de: *Genetic Resources Information System for Wheat and Triticale*, 2017]. Disponível em: >http://wheatpedigree.net/sort/renderPedigree/61662< [Acesso em: 14 de maio de 2017]

FIGURA 4. Reação de Toropi à (A) *Puccinia triticina* em planta adulta e (B) ao alumínio tóxico em plântula.

Os estudos para elucidar a genética da resistência de Toropi iniciaram por Barcellos *et al.* (2000), os quais determinaram que a RPA era devido a presença de dois genes recessivos e complementares. Tais estudos foram realizados utilizando progênies em F_2 dos cruzamentos entre Toropi x IAC13-Lorena (Ciano 67/IAS 51) (Figura 5). Mais tarde, Da Silva *et al.* (2012), usando a mesma população de linhas recombinantes de Toropi x IAC13,

porém em F$_7$, mapearam esses dois genes nos cromossomos 1AS e 4DS. Estes dois genes receberam a denominação temporária de *Trp-1* e *Trp-2* (McIntosh *et al.*, 2017). No mesmo ano, Rosa (2012) identificou dois novos genes diferentes de *Trp-1* e *Trp-2*, um deles conferindo resistência do tipo raça-específica, o que até então era desconhecido no *background* de Toropi. Localizado no cromossomo 3D, este gene recebeu a denominação temporária de *Trp-Se*, estando mapeado dentro de um grupo de ligação composto por 22 marcadores microssatélites. De acordo com a autora, este gene é uma importante fonte de resistência à ferrugem da folha quando associado a outros genes *Lr*, principalmente na Nova Zelândia, onde foi possível observar respostas de quase imunidade às raças testadas. No entanto, em ensaios de campo realizados no Brasil e Canadá, este gene não foi efetivo, provavelmente devido à diferente combinação de virulência apresentada pelas raças de *P. triticina* destes dois países. O segundo gene de resistência descoberto por Rosa (2012) recebeu o nome temporário de *Trp-3* e foi mapeado no cromossomo 4BL sendo flanqueado pelos marcadores *gpw4079* (0,8 cM – região distal) e *gwm149* (2,4 cM – região proximal) (Rosa, 2012).

Diversas regiões do genoma desta cultivar podem estar envolvidas no processo de resistência. Foram identificados até o momento QTLs nos cromossomos 2B, 5AL, 5D, 4B e 7A, em anos e locais diferentes (Rosa, 2012). A região denominada como *QLr.crc-5AL* merece destaque, pois apresentou RPA não só para a ferrugem da folha, mas também para a ferrugem amarela (*QSr.crc-5AL*) (Rosa, 2012). *QLr.crc-5AL* ou *QSr.crc-5AL*, foi identificado no braço longo do cromossomo 5A, sendo altamente significativo para ferrugem da folha no Canadá, explicando de 22 a 39% da variação fenotípica. Para a ferrugem amarela o mesmo explicou aproximadamente 13% da variação fenotípica em ensaios conduzidos na Nova Zelândia (Rosa, 2012). Esta região do genoma de Toropi pode ancorar um novo gene RPA uma vez que, de acordo com McIntosh *et al.* (2017), não há relato de genes *Lr* mapeados no cromossomo 5A de trigo. Estes estudos sugerem que a resistência de Toropi é altamente complexa e pode ser condicionada por pelo menos cinco genes. Foram identificados até o momento dois genes de planta adulta, designados como *Trp-1* e *Trp-2,* um gene de plântula *Trp-Se* e o gene *Trp-3*, este último de menor efeito, raça-específico e de planta adulta, além da região *QLr.crc-5AL,* que devido ao seu efeito poderá ser alvo de estudos genéticos posteriores. Mesmo assim, não se descarta a hipótese de que Toropi apresente outras regiões com resistência ainda não identificadas, posto que a identificação da resistência é fortemente influenciada pelas condições ambientais e, principalmente, pelo tamanho das populações em estudo (Kumar *et al.*, 2013).
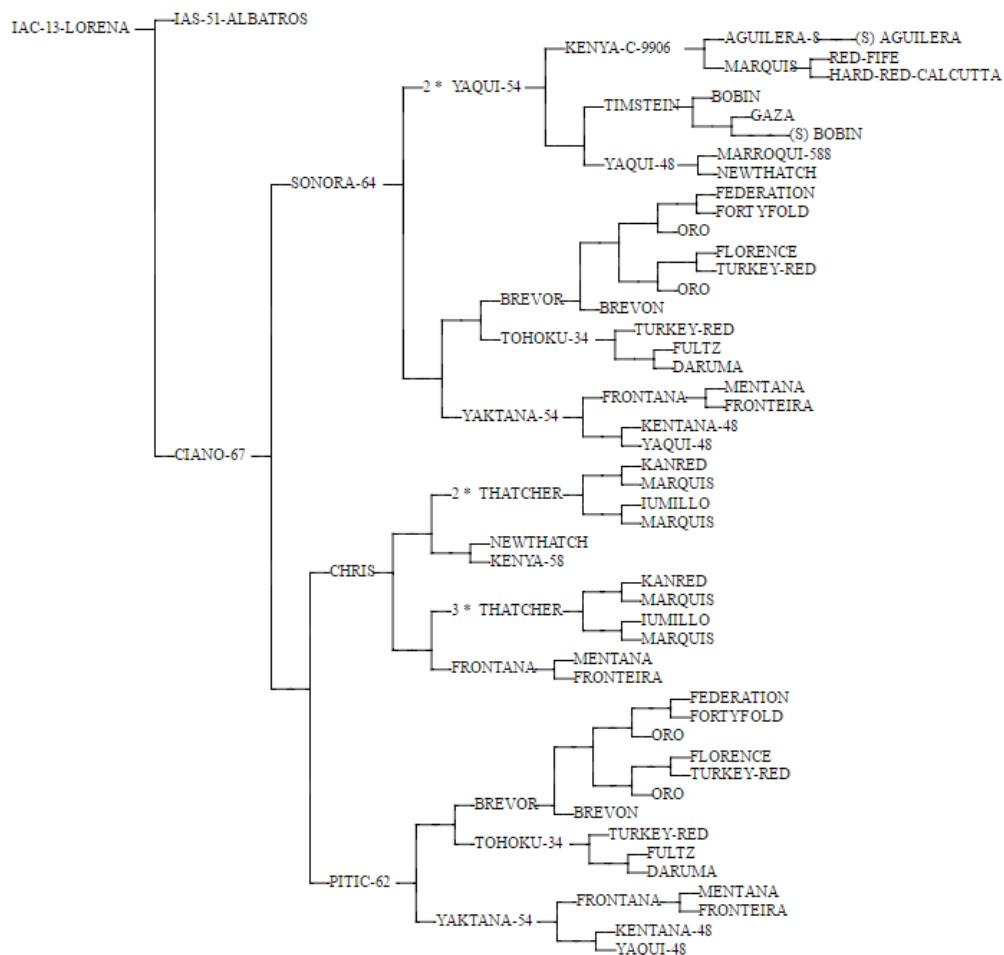
FIGURA 5. Genealogia da cultivar IAC13. [Adaptado de: *Genetic Resources Information System for Wheat and Triticale*, 2017]. Disponível em: >http://wheatpedigree.net/sort/renderPedigree/61662< [Acesso em: 14 de maio de 2017]

Ensaios de campo conduzidos no Quénia de 2005 a 2011 constataram que Toropi também possui RPA à ferrugem do colmo (*dados não publicados*). A resistência a múltiplas doenças é uma das características mais importantes dos genes de planta adulta. Este potencial efeito pleiotrópico tem sido estudado com detalhes nos genes *Lr34, Lr46*, *Lr67* e *Lr68* e, mais recentemente, em *Lr75* (Singla *et al.*, 2017). Estudos recentes utilizando marcadores moleculares diagnósticos comprovaram que Toropi não possui nenhum dos genes citados acima (*dados não publicados*), o que faz da resistência genética desta cultivar, uma possível nova fonte para programas de melhoramento. A resposta de Toropi à *P. triticina* assemelha-se às respostas do gene *Lr34*. Porém, recentes ensaios conduzidos, comprovam que Toropi apresenta fenótipo com menor índice de doença quando comparado a linhas carregando *Lr34*. Portanto, levanta-se a hipótese de que um ou mais genes de Toropi podem apresentar mecanismos de resistência semelhante ao do *Lr34*.

Sabe-se que a resistência de Toropi é não-específica às raças de *P. triticina*. Por isso, foram conduzidos estudos histopatológicos entre três genótipos de trigo e o patógeno com o intuito de conhecer os mecanismos estruturais e bioquímicos e a natureza espaço-temporal do seu desenvolvimento, o que representa a etapa intermediária essencial para relacionar o fenótipo com o genótipo. Observou-se que houve uma redução drástica na formação de apressórios e de todas as outras estruturas fúngicas subsequentes do processo infeccioso em Toropi, quando comparado aos outros dois genótipos BRS194 e RL6010 *Lr9* (Wesp-Guterres *et al.*, 2013). Durante as várias tentativas de penetração pelo patógeno, observou-se que ocorreu produção de compostos autofluorescentes e morte celular tardia, porém não houve formação de peróxidos. Acima de tudo, os eventos iniciais de infecção foram significativamente reduzidos antes da formação do haustório, o que caracteriza esta resistência como um mecanismo do tipo pré-haustorial (Wesp-Guterres *et al.*, 2013). Esta resistência restringe a formação de estruturas primárias de infecção, como o apressório sobre o estômato, a vesícula subestomatal e a célula mãe do haustório. Esse mecanismo pode ser um dos responsáveis pela manutenção da doença em baixos níveis de severidade, mesmo em alta pressão de inóculo.

As alterações no transcriptoma de Toropi durante a infecção de plantas adultas por *P. triticina* foram estudadas por Casassola *et al.* (2014). Nestas análises, as observações prévias de Wesp-Guterres *et al.* (2013) de que a resposta de defesa em Toropi se inicia nos primeiros momentos de contato com o patógeno foram confirmadas. Os autores verificaram ainda que há maior expressão gênica no momento da formação do haustório. As principais rotas metabólicas alteradas durante o processo de infecção por ferrugem da folha foram: geração de precursores metabólicos e energia; catabolismo; resposta a estímulos bióticos e abióticos e ao estresse; e transporte. Mecanismos clássicos de defesa como lignificação, estresse oxidativo, geração de energia, fluxo de água e lipídeos e ciclo celular, foram alterados durante o processo de infecção, bem como pode haver uma modulação do metabolismo da planta pelo patógeno visando beneficiar seu desenvolvimento. Genes relacionados com a patogênese (PR) também tiveram expressão em diferentes tempos pós-inoculação. Observou-se que em 24 h após a inoculação é o momento em que mais genes são diferencialmente expressos. Os genes envolvidos no reforço da parede celular, na degradação de estruturas fúngicas, produção de ROS, detoxificação celular, geração de energia e absorção de água e nutrientes parecem estar envolvidos na resistência pré-haustorial de Toropi. Vários *contigs* não apresentaram resultado de BLAST, podendo representar novos genes a serem utilizados no melhoramento genético, assim como outros genes com função conhecida, mas ainda não utilizados. Doze *contigs* foram expressos somente na presença do patógeno e não possuem homologia na variedade de trigo Chinese Spring. Quatro *contigs* dentre os 12 não

possuem anotação funcional e podem representar uma nova visão no entendimento de mecanismos ainda não descritos envolvidos na defesa de trigo contra a ferrugem da folha.

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3 ARTIGO 1

Quantitative Traits Loci for leaf rust resistance in wheat[3]

Quantitative Traits Loci for leaf rust resistance in wheat

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ABSTRACT: Leaf rust, caused by the fungal pathogen *Puccinia triticina* is a major threat to wheat production in many wheat growing regions around the world. The introduction of leaf rust resistance genes into elite germplasm is the preferred method of disease control and crucial to sustained wheat production. Consequently, there is considerable value in identifying and characterizing new sources of leaf rust resistance. While many major leaf rust resistance genes are known for wheat, a growing number of potentially valuable quantitative sources of resistance are reported. This review presents the progress that has been made in the genetic mapping of quantitative trait loci (QTL) for leaf rust adult plant resistance, while assessing their potential value for wheat leaf rust resistance breeding. Here we report 188 leaf rust resistance QTL identified using bi-parental mapping populations, 165 identified in genome-wide association studies (GWAS) and 35 Meta-QTL (MQTL). The MQTL are consensus genomic regions obtained by QTL meta-analysis. The leaf rust resistance loci reported here provide an overview of the diversity present in wheat genotypes across the world. Leaf rust resistance QTL were found on all 21 chromosomes of hexaploid wheat, with a concentration of resistance QTL on chromosomes 1B and 2B, with 28 and 23 QTL identified, respectively.

Key-words: Leaf rust resistance; QTL; MQTL; APR genes; *Puccinia triticina*; *Lr* genes.

Wheat leaf rust resistance

Hexaploid bread wheat (*Triticum aestivum* L.; AABBDD) is believed to have originated some 10,000 years ago (TANNO & WILLCOX, 2006) from a hybridization between the tetraploid emmer wheat (*Triticum dicoccoides;* AABB) and the diploid grass (*Aegilops tauschi*; DD) (NESBITT & SAMUEL, 1996). Wheat provides 1/5th of the calorie

intake of the world's population and therefore is a major contributor to global food security (FAO, 2015). Disease is a major factor impacting on crop production, and for wheat the three rusts, leaf (brown) rust, stripe (yellow) rust and stem (black) rust caused by *Puccinia triticina*, *P. striiformis* f. sp. *tritici* and *P. graminis* f. sp. *tritici*, respectively, are considered wide global problems. Rust disease in wheat have been recorded for thousands of years (KOLMER et al., 2009), with rust epidemics being exacerbated by growing monocultures of varieties with limited resistance genetic diversity. While stem and stripe rust are in general considered more damaging than leaf rust, leaf rust is more persistent and widespread, making it a significant problem year-on-year (HUERTA-ESPINO et al., 2011). Currently fungicides are widely used to control wheat rusts, however in many countries access to appropriate fungicides, and inappropriate application methods can have serious consequences on the environment and human health (KUMAR et al., 2013), as well as increasing production costs. Introduction of effective and potentially durable genetic resistance into new wheat varieties is by far the preferred approach.

Many loci conferring resistance to the three rust pathogens of wheat have been reported, and for leaf rust 77 loci have been given a designated *Lr* number (McINTOSH et al., 2017). Many of these *Lr* genes represent resistance that is effective throughout the life of the wheat plant, commonly referred to as all-stage-resistance (ASR). However, ASR is often race-specific, the resistance only being effective against *P. triticina* isolates carrying the corresponding avirulence gene (NSABIYERA et al., 2016; KOLMER et al., 2005). Consequently, due to high levels of virulence diversity present within *P. triticina* populations, long-lasting resistance within wheat varieties has been hard to achieve (KOLMER, 2013), many newly introduced *Lr* genes becoming ineffective within a few years of introduction. However, some *Lr* genes are known that have remained effective for many years, conferring non-race-specific resistance. In contrast to most race-specific *Lr* genes these non-race-specific *Lr* genes confer quantitative or partial resistance, and are often expressed at later stages in the plants development, being referred to as adult plant resistance (APR). APR generally tends to result in the production of smaller pustules and fewer spores, and when associated with an increased latent period is often termed slow-rusting resistance.

Here, we provide a concise review of quantitative trait loci (QTL) associated to APR leaf rust resistance in hexaploid bread wheat (*T. aestivum*). We reviewed 49 studies published between 1971 and 2017 and we reference 188 leaf rust resistance QTL identified in wheat. We also included results obtained with QTL meta-analysis and genome-wide association studies (GWAS) published between 2015 and 2017. Thus, the goals of this review are to: i) summarize the main molecular technologies that have been used on mapping population and QTL analysis, and ii) summarize APR QTL and meta-QTL for leaf rust.

Quantitative trait loci (QTL) and Meta-QTL (MQTL)

Resistance that expresses a quantitative phenotype is commonly associated with several genes of partial effect referred to as quantitative trait loci (QTL), although observed phenotypic variation can also be attributed to external, environmental variables that affect the phenotypic penetrance of the QTL (St CLAIR, 2010). Therefore, in quantifying the resistance effect of a QTL, and its value to rust resistance breeding, it is important to consider the QTL expression over a range of environments, across seasons and under different levels of disease pressure. It must also be remembered that loci conferring partial rust resistance can also be race-specific (SØRENSEN et al., 2014).

Several statistical methods have been developed to detect QTL (HACKETT, 2002). A QTL is described by its position on a linkage map, r^2 (percentage of phenotypic variation explained or PVE, %) and LOD (Logarithm of the Odds) score. The value of r^2 shows the influence of the QTL on the trait, being the percent of the total trait phenotypic variance that is accounted for by the QTL associated markers (WRAY et al., 2013). The LOD score is the statistical strength of evidence for a QTL at a given location (BROMAN, 2001). The term "major QTL" is often used to indicate that a QTL has a large effect on the phenotype and is usually detected in more than one environment. Some authors consider a QTL as "major" when it contributes 15 to 20% to the phenotypic variation segregating in the test population (LIUSHA et al., 2016; DU et al., 2015). For example, the major QTL *Lr34* has been shown to contribute up to 73.1% of the leaf rust resistance phenotypic variation in the wheat bread line "Saar" (LILLEMO et al., 2008). However, most studies show *Lr34* to account for, on average, 30 to 40% of the phenotypic variation (TSILO et al., 2014; KUMAR et al., 2013; SCHNURBUSCH et al., 2004; SUENAGA et al., 2003).

Four leaf rust APR loci, *Lr34* (LAGUDAH et al., 2009; SUENAGA et al., 2003), *Lr46* (SINGH et al., 2013; 1998), *Lr67* (HERRERA-FOESSEL et al., 2014; HERRERA-FOESSEL et al., 2011) and *Lr68* (HERRERA-FOESSEL et al., 2012) have been extensively used, and studied. Recently, a new APR gene, *Lr75* was described, but little information is available so far (SINGLA et al., 2017). An interesting feature of some of these APR genes is that they provide resistance to multiple pathogen species. *Lr34* (also known as *Yr18*, *Sr57, Pm38*), *Lr46* (*Sr58*, *Yr29, Pm39*) and *Lr67* (*Sr55, Yr46, Pm46*) provide partial resistance to all three rust species, and to powdery mildew caused by *Blumeria graminis.*

*Lr3*4 was the first APR gene to be cloned (KRATTINGER et al., 2009). It encodes an ATP-binding cassette (ABC) transporter and currently two predominant alleles of *Lr34* have been found in the wheat genome on chromosome 7D differing by only two exon-polymorphisms (LAGUDAH et al., 2009; KRATTINGER et al., 2009). Studies suggest that

the resistant haplotype is evolutionary younger than the susceptible, and that it is unique to the wheat D-genome. Cloning of *Lr34* has enabled specific-DNA markers to be developed; *cssfr5* (LAGUDAH et al., 2009), *wMAS000003* and *csLV34* (LAGUDAH et al., 2006), providing a fast and powerful tool for Marker Assisted Selection (MAS), allowing *Lr34* to be combined with other QTL and/or genes of interest.

The leaf rust APR gene *Lr67* has also been cloned and encodes a predicted hexose transporter (MOORE et al., 2015). A bacterial artificial chromosome (BAC) clone carrying the linked *gwm165* maker (0.4 cM) on chromosome 4DL was used to mapped and to clone *Lr67* (HERRERA-FOESSEL et al., 2011). The protein coded by the resistant allele of *Lr67* (*LR67res*) differs from the susceptible allele (*LR67sus*) by two amino acids in a region conserved across hexose transporter proteins. Although both alleles contain identical promoter regions and are upregulated upon rust infection, only *LR67res* confers APR, possibly by altering wheat sugar levels and signals that may limit the growth of biotrophic fungi (MOORE et al., 2015).

Lr46 was the second slow rusting gene characterized after *Lr34*, and it is present in diverse wheat germplasm derived from CIMMYT (WILLIAN et al., 2003). This gene was located on chromosome 1BL of wheat cv. Pavon 76 (SINGH et al., 1998). *Lr46* was mapped distal to marker *Xwmc44,* approximately 5 to 15 cM, and proximal to *Xgwm259* (SUENAGA et al., 2003). *Lr46* presents similar mechanism to *Lr34* and *Lr67*, as multiple-pathogens resistance (LILLEMO et al., 2008; WILLIAN et al., 2003; MARTÍNEZ et al., 2001). However, the effects of *Lr46* are not as pronounced as *Lr34* in seedling stages (MARTÍNEZ et al., 2001). Additionally, it has been shown that *Lr46* has an addictive effect on leaf rust resistance when combined with *Lr34* (LILLEMO et al., 2008).

Lr68 is an APR that was mapped on chromosome 7BL (HERRERA-FOESSEL et al., 2012). Its origin was traced back to the Brazilian cultivar Frontana, a cultivar that also possesses *Lr34* and *Lr46*. The markers *Psy1-1* (0.5 cM) and *gwm146* (0.6 cM) were reported to flank the *Lr68* locus, but were not suitable for MAS (HERRERA-FOESSEL et al., 2012). The co-dominant marker *cs7BLNLRR* (0.8 cM) and the dominant marker *csGS* (1.2 cM) have subsequently been developed for *Lr68* (HERRERA-FOESSEL et al., 2012).

Lr75 is a partial APR gene mapped to chromosome 1BS in the Swiss cultivar Forno (SINGLA et al., 2017). Schnurbusch et al. (2004) reported six leaf rust resistance QTL in Forno, including *Lr34*. The locus *QLr.sfr-1BS* (now *Lr75)* explained from 28 to 32% of the phenotypic variation when grown in Switzerland and is not associated with a leaf tip necrosis phenotype. A re-evaluation of the original population from the cross Arina x Forno resulted in the designation of *QLr.sfr-1BS* as *Lr75* (SINGLA et al., 2017).

While APR genes often confer partial resistance, in some combinations with other APR or ASR genes strong resistance can be achieved. For example, the ASR genes *Lr16*

and *Lr23,* in combination with *Lr34* have conferred effective leaf rust resistance for several years in the hard red wheat "Norm", even though alone these genes do not provide a significant level of resistance (KOLMER, 2015; TSILO et al., 2014). While the APR gene *Lr46* (1BL chromosome) in combination with the QTL *QLr.cdl*-5BL exhibits enhanced, additive resistance (KOLMER, 2015). A complementary combination of ASR and APR genes is therefore considered the most desirable for providing effective leaf rust resistance in wheat, while potentially maintaining resistance durability (ELLIS et al., 2014; SINGH et al., 2011; LAGUDAH et al., 2011; McINTOSH, 1998).

Goffinet & Gerber (2000) developed a method of meta-analysis aimed at combining independent QTL studies. The method involves the building of a consensus map, the projection of QTL on to this map and the estimation of consensus- or meta-QTL (MQTL). Meta-QTL analysis is therefore an approximation of the real number of independent QTL for the trait under study. Meta-analysis of QTL allows identification of regions that are repeatedly found in a target trait analysis, providing confidence in QTL locations and effects, and eliminates potential false QTL (SORIANO & ROYO, 2015; GOFFINET & GERBER, 2000). Combining data from different populations is a valuable approach to know whether the leaf rust resistance QTL identified in one mapping population corresponds to those found in other population, but it is important to remember that the outcomes of a meta-analysis are strongly affected by the accuracy of the primary QTL mapping (GOFFINET & GERBER, 2000).

A meta-analysis carried out by Soriano & Royo (2015) incorporated 144 leaf rust resistance QTL, obtained from 20 mapping populations and 33 parental lines, in bread and durum wheat, from studies undertaken between 1999 and 2015 in 52 environments. This meta-QTL analysis identified 48 regions contributing to leaf rust resistance, over 17 wheat chromosomes, of which 35 MQTL contained at least two QTL, while the other 13 regions contained unique QTL. Seven of these MQTL were found to co-localize with the genes *Lr10*, *Lr13*, *Lr14*, *Lr19*, *Lr23*, *Lr27*, *Lr34*, *Lr46*, *Lr67*, *Lr68* and *Lr71*.

Technologies for mapping and characterizing QTL

With the advances in DNA marker technologies it is now possible to obtain good genetic maps for hexaploid wheat, with good marker coverage of most chromosomes, although the D-genome is still underrepresented due to low levels of DNA polymorphism (GAO et al., 2016; LI et al., 2015; KERTHO et al., 2015).

On the 49 studies published between 1971 and 2017 analyzed we observed that SSR was the marker most widely used on leaf rust QTL analysis (**Table S1** – supplementary

material). A total of 38 mapping populations were developed using SSR, of which 12 studies used only SSR, 13 combined SSR and DArT, two others also combining SSR and DArT-Seq. Microsatellites are also used in others 11 studies combined with different markers as restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), cleaved amplified polymorphic sequences (CAPS) and single nucleotide polymorphisms (SNP). DArT and DArT-Seq were used on three and two others studies, respectively (**Table S1**).

Simple Sequence Repeat (SSR) markers are still a popular choice in wheat due to their good coverage of the genome, codominant inheritance, high information content, simple detection and reproducibility (COLASUONNO et al., 2013). SSR markers are also a good marker to compare between genetic maps from different crosses. Diversity Arrays Technology (DArT) is a high throughput whole-genome genotyping technique initially applied on rice (JACCOUD et al., 2001) that has been applied successfully to species with large and complex genomes, including polyploid as wheat, oat and strawberry (TINKER et al., 2009; SINGH et al., 2014; SANCHEZ-SEVILLA et al., 2015).The DArT method based on hybridizations allows for simultaneous detection of several thousand DNA polymorphisms by scoring the presence or absence of DNA fragments in genomic representations generated from genomic DNA samples through a process of complexity reduction without require any DNA sequence information (JACCOUD et al., 2001). As Next-Generation Sequencing (NGS) technologies become more affordable the use of Single Nucleotide Polymorphism (SNP)-based marker platforms has increased, including in wheat. Mapping SNP in wheat can help to develop high throughput molecular markers for important traits. One example is the SNP identification platform DArT-Seq (DArT technology, Canberra, Australia; http://www.diversityarrays.com), which combine the complexity reduction of the DArT method with NGS, that was also originally developed for rice (KILIAN, 2009), but has now been used for several crops, including wheat (DU et al., 2015; KOLMER, 2015; LAN et al., 2015), barley (WENZL et al., 2004), oat (COVER, 2010) and sugarcane (HELLER-USZYNSKA et al., 2011). As consequence of improvement in high-throughput genotyping technologies and statistical programs, the Genome-Wide Association Studies (GWAS) has been utilized as an alternative approach to bi-parental mapping (ZHU et al., 2008). In this sense, diverse technologies have been implemented to SNP genotyping in wheat, KASPar technology (ALLEN et al., 2013), Illumina GoldenGate (AKHUNOV et al., 2009), and Illumina iSelect 9 and 90K (WANG et al., 2014; CAVANAGH et al., 2013). The wheat 90K SNP array is one of the highest density genotyping platforms available for wheat and has a better genome coverage than 9K SNP array (WANG et al., 2014).

Many leaf rust resistance QTL identification studies have used bi-parental mapping populations, with good results. More recently association mapping populations have been

used to widen the germplasm search for leaf rust resistance QTL (KERTO et al., 2015). Genome-Wide Association Studies of leaf rust resistance have utilized the iSelect 9K (TURNER et al., 2017; AOUN et al., 2016; KERTHO et al., 2015) and iSelect 90K Illumina SNP technologies (GAO et al., 2016). Association mapping has an enormous potential to process large collections of diverse germplasms, and provides better mapping resolution compared to bi-parental mapping populations (ZHU et al., 2008), however it is becoming increasingly apparent that subsequent validation of the marker-trait associations identified in GWAS is required (PRINS et al., 2016).

Leaf rust resistance QTL

In this review we reference 188 leaf rust resistance QTL identified across all 21 wheat chromosomes by bi-parental mapping (**Table 1** and **Table S1**). We reviewed 49 studies published between 1971 and 2017, representing 49 mapping populations and 61 different donor lines (**Table 1**). Li et al. (2014) reported only 80 leaf rust APR loci across 16 chromosomes, showing a considerable effort to increase the identification of new resources for leaf rust genetic resistance. In addition we list the 35 Meta-QTL (**Table S2**) identified by Soriano & Royo (2015) and 165 QTL and *Lr* genes identified across all 21 wheat chromosomes in five GWAS published between 2015 and 2017, undertaken in 2163 winter wheat landraces (LI et al., 2016; KERTHO et al., 2015), 2580 spring wheat genotypes (TURNER et al., 2017; GAO et al., 2016) and 496 durum wheat (AOUN et al., 2016) (**Table S3**). However, this review will now focus only on the leaf rust resistance QTL identified by bi-parental mapping, to provide a valuable update for both pathologists and wheat breeders on the diversity of leaf rust resistance available in wheat germplasm.

The B genome carries the greatest number of leaf rust resistance QTL (**Table 2**), having 105 QTL (approximately 56%) out of 188 QTL and 20 MQTL, with chromosome 2B being most significant (**Table 3**). Li et al. (2014) also observed the B genome contains more leaf rust APR QTL than A or D genomes. The A genome carries the fewest leaf rust resistance QTL, with chromosomes 4A, 5D and 6D being the least represented (**Table 1**). The group 2 chromosomes carry 50 leaf rust resistance QTL and 6 MQTL (**Table 3**), and are well known for carrying disease resistance loci to all three rusts and powdery mildew (McINTOSH et al., 2017). Wide ranging phenotypic variation was observed between the 49 studies, with reports of leaf rust resistance QTL representing 0.8 to 76.9% of the phenotypic variation present within the bi-parental mapping populations (**Table S1**).

Table 1. The number of leaf rust resistance QTL found on each hexaploid wheat chromosome identified in bi-parental mapping studies

Chr[1]	QTL[2]
1A	9
1B	28
1D	4
2A	10
2B	23
2D	17
3A	5
3B	10
3D	5
4A	1
4B	10
4D	6
5A	4
5B	10
5D	1
6A	5
6B	7
6D	2
7A	2
7B	17
7D	12
Total	**188**

[1]Chr: chromosome. [2]Some QTL reported here may be reported more than once, as different markers could have been used to map the same location on chromosome.

Table 2. Leaf rust resistance QTL found in each wheat genome from the bi-parental mapping and QTL meta-analyses

Genome	Bi-parental	MQTL
A	36	8
B	105	20
D	47	7

The ancestor of the wheat B genome is probably extinct, the nearest relative being thought to be *Aegilops speltoides*. The B genome is the largest of the wheat genomes, and the large degree of changes at the DNA level makes it difficult to establish its donor (GUSTAFSON et al., 2009). Therefore, a direct comparison of the leaf rust resistance diversity on the wheat B genome donor with its progenitor is not possible. In the case of the D genome the donor species, *Aegilops tauschii* is still available. The limited DNA polymorphism associated with the D genome in hexaploid wheat suggests limited polyploidization events during the evolution of hexaploid wheat. Therefore *Ae. tauschii* still represents a wide potential source of genetic diversity that could be introduced into

hexaploid wheat, and to this end is used in wheat synthetic breeding. The progenitor of the A genome is the diploid einkorn wheat *Triticum urartu*, which was cultivated more extensively than *Ae. speltoides* and *Ae. tauschii* (LING et al., 2013). Different approaches have reported a wide-ranging loss of genes in the hexaploid A genome, when compared to its diploid progenitor (LING et al., 2013; HERNANDEZ et al., 2012; BRENCHLEY et al., 2012). This genes loss may explain why the A genome has less *Lr* resistant loci than the B and D genomes in the hexaploid wheat.

Table 3. The number of QTL and MQTL for leaf rust resistance found on each chromosomal group

	Bi-parental	MQTL
Group 1	41	5
Group 2	50	6
Group 3	20	5
Group 4	17	5
Group 5	15	2
Group 6	14	5
Group 7	31	7

In the following sections we discuss in detail the 188 leaf rust resistance QTL identified in bi-parental mapping studies (**Table S1**) and the 35 MQTL (**Table S2**) by chromosome group. Despite some QTL are grouped in the equivalent position, we cannot postulate here which ones among them are the same.

Group 1 chromosomes

The group 1 chromosomes provide 41 QTL and five MQTL, of which nine QTL are located on chromosome 1A, 28 on chromosome 1B and four on chromosome 1D (**Table 1**). From the MQTL, one was found in 1A and four on 1B (**Table S2**). The nine QTL and the MQTL1 reported on the chromosome 1A, are derived from the wheat genotypes Syn022L, Chapio, Shanghai 3 x Catbird, Luke, Apache and Sujata (DU et al., 2015; LAN et al., 2015; AZZIMONTI et al., 2014; ZHOU et al., 2014; KUMAR et al., 2013). An interesting APR QTL has been identified on the short arm of chromosome 1A, *QLr.cau*-1AS found in the genotype Luke, which explains from 44.3 to 52.6% of the phenotypic variance in this wheat genotype for leaf rust resistance (DU et al., 2015). The RILs containing *QLr.cau*-1AS were capable of reducing final leaf rust severity by 55.5%, on average, when grown in Beijing, Gansu, and Shandong/China. RILs possessing *QLr.cau*-1AS in combination with the *Lr34* gene showed around a 78.5% reduction in final rust severity (DU et al., 2015). The MQTL1 was mapped at 27.64 cM on chromosome 1A co-localizing the ASR *Lr10* gene (SORIANO & ROYO, 2015). The adult plant leaf rust resistance QTL *Trp-1* from the Brazilian wheat variety Toropi

was also mapped on chromosome 1A (McINTOSH et al., 2017; DA SILVA et al., 2012). However, evidence suggests that a second QTL, *Trp-2,* found on chromosome 4D, is required for the phenotype seen in Toropi (DA SILVA et al., 2012; BARCELLOS et al., 2000).

Lr46 on chromosome 1BL has been widely used in CIMMYT germplasm (LAN et al., 2014; ROSEWARNE et al., 2012). The *Lr46* locus has been shown to confer broad-spectrum resistance to four biotrophic pathogens: *P. triticina* (*Lr46*), *P. striiformis* (*Yr29*), *Blumeria graminis* f. sp. *tritici* (*Pm39*) (LILLEMO et al., 2008) and *P. graminis* f. sp. *tritici* (*Sr58*; SINGH et al., 2013). It is also associated with premature senescence of leaf tips, commonly denoted as leaf tip necrosis (*Ltn2;* ROSEWARNE et al., 2006). Meta-QTL analysis also reported that the MQTL5 is colocalized with *Lr46* and was mapped at 113.52 cM (Table S2; SORIANO & ROYO, 2015). However, the effectiveness of *Lr46* depends upon the environment and the genetic background (LAN et al., 2015), being less effective at higher temperatures (ROSEWARNE et al., 2015). *Lr46* explained from 16 to 55% of the total of phenotypic variation in different mapping population and experimental conditions (LAN et al., 2015, 2014; ROSEWARNE et al., 2015). The APR *Lr75* also mapped to chromosome 1B, but to the short arm, it is flanked by SSR markers *gwm604* and *swm27* (SINGLA et al., 2017). The *Lr75* (*QLr.sfr-1BS*) explained from 28 to 32% of the phenotypic variation when grown in Switzerland and is not associated with a leaf tip necrosis phenotype (SCHNURBUSCH et al., 2004).

Four QTL have been reported on chromosome 1D in three studies, derived from the wheat genotypes Syn022L, Quaiu 3 and Kenya Kongoni (CALVO-SALAZAR et al., 2015; BASNET et al., 2014; NAZ et al., 2008), being two of them identified as the ASR *Lr21* and *Lr42* genes (BASNET et al., 2014; NAZ et al., 2008). Studies using exotic alleles or synthetic accession are currently a great source to novel QTL and genes to wheat diseases. Working with a backcross population derived by the synthetic wheat Syn022L, developed from hybridization of *T. turgidum* ssp. *dicoccoides* and *T. tauschii*, Naz et al. (2008) found an interesting QTL mapped on chromosome 1D at marker locus *Xbarc149*. In their work, *QLrs.B22*-1D (ASR) or *QLr.B22*-1D (APR) presented the strongest effect compared with other QTL, this exotic allele being active in seedling and field assays reducing leaf rust symptoms by 46.3 and 43.6%, respectively. These two QTL explains 49 and 14.6% of the phenotypic variation, respectively (NAZ et al., 2008).

Group 2 chromosomes

The group 2 chromosomes provide the greatest number of leaf rust resistance genes, 50 QTL and six MQTL having been reported, with 10 QTL on chromosome 2A, 23 on chromosome 2B and 17 on chromosome 2D (**Table 1** and **Table S1**). To date, 20

designated *Lr* genes have been mapped to group 2 (McINTOSH et al., 2017). The ASR genes *Lr11*, *Lr17* (alleles a and b), *Lr37*, *Lr38, Lr45* and *Lr65* have been mapped on 2A (McINTOSH et al., 2017). The APR QTL *QLr.inra-2Ab* was identified co-located with *Lr37* gene and with *St38,* a *Septoria tritici* blotch resistance locus in the cv. Apache (AZZIMONTI et al., 2014). *QLr.inra-2Ab* is a minor QTL that explains from 5.5 to 12.2% of the phenotypic variation and was mapped in a region of 0 to 31.5 cM (AZZIMONTI et al., 2014). Wang et al. (2015) located the *QLr.hbau-2AS,* an APR locus close to *Lr37*, but they assert that the genes are not the same. *QLr.hbau-2AS* is derived from cv. Weimai 8, explains from 25.79 to 60.72% of the phenotypic variation and was mapped between the markers *Xcfd36* and *Xbarc1138*, with an interval of 2.58 cM (WANG et al., 2015).

There are six designated *Lr* genes on chromosome arm 2BS, three APR (*Lr13, Lr35* and *Lr48*) and three ASR genes (*Lr16, Lr23* and *Lr73*) (McINTOSH et al., 2017). *Lr35* has been located near the chromosome 2B centromere and *Lr48* is a recessive hypersensitive APR gene flanked by SSR markers *Xgwm429* and *Xbarc7* (BANSAL et al., 2008). While, *Lr13* is described as conferring race-specific APR (DYCK et al., 1966), and was widely used. It continues to contribute for resistance in Australia and Canada (ZHANG et al., 2016), despite being no longer effective in Mexico and South America (SINGH & RAJARAM, 1992). Recently *Lr13* was found to be association with the hybrid necrosis gene *Ne2m* (ZHANG et al., 2016). A major QTL, *QLr.hebau-2BS* is also found on 2BS which does not map to the location of these six *Lr* genes (ZHOU et al., 2014). *QLr.hebau-2BS* was mapped by ZHOU et al. (2014) on Shanghai 3 x Catbird, it was flanked by *XwPt8548* and *XwPt2314* and explained from 15.3 to 37.4% of the phenotypic variance. The MQTL8 is a result of seven studies utilizing cv. Apache and Creso, and it is likely to be associated to the genes *Lr13* and *Lr23* (SORIANO & ROYO, 2015). A minor APR *QLr.hbau-2BS* was identified in cv. Lantian 9, but its relationship with the genes *Lr13* and *Lr48* still requires furthers studies (LIUSHA et al., 2016).

Chromosome arm 2BL have two genes, the APR *Lr50* and the ASR *Lr58* (McINTOSH et al., 2017). Each one coming for a different wheat species, *Lr48* was mapped in *T. aestivum*, *Lr50* in *T. timopheevii* and *Lr58* in *Ae. triuncialis* (McINTOSH et al., 2017). Only one major QTL have been identified on 2BL, *QLr.inra-2B* explains from 15 to 35.6% of the phenotypic variation on cv. Apache (AZZIMONTI et al., 2014). This QTL might represent a good source of resistance since it possibly co-localizes with the *Yr7* gene (MALLARD et al., 2005).

Chromosome 2D has eight designated *Lr* genes, of which two are APR genes; *Lr22a* derived from *Ae. tauschii* and *Lr22b* derived from *T. aestivum* (McINTOSH et al., 2017). From the 17 QTL located on chromosome 2D the most effective were *QLrlp.osu-2DS* and *Qlr.inra-2D*, each one explains 42.8% and from 12.4 to 46.4% of the phenotypic variance,

respectively (AZZIMONTI et al., 2014; XU et al., 2005). The locus *QLr.inra-2D* is derived by the cv. Balance and co-locates with a *S. tritici* blotch resistance locus and *Rht8* (AZZIMONTI et al., 2014). *QLrlp.osu-2DS* is derived by wheat line CI 13227, this loci has effect on prolonging the latent period and was mapped about 2.5 cM from *xactg.gtg185* and 2.0 cM from *xbarc124* (XU et al., 2005). Selected RILs possessing *QLrlp.osu-2DS* had a latent period mean of 13 days, while the lines without the QTL had latent periods of 7.5 days on average (XU et al., 2005).

Group 3 chromosomes

This group presents seven designated *Lr* genes, five being ASR genes (*Lr24*, *Lr27*, *Lr32*, *Lr63* and *Lr66*) and the two APR *Lr74* and *Lr77* (McINTOSH et al., 2017). There are 20 QTL and five MQTL associated with this group, five QTL on chromosome 3A, ten on chromosome 3B and five on chromosome 3D (**Table 3; S1** and **S2**). *QLr.ubo-3A* is a major QTL mapped on 3A, it explains from 24.8 to 31.1% of the phenotypic variation and is derived by the cv. Lloyd (MACCAFERRI et al., 2008). *QLr.fcu-3AL* derived by synthetic hexaploid wheat line TA4152-60, also presented major effects, in this case on field experiments and seedling tests (CHU et al., 2009). *QLr.fcu-3AL* was mapped from 99.4 to 129.6 cM and present effects ranging from 10 to 18% of the phenotypic variation (CHU et al., 2009). *QLr.hbau-3A* mapped on cv. Lantian 9 explains only 5.6% of phenotypic variation, it is located near to two previously reported loci, *QLr.ubo-3A* (MACCAFERRI et al., 2008) and *QLr.sfsf.3AL* (LIUSHA et al., 2016; MESSMER et al., 2000). According to Maccaferri et al. (2008), the 3A QTL found in their study and that one found by Messmer et al. (2000) may be identical.

The leaf rust resistance QTL identified on chromosome 3B explain from 1.7 (*QLr.cim-3BS*) to 36.7% (*QLr.sun-3BS*) of the total phenotypic variation (CHHETRI et al., 2016; ROSEWARNE et al., 2015). *QLr.cin-3BS* was mapped by the marker *Xgwm533* on cv. Chapio at 14.2 cM, and may be a pleiotropic effect of *Sr2* (ROSEWARNE et al., 2015). *QLr.sun-3BS* derived from BT-Schomburgk Selection (BTSS), was the last QTL mapped on this chromosome (CHHETRI et al., 2016). The Chhetri et al. (2016) study was the first one on QTL identification in leaf rust resistance published with DArT-Seq markers. Additionally, there are others two QTL on 3B, *QLr.cim-3BS.1* derived from cv. Francolin (LAN et al., 2014) and *QLr.sfrs-3B* derived by the spelt winter line Oberkulmer (MESSMER et al., 2000). These studies have used different marker systems and the relative map locations of these two QTL plus *QLr.sun-3BS* suggest that the same QTL has been identified in different mapping populations (CHHETRI et al., 2016).

QLr.cim-3DC is the unique major APR loci on chromosome 3D, it explains from 17.8 to 25.4% of the phenotypic variation (LAN et al., 2014). This QTL was detected near the

centromere based on the DArT markers *wPt-732670* and *wPt-742537* (LAN et al., 2014). While *QLr.tam-3D* derived from Quaiu 3, explained 11.1% to APR across two years and is co-located with *QYr.tam-3D* (BASNET et al., 2014). *QLr.inra-3Db* is an ASR QTL explaining from 7.4 to 15.1% of derived by cv. Apache (AZZIMONTI et al., 2014).

Group 4 chromosomes

The homeologous chromosomes of group 4 are responsible for anchoring 17 QTL and five MQTL. One QTL is anchored on chromosome 4A, 10 on chromosome 4B and six on chromosome 4D (**Table 3; S1** and **S2**). Chromosome 4A is the poorest represented since there is only two designated *Lr* gene, the ASR *Lr28* derived from *Ae. speltoides* and *Lr30* from *T. aestivum* (McINTOSH et al., 2017). This chromosome also has the MQTL17 associated, being derived by cv. Opata 85 located at 54.67 cM (SORIANO & ROYO, 2015).

There only minor QTL on chromosome 4B, which ranging from 0.8 (*QLr.cim-4BS*) to 10.7% (*QLr.sfr-4BS*) of the phenotypic variation (ROSEWARNE et al., 2015; SCHNURBUSCH et al., 2004) mapped until now. *QLr.cim-4BS* is derived from cv. Chapio and was mapped at 26.8 cM (ROSEWARNE et al., 2015). The marker associated with *QLr.cim-4BS* (*wPt-6209*) have been previously mapped on a consensus map '4B Con November 2011' (http://ccg.murdoch.edu.au/cmap/ccg-live/) at 0.3 cM flanking by *Xgwm251* and *Xgwm149*, which were identified as flanking markers to *Lr12* (SINGH & BOWDEN, 2011). While *QLr.sfr-4BS* was mapped in cv. Forno being associated to leaf tip necrosis too (SCHNURBUSCH et al., 2004). In addition to *Lr12* also are mapped on this chromosome the genes *Lr25, Lr31* and *Lr49* (McINTOSH et al., 2017). The MQTL18 and MQTL19 belongs to 4B and had no association with the described genes (SORIANO & ROYO, 2015).

The chromosome 4D possesses *Lr67* an important APR gene which was originally transferred from PI250143, a Pakistani wheat accession, in the Thatcher-derived line RL6077 (DYCK & SAMBORSKI, 1979). *Lr67* possesses the physiological marker *Ltn3* (HERRERA-FOESSEL et al., 2014) and co-localizes with the MQTL20, which is originated from three QTL involving cv. Sujata and the synthetic line TA4252-60 (SORIANO & ROYO, 2015). In recent field work, *Lr67* explained from 33.6 to 57.9% of the total phenotypic variation (LAN et al., 2015). Three other QTL with minor effects have been identified on this chromosome being them *QLr.inra-4Da*, *QLr.sfrs-4DL* and *QLr.fcu-4-DL* (AZZIMONTI et al., 2014; CHU et al., 2009; MESSMER et al., 2000).

Group 5 chromosomes

Sixteen QTL and two MQTL have been found on the group 5 wheat chromosomes, four QTL on 5A, ten on 5B and one on 5D (**Table 1**). There is no *Lr* gene cataloged on 5A

yet (McINTOSH et al., 2017), on the other hand exist minor loci mapped on it (CALVO-SALAZAR et al., 2015; ROSEWARNE et al., 2012; SINGH et al., 2009; MESSMER et al., 2000). Two QTL derived from cv. Avocet have been identified, being *QLr.cim-5AC* an APR QTL located at centromeric region and explains 5% of the phenotypic variation (CALVO-SALAZAR et al., 2015). While *QLr.cimmyt-5AL* explains from 6.8 to 7.4% of the phenotypic variation (ROSEWARNE et al., 2012). There is a great possibility of these two loci be the same QTL since they coming from the same genotype and were closely located. *QLr.pbi-5AS* and *QLr.sfrs-5AS* are both located at short arm, explaining 11.2 and 7.7% of the phenotypic variation, respectively (SINGH et al., 2009; MESSMER et al., 2000). *QLr.pbi-5AS* is derived from cv. Beaver and was mapped from 11 to 16 cM (SINGH et al., 2009), and *QLr.sfrs-5AS* coming from cv. Forno and was mapped at 50 cM (MESSMER et al., 2000).

From a study by Kumar et al. (2013) two consistent APR QTL were found on chromosome 5B, that were observed in more than one environment and year. *QLr.ccsu-5B.4* and *QLr.ccsu-5B.5* are minor loci derived by the mapping population (CIGM86.940 x Altar 84) x Opata 85, conditioning leaf rust resistance in India environments (KUMAR et al., 2013). Both are minor loci which explain from 2.5 to 8.37% (*QLr.ccsu-5B.4*) and 4.38 to 7.34% (*QLr.ccsu-5B.5*) of the phenotypic variation (KUMAR et al., 2013). According to Azzimonti et al. (2014) there is a new QTL derived by cv. Balance called *Qlr.inra-5Bb/7Bb,* but it was no possible to assert the exact position, if it belongs to 5B or 7B chromosomes, because the SSR marker used was previously mapped on both chromosome. There are two gene, *Lr18* and *Lr52* both being ASR derived by *T. timopheevi* and T*. aestivum*, respectively (McINTOSH et al., 2017). MQTL22 and MQTL23 were also associated to 5B, the first located on 47.66 cM and the second 140.81 cM (SORIANO & ROYO, 2015).

Lr1, Lr57, Lr70 and *Lr76* are the only designated *Lr* genes reported to 5D, but only *Lr1* was associated to the short arm (McINTOSH et al., 2017). Just *QLr.sfrs-5DL* have been associated to this chromosome, it explains 8.9% of the phenotypic variation in the cv. Oberkulmer (MESSMER et al., 2000). Additionally, Soriano & Royo (2015) did not find any MQTL on chromosome 5D.

Group 6 chromosomes

This chromosomal group is the poorest to leaf rust resistance QTL, presenting only 14 QTL and five MQTL. The QTL from group 6 come from the genotypes Balance, Bairds, Syn022L, Avocet, Pavon 76, Pastor, Bainong 64, Jingshuang 16 and TA4152-60 (REN et al., 2017; LAN et al., 2017; AZZIMONTI et al., 2014; REN et al., 2012; ROSEWARNE et al., 2012; CHU et al., 2009; ZHANG et al., 2009; NAZ et al., 2008; WILLIAM et al., 2006). Five

QTL are on chromosome 6A, seven on chromosome 6B and two on chromosome 6D (**Table 1**).

From the five QTL on 6A, three coming from cv. Avocet (REN et al., 2017; ZHANG et al., 2009; WILLIAM et al., 2006) and might be probably the same. Despite Avocet being used as susceptible parent on several crosses, it presents some minors loci contributing to leaf rust resistance. *QLr.hbau-6AL* explains 7% (ZHANG et al., 2009), *QLr.cimmyt-6AL* explains from 4.2 to 6.3% (WILLIAM et al., 2006) and *QLr.cim-6AL* from 8.5 to 12.6% (REN et al., 2017) of the phenotypic variation. While *QLr.inra-6Aa* and *QLrs.B22-6A* coming from cv. Balance and Syn022L, respectively (AZZIMONTI et al., 2014; NAZ et al., 2008). *QLr.inra-6Aa* explains from 8 to 21.5% (AZZIMONTI et al., 2014) and *QLrs.B22-6A* explains 3.7% (NAZ et al., 2008) of the phenotypic variation.

Among the seven QTL present on chromosome 6B, all of them are coming from different donors and are all of minor effect. It is worth to mention that the *QLr.inra-6B* locus, derived by cv. Balance, is responsive to seedling rust resistance and explains from 3.4 to 29.2% of the phenotypic variation (AZZIMONTI et al., 2014). This QTL was mapped by the flaking markers *wPt4716* / *wPt4388* and co-located with *QYr.sun-6B* to yellow rust resistance (AZZIMONTI et al., 2014; BARIANA et al., 2010). All *Lr* genes mapped on 6B are ASR with only *Lr3* (alleles a, bg and ka) located on the long arm and *Lr9, Lr36, Lr53* and *Lr61* located on short arm (McINTOSH et al., 2017).

Up to now only one designated *Lr* gene has been found on chromosome 6D, *Lr38* presents an ASR gene derived from *Agropyron intermedium* (McINTOSH et al., 2017). The synthetic Syn022L is the unique wheat accession presenting resistance on chromosome 6D (NAZ et al., 2008), demonstrating the importance of synthetic wheat in order to achieve new sources of resistance. *QLrs.B22-6D* is an ASR loci derived by Syn022L and positioned at 34.6 cM, which explains only 2% of the phenotypic variation (NAZ et al., 2008). Naz et al. (2008) also identified the APR QTL *QLr.B22*-6D, located at 25 cM and explaining 3.2% of the phenotypic variation. According to Naz et al. (2008) both loci may be correspondent to a QTL previously mapped by Nelson et al. (1997).

Group 7 chromosomes

Group 7 contains two of the most important APR genes, *Lr34* and *Lr68*, and six ASR *Lr* genes (McINTOSH et al., 2017). It presents 31 QTL and seven MQTL, with two QTL on chromosome 7A, 17 on chromosome 7B and 12 on chromosome 7D (**Table 1**). Using SSR and DArT markers in a double haploid mapping population, Azzimonti et al. (2014) were able to find a major QTL for ASR on 7A, designated as *Lr20* or *QLr.inra*-7Aa, derived from cv. Balance and explaining 69.4% of the phenotypic variation in this cultivar. This locus also is co-located with a QTL to vernalization (LE GOUIS et al., 2012) and *S. tritici* blotch

resistance (GOUDEMAND et al., 2013). According to studies conducted by Tsilo et al. (2014) there is a minor QTL on 7A derived by the breeding line MN98550-5, which explains 4.93% of the phenotypic variation.

A number of loci for leaf rust resistance are located on 7B (**Table S1**). *QLr.sfrs-7B.2* and *QLr.ubo-7B.2* are the major APR QTL on this chromosome, each one explaining 35.8% and from 48.9 to 76.9% of the phenotypic variation, respectively (MACCAFERRI et al., 2008; MESSMER et al., 2000). *QLr.sfrs-7B.2* is derived by cv. Forno (MESSMER et al., 2000), which also possesses *Lr34*, *Lr14a* and *Sr17*, and is considered an important accession for breeding programs. *QLr.ubo-7B.2* was also significant to leaf rust seedling resistance and it is derived by cv. Colosseo, a *T. durum* accession (MACCAFERRI et al., 2008). *QLr.ubo-7B.2* localizes at 209 to 214 cM (MACCAFERRI et al., 2008) and none loci to leaf rust resistance have been mapped close to this region. *Lr68* has been mapped to the long arm of chromosome 7B, this gene is associated to *Ltn* and although it has smaller effect than others genes conferring slow rusting, it might share similar defense mechanism (HERRERA-FOESSEL et al., 2012). The MQTL33 was associated to chromosome 7B, it is formed by 11 QTL that originate from cvs. Colosseo, Creso and Sujata (SORIANO & ROYO, 2015). It co-localizes with *Lr14, Lr19* and *Lr68* and is the Meta-QTL with more QTL than any other found in the study by SORIANO & ROYO (2015).

Lr34 is a major QTL on 7DS which has been reported in a number of studies using many different mapping populations and methods (ZHANG et al., 2017; ROSEWARNE et al., 2015; TSILO et al., 2014; KUMAR et al., 2013; LILLEMO et al., 2008; NAZ et al., 2008; SCHNURBUSCH et al., 2004; SUENAGA et al., 2003; FARIS et al., 1999). This indicates the widespread use of *Lr34* in many wheat cultivars. *Lr34* was first described in the Brazilian cultivar Frontana and was traced to the Italian variety Mentana. Recently, *Lr34* has been cloned (KRATTINGER et al., 2009), however its biological function has yet to be determined. Apart from *Lr34*, only two minor APR QTL have been mapped on 7D, *QLr.cim-7DS* derived by Francolin#1 (LAN et al., 2014) and *QLr.hebau-7DS* derived by Naxos (ZHOU et al., 2014). *QLr.cim-7DS* was positioned at 2 cM (flanked by *wPt-744857* / *Xgwm295*) and explains from 3.3 to 4.2% of the phenotypic variation (LAN et al., 2014). *QLr.hebau-7DS* was mapped by the marker intervals *Xgwm1220* / *Xswm10* and *Xtpt7755* / *Xbarc128a*, which explained 4.4 and 6.0% of the phenotypic variation, respectively (ZHOU et al., 2014). According to Zhou et al. (2014) *QLr.hebau-7DS* is possible a new QTL with potential pleiotropic to leaf rust and powdery mildew resistance.

CONCLUSION

This analysis also showed that the genome B has been proved to be the most important genetic resource against leaf rust, because it anchors the major number of QTL mapped and reported in scientific publications (55.85%), followed by Genome D (25%) (**Table 2**). In the same way, the Group 2 chromosomes is the richest, having been reported in total 50 QTL (26.5%), while Groups 6 was the poorest with only 14 QTL (7.5%).

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4 ARTIGO 2

QTL mapping of wheat leaf rust to adult plant resistance in the Brazilian cultivar Toropi[4]

QTL mapping of wheat leaf rust to adult plant resistance in the Brazilian cultivar Toropi

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ABSTRACT: Leaf rust caused by *Puccinia triticina* is one of the most destructive foliar disease on wheat around the world. The effective and durable leaf rust resistance presented by the Brazilian cultivar Toropi makes it an excellent choice to the genetic resistance study, since it does not possess any of the main adult plant resistance (APR) gene designated up to now. The basis of resistance was investigated in a population of 86 F_{12} recombinant inbreed lines (RILs) derived from the cross Toropi x IAC13. The parents and RILs were phenotyped in three greenhouse assays to leaf rust in 2014 in Porto Alegre, Brazil. The population was genotyped with simple sequence repeat (SSR) and Diversity Arrays Technology (DArT-Seq) markers. A total of 3020 markers were mapped onto 36 linkage groups corresponding to more than 21 chromosomes on wheat. The total length of the genetic map is 2,598.77 cM, with an average distance of 1.81 cM between markers. The length of individual chromosomes ranged between 1.29 cM for chromosome 4D and 229.27 cM for chromosome 6B. Significant effects for reduction on leaf rust severity were found on chromosomes 1A, 2A, 2D, 3B, 5A, 6B and 7D. The loci *QLr.ufrgs-1A*, *QLr.ufrgs-2A* and *QLr.ufrgs-3B* were associated to cv. Toropi. *QLr.ufrgs-2D*, *QLr.ufrgs-5A*, *QLr.ufrgs-6B* and *QLr.ufrgs-7D* had association with the susceptible cv. IAC13.

Key-words: Toropi; APR; leaf rust resistance; QTL; DArT-Seq.

INTRODUCTION

Wheat (*Triticum aestivum* L.) provides above 20% of the calorific consumption for nearly 2/3 of the human population (HAWKESFORD et al., 2013). It is expected that the global population reaches from 9 to 10 billion by the year 2050, and because of that, the

world food security is so fundamental (RIAZ et al., 2016). The Brazilian wheat production is insufficient to suit the internal consumption, reaching in average 6 to 7 million tons per year, which correspond to 50% of the population consumption demand. The yield was only 3.17 and 2.61 t/ha, in 2016 and 2017 seasons, respectively (CONAB, 2017).

In addition, the cultivated area in Brazil has diminished recently due to the market situations, that is not favourable to wheat grains commercialization. Besides the environment conditions with is favourable to several diseases, among them, the leaf rust is one of the most threatening in South America. Wheat leaf rust, caused by *Puccinia triticina* Eriks., has been characterized as one of the most predominant disease in wheat-producing areas (KOLMER, 2005). Many races of *P. triticina* are present in the Southern Cone of America (Argentina, Brazil, Chile, Paraguay and Uruguay) every year, making the pathogen populations extremely dynamic, leading to the short life of resistance in commercial cultivars (GERMÁN et al., 2017). *Puccinia triticina* has evolved and adapted to a range of environments, increasing its ability to infect wheat wherever it is cultivated (WINZELER et al., 2000). This disease has the capacity to cause significant yield loss reaching up to 50%, varying with the wheat cultivar and its stage of development at the moment of infection (HUERTA-ESPINO et al., 2011; ROELFS et al., 1992).

The control of leaf rust pathogens can be achieved by fungicides application and/or by commercial cultivars contained genetic resistance. Utilization of resistant cultivars is desirable over fungicides because it is economic and eco-friendly (SINGH et al., 2013). Wheat breeding focusing on genetic resistance to leaf rust is a cost-effective approach to control the disease. Its benefit is estimated at a ratio of 27:1 (MARASAS et al., 2004).

Genes to fungal disease resistance can be classified according to their specificity and durability. McIntosh et al. (2017) described more than 77 *Lr* (Leaf rust) resistance genes, most being all-stage-resistance (ASR), which are effective in the whole life of wheat, although only few of them remain effective for long periods of time. Contrasting with ASR genes, the race-non-specific genes constitute a more durable resistance because they are mainly effective in plant adult stages, also termed as adult plant resistance (APR) genes. In general APR genes are characterized by conferring partial resistance and a slow-rusting development associated with small and restricted uredia that possess fewer amount of spores.

Five APR genes have been currently characterized and usually present multi pathogens resistance. They are *Lr34* (also known as *Yr18*, *Sr57* and *Pm38*) (LAGUDAH et al., 2009), *Lr46* (*Sr58*, *Yr29* and *Pm39*) (HERRERA-FOESSEL et al., 2011; SUENAGA et al., 2003)*, Lr67* (*Sr55*, *Yr46* and *Pm46*) (HERRERA-FOESSEL et al., 2011), *Lr68* (HERRERA-FOESSEL et al., 2012) and *Lr75* (SINGLA et al., 2017). From these genes only *Lr34*, located at 7DS chromosome, and *Lr67* on 4DL, have been cloned and characterized

up to now. *Lr34* encodes an ATP-binding cassette (ABC) transporter (KRATTINGER et al., 2009) and *Lr67* encodes a predicted hexose transporter (MOORE et al., 2015). From their cloning, specific-DNA markers were developed being *cssfr5* (LAGUDAH et al., 2009), *wMAS000003* and *csLV34* (LAGUDAH et al., 2006) associated to *Lr34*, and the *gwm165* maker (0.4 cM) linked to *Lr67* (HERRERA-FOESSEL et al., 2011).

Lr46 is located on chromosome 1BL and was mapped distal to marker *Xwmc44* (5 - 15 cM), and proximal to *Xgwm259* (SUENAGA et al., 2003). *Lr68* is an APR gene derived by Frontana that was mapped to chromosome 7BL using the markers *cs7BLNLRR* (0.8 cM) and *csGS* (1.2 cM) (HERRERA-FOESSEL et al., 2012). Additionally, *Lr75* was the last APR gene described and was mapped on chromosome 1BS (SINGLA et al., 2017).

The Brazilian cultivar Toropi released in 1965 was commercially cultivated for 15 years, expressing a high level of resistance to leaf rust. Its resistance has been classified as APR and is effective up to now in experimental fields. Toropi is originated from the cross Frontana 1971.37/Quaderna//Petiblanco 8. It is known that Frontana possesses the APR genes *Lr13* and *Lr34*. In Toropi, on the other hand, several assays demonstrated that *Lr13* (DA SILVA et al., 2012), *Lr34* (DA SILVA et al., 2012; ROSA, 2012; BARCELLOS et al., 2000) and *Lr68* (*see results*) are absent. This information suggests that Toropi could anchors novel loci to APR, being an important genetic source to leaf rust resistance.

Toropi APR started to be studied by Barcellos et al. (2000), who identified two recessives genes to resistance using a F_2 population from a cross between Toropi and IAC13. After, in a study using monosomic families from Toropi with Cappele-Desprez, Da Silva et al. (2012) also observed segregation indicating the presence of two recessive genes (7 resistant:9 susceptible) as observed by Barcellos et al. (2000). The authors also identified distorted segregation ratios in the monosomic families of chromosomes 1A and 4D, indicating that the APR genes in Toropi are located on these chromosomes. Amplified fragment length polymorphism (AFLP), sequence tagged site (STS) and single sequence repeats (SSR) markers were used to map the leaf rust Toropi loci using F_7 recombinant inbred lines (RILs) derived from the cross Toropi x IAC13 (DA SILVA et al., 2012). The genes, temporarily named *Trp-1* and *Trp-2*, were mapped on the short arm of chromosome 1A and 4D, respectively (DA SILVA et al., 2012). Additionally, these authors ruled out the presence of *Lr10* on 1AS chromosome and *Lr47* on 4D. In the same year Rosa (2012), using a double-haploid (DH) population derived from Toropi x Thatcher, found four genes related to leaf rust resistance: *Trp-1*, *Trp-2*, *Trp-3* and *Trp-Se*. *Trp-Se* was never reported before for Toropi and was mapped on 3DL chromosome, this gene was effective only in New Zealand environments, where presented near immunity response (ROSA, 2012). *Trp-3* is a race-specific adult plant gene mapped on chromosome 4BL. Seedlings carrying *Trp-3* showed mesothetic reaction in New Zealand, intermediate to susceptible in Canada and

susceptible in Brazil (ROSA, 2012). Rosa (2012) observed a significant QTL (named: *QLr.crc-5AL.1* and *QStr.crc-5AL.1*) on 5AL chromosome, which confers leaf and stripe rust resistance. The resistance gene located on chromosome 5AL, which was named *Trp-1* according to Rosa (2012), is a novel source of resistance since there is no *Lr* gene described in this location (McINTOSH et al., 2017). The second adult plant race non-specific resistance gene identified by the author (*Trp-2*), was not assigned to a chromosome, although regions on chromosomes 2B, 5D and 7A are possible locations (ROSA, 2012). Inconsistences among gene locations found by the cited authors in relationship to *Trp-1* and *Trp-2*, show the necessity of new genetic studies in Toropi.

Researches using quantitative trait loci (QTL) have helped to identify specific regions on chromosomes that are associated with agronomic quantitative trait of interest. Several QTL studies have been carry out to identify genomic regions with large effect with leaf rust diseases (DU et al., 2015; LAN et al., 2015). Currently, several approaches have been used to find important QTL on wheat genome, such as SSR and DArT-Seq markers, which are possible to construct high-density genetic maps. DArT-Seq combines the complexity reduction of the DArT method (ALTSHULER et al., 2000) with next generation sequencing. DArT-Seq can also detect and genotype DNA variations at hundreds of genomic loci in parallel without any previous sequence information (ZHOU et al., 2015), and is rapidly gaining popularity as a preferred method of genotyping by sequencing (KILIAN et al., 2012).

A population of recombinant inbred lines (RILs) developed from Toropi (female) and IAC13 (male) was used in our work with the objectives of i) investigate the genetic basis of leaf rust resistance in Toropi, ii) identify APR loci conferring resistance using DArT-Seq and SSR markers, iii) evaluate the individual effects of QTL on leaf rust resistance at the adult plant stage and iv) exclude the possibility of Toropi possessed the APR genes *Lr34, Lr46, Lr67* and *Lr68*.

METHODS

Plant material

The bi-parental mapping population of 86 RILs F_{12} derived from a cross between Toropi (Frontana 1971.37/Quaderna//Petiblanco 8) and IAC13-Lorena (Ciano 67/IAS51) was used to generate the linkage map. Toropi was used as the female parent and the cultivar IAC13 was the pollen donor. These genotypes were chosen as parents for map construction because of their different rust resistance pattern. Toropi has shown excellent levels of leaf (ROSA et al., 2016; CASASSOLA et al., 2014; DA SILVA et al., 2012; BARCELLOS et al., 2000), stripe (ROSA et al., 2016) and stem rust (*data unpublished*)

resistance on several field and greenhouse assays. On the other hand, IAC13 is susceptible to all Brazilian *P. triticina* races (BARCELLOS et al., 2000).

Greenhouse experiments

The F_{12} RILs and parents were evaluated for wheat leaf rust APR in three greenhouse experiments conducted in April, August and September 2014. For each line, three flag leaves completely expanded were inoculated with *P. triticina* uredinospores of MFT-MT race. Inoculations were done with suspensions of fresh spores in distilled water + Tween 20 (0,02%), at concentration of 2.13×10^5, 3.12×10^5 and 4.27×10^5 spores per mL, on April, August and September experiments, respectively. The suspensions were sprayed on the adaxial surface of three flag leaves and the plants were incubated in a dark wet growth chamber (100% UR) for 12h to allow spore germination and infection. After, the inoculated plants were kept at 20 °C with 14h light and 10h dark until the first pustules showed up (± 8 days) and then were transferred to greenhouse until the readings to be done, at 11 days in April and 14 days after inoculation (d.a.i.) to August and September. Under these new conditions, the mean temperature in the green house were 30, 22 and 24 °C, respectively for the first, second and third experiment until the readings of severity and type of infection.

The disease severity percentage was evaluated according to the modified Cobb scale (PETERSON et al., 1948), where the percentage of tissue affected was visually estimated. The disease severity percentage and host response or infection type (IT) were pooled in a single value, the coefficient of infection (CI), which was obtained by multiplying the percentage of severity by the host response, where: resistant – R = 0.2, moderately resistant – MR = 0.4, moderately susceptible – MS = 0.8 and susceptible – S = 1.0 (ROELFS et al., 1992). Low amounts of leaf rust were identified by trace (T) and this was added up in the results of the multiply percentage of severity by the host response, where TR = 0.02; TMR = 0.04; TMS = 0.08 and TS = 0.1 (ROELFS et al., 1992). At the moment of evaluation all the inoculated flag leaves were photographed and frozen to keep them as record for possible further analysis if necessary.

To determine the frequency distribution of each RIL the number of classes was estimated according the equation proposal by Steel and Torrie (1980): i = A/K. Where: i = class midpoint; A = range of severity; K = number of classes =√n; n = number of RIL.

Statistical analysis

Analysis of variance (ANOVA) of percentage of severity of disease, infection type and coefficient of infection of leaf rust response was conducted using the linear mixed model (REML), with lines as fixed effect and experiments and replicates as random effect. Predicted means for the RIL population were extracted from the REML analyses for each

phenotypic data set. We also used transformed data using Log 10 to achieve near normality. All analyses were performed using the software GenStat (GenStat 16th Edition, Rothamsted Experimental Station, Harpenden, UK).

Molecular analysis

Genomic DNA from 86 RILs and parents was extracted from seedlings using the hexadecyltrimethylammonium bromide (CTAB) buffer protocol (LEFORT & DOUGLAS, 1999). The quantity and quality of DNA was assessed using Nanodrop spectrophotometer (Nanodrop Technologies, Oxfordshire, UK). The population was screened using 90 SSR primers (Table S1, supplementary material), as well as KASP marker *wMAS000003,* associated with *Lr34/Yr18/Pm38*. Four SSR markers used are associated with three leaf rust genes: *Lr46* (*wmc44*; SUENAGA et al., 2003), *Lr67* (*cfd23* and *cfd71*; HIEBERT et al., 2010) and, *Lr68* (*cs7BLNLRR*; HERRERA-FOESSEL et al., 2012). After that, a high-throughput genotyping method using the DArT-Seq™ technology (SANSALONI et al., 2011; Triticarte Pty Ltd, Canbera, Australia. http://www.triticarte.com.au) was employed to genotype the F_{12} RIL population to investigate polymorphisms potential. In addition, the RIL population was screened with 14 chromosome 4D KASP markers (Table S1, supplementary material) for improving marker coverage of the chromosome 4D. Each marker scores for each sample were converted into "A" (IAC13), "B" (Toropi) and "NA" (missing data) by comparison against parental scores (Table S3, supplementary material). The SNP data that identified heterozygotes profile in some of the parents as well as proportion of missing data greater than 80% were discarded. Markers of unknown parental origin were also removed from the dataset.

Linkage map construction and QTL analysis

For each segregating marker, a chi-square test was performed to test for deviation from the 1:1 expected segregation ratio at a 5% level of significance and any locus showing significant distortion was removed from the data set before constructing the linkage groups. Linkage groups were established using a minimum likelihood odds (LOD) score of 5.0 and a maximum recombination frequency threshold of 0.30. Segregation data were analysed with MapDisto v. 1.7 (LORIEUX, 2007), the map construction was carried out using R/qtl package version 1.40-8 (BROMAN et al., 2009), and drawn in MapChart v. 2.3 (VOORRIPS, 2002). The Kosambi mapping function (KOSAMBI, 1944) was used to calculate map distances (cM) from recombination frequency.

In order to assign linkage groups to physical chromosomes, the linkage groups were aligned with existing wheat consensus maps. We validated our map against published wheat genome sequences (International Wheat Genome Sequencing Consortium, 2014).

Flanking sequences of SNP markers in our map were initially blasted against the published genome using BLASTn (ALTSCHUL., 1990) with an *e*-value cut-off of 1e-20 and ≥95 identity. In total 77.9% of the mapped DArT-Seq markers were assigned a chromosomal location by reference to the consensus *Chinese Spring* wheat genome (International Wheat Genome Sequencing Consortium, 2014).

QTL analyses were carried out using the QTL package R/qtl, version 1.40-8 (BROMAN et al., 2009). Predicted means were obtained for each phenotypic data set from the REML analysis on GenStat software, and used as additional data set in the QTL analyses. Single marker regression (SMR), standard interval mapping (IM), Harley-Knott regression (HK) and composite interval mapping (CIM) were performed with all leaf rust phenotypic data set. Significance thresholds of 10 and 5% LOD were obtained using 1000 permutation tests for each phenotypic data set.

RESULTS

Evaluation of leaf rust in the RIL population

All the inoculations resulted in successful infection. Differences between the resistant and susceptible parents are clearly seen on Figure 1A and 1B. Leaf rust severity on some RILs transgressed their parents, ranging from near immunity (Figure 1C) to highly susceptible (Figure 1D) in all greenhouse experiments.

| **A** Toropi | **B** IAC13 |
| **C** Line 710 | **D** Line 753 |
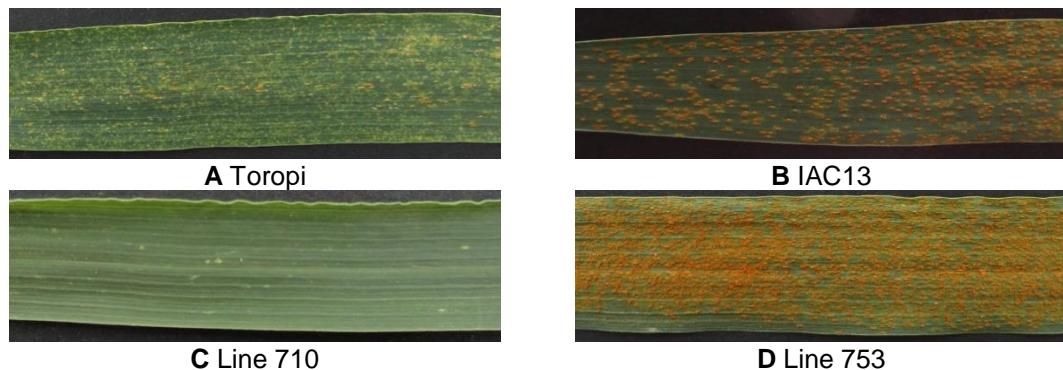
Figure 1. Development of pustules on the parents Toropi (A) and IAC13 (B) and on the lines 710 (C) and 753 (D) after inoculation with *Puccinia triticina* uredinospores of MFT-MT race in greenhouse experiments, as examples of reactions of resistance and susceptibility, respectively.

The frequency distribution of severity ranged from 1 to 50% (Figure 2) among the experiments, while the CI from 0.1 to 48 (Figure 3). Experiment reading in April had less % of severity and CI than those from August and September. ANOVA among the three experiments showed statistically significant differences ($P \leq 0.001$). The range of scores, %

of severity and CI were also lower on April experiment, contrasting with those obtained in September assay which showed higher ranges.

The parental genotype Toropi showed scores ranging from 1SMS to 20R5MS, and IAC13 ranged from 5SMS to 45SMS (Table 1). The highest percentage of severity was observed on August experiments, that ranched 65%. September experiment had 50% of severity as the highest score and April only 30%. The low range severity had a value of 1% on April and September, and 3% on August.

ANOVA tests indicated that the percentage of disease severity, infection type and CI scores data were statistically no significant between the experiments 2 and 3 ($P \leq 0.05$). The difference was restricted to the RILs. However, ANOVA showed statistically significant difference between phenotype experiment 1 and 2, as well as 1 and 3 ($P \leq 0.001$).

Table 1. Summary of final leaf rust severities in recombinant inbreed lines from Toropi x IAC13 population evaluated in Brazil during April, August and September 2014.

Severity (%)	April			August			September		
Toropi	3	3	1	8	8	5	5	8	12
IAC13	8	5	-	45	45	25	45	45	25
RIL means	10.61			16.67			16.15		
Low range	1			3			1		
High range	30			65			50		

Genetic map construction

The final genetic linkage map consisted of 3020 bin markers identified from DArT-Seq, SSR and KASP genotyping divided on 36 linkage groups for the Toropi x IAC13 cross, giving a total map length of 2,598.77 cM, with individual linkage groups ranging from 1.29 cM for chromosome 4D to 229.27 cM for chromosome 6B (Table 2; Figure S1, supplementary material). The number of markers per linkage group ranged from 3 for chromosome 4D and 7D to 320 for chromosome 7B (Figure S1, supplementary material). Genome D was poorly represented, and no linkage group was identified for chromosome 5D (Table 2). We removed 53 unlinked markers (32 SNPs, 18 SSR and 3 KASP) from map draw. DArT-Seq markers associated to the same position were excluded to the map draw as well, aiming a clearer picture of the linkage groups.

Analysis and QTL detection

The QTL analyses were performed independently for each greenhouse experiment and traits. Using single regression analysis, we detected seven QTL for leaf rust resistance in 86 RILs derived from Toropi x IAC13 population. The QTL reported are those identified using the predicted means and having a LOD value above the 10% global thresholds given in Table 3.

Severity
April, 2014
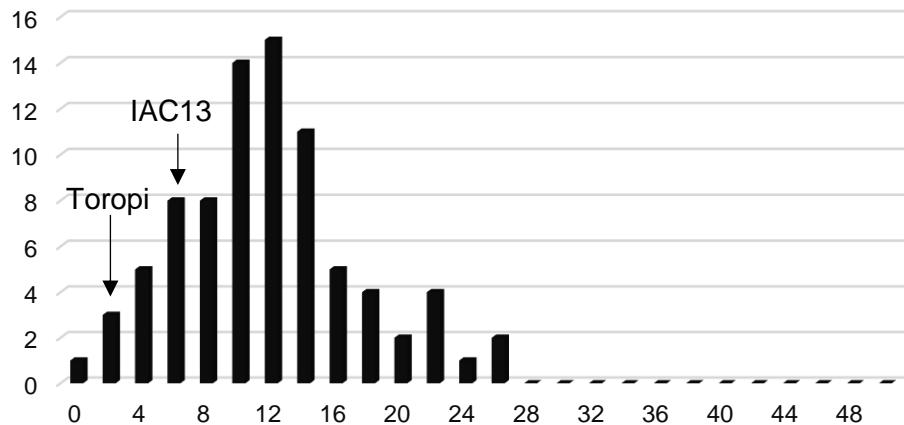
Severity
September, 2014
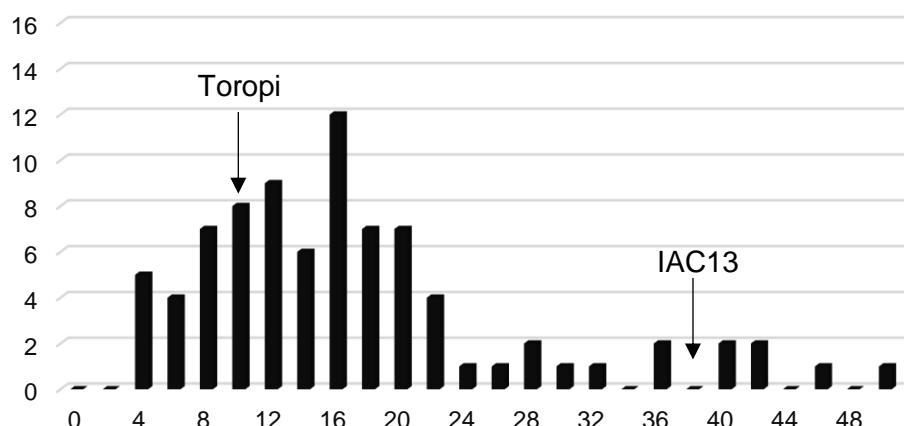
Severity
October, 2014
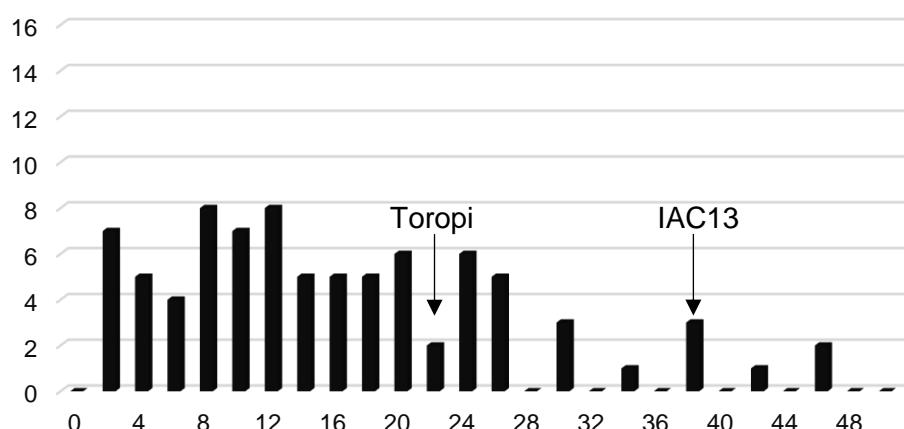
Figure 2. Frequency distribution of recombinant inbreed lines from Toropi x IAC13 population for adult plant leaf rust severity in three greenhouse assays.

Coefficient of infection
April, 2014

Coefficient of infection
September, 2014

Coefficient of infection
October, 2014
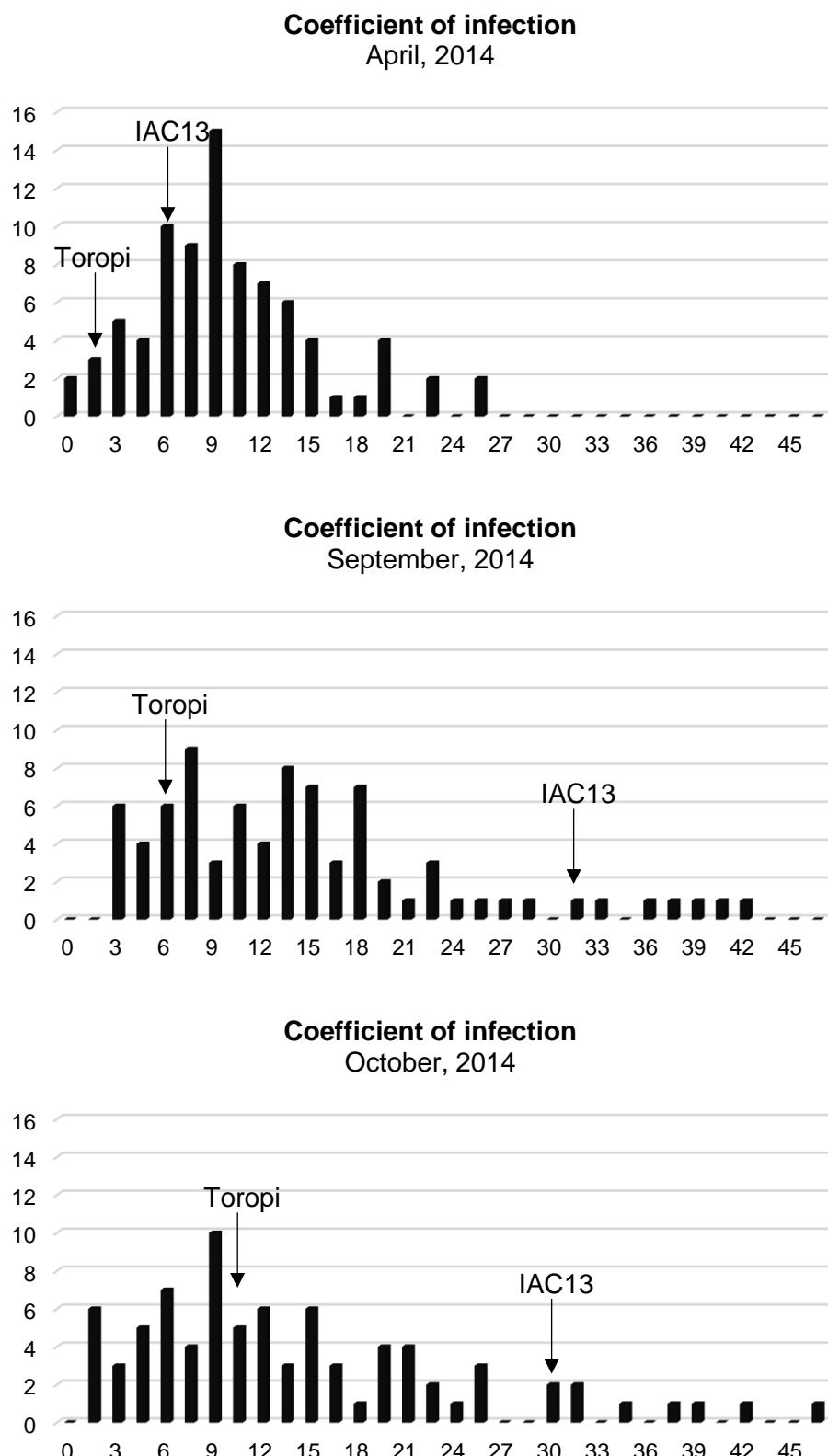
Figure 3. Frequency distribution of recombinant inbreed lines from Toropi x IAC13 population for adult plant leaf rust coefficient of infection in three greenhouse assays.

Table 2. Linkage groups developed for the 86 recombinant inbreeding lines from Toropi x IAC13. DArT-Seq, single sequence repeat (SSR) and KASP markers were analysed. The genetic distance between markers is in centiMorgans (cM). Min – minimum and Max – maximum distance between markers.

Chr	Linkage group	Map size (cM)	N° markers	Distance (cM)	
				Average	Min. – Max.
1A	10	175.22	108	1.64	0 – 33.11
1B	4	124.88	209	0.60	0 – 28.45
	24	25.79	25	1.07	0 – 19.47
1D	18	26.71	63	0.42	0 – 9.82
2A	3	181.27	299	0.61	0 – 29.62
2B	2	164.74	176	0.93	0 – 24.66
	15	52.77	79	0.68	0 – 11.86
2D	19	26.81	53	0.52	0 – 11.28
3A	5	91.89	196	0.47	1 – 7.96
	29	10.06	12	0.91	0 – 8.84
3B	11	103.77	107	0.98	0 – 15.71
	13	57.68	96	0.61	0 – 17.12
3D	33	17.15	4	5.72	4.94 – 12.12
4A	2	140.01	137	1.04	0 – 27.10
	12	61.12	104	0.59	0 – 16.28
4B	16	29.39	70	0.43	0 – 7.06
	26	72.42	14	5.57	0 – 19.59
4D	27	8.42	14	0.65	0 – 4.07
	35	1.29	3	0.65	0 – 1.29
5A	6	216.09	159	1.37	0 – 78.49
	23	31.39	26	1.26	0 – 8.84
	31	13.62	6	2.72	0 – 7.93
5B	14	79.52	90	0.89	0 – 22.74
	21	127.07	48	2.70	0 – 46.87
5D	NO	-	-	-	-
6A	17	33.09	67	0.50	0 – 10.66
	25	9.75	24	0.42	0 – 4.29
	30	25.28	10	2.81	0 – 10.60
6B	7	229.27	138	1.67	0 – 48.49
6D	22	18.19	43	0.43	0 – 5.69
7A	8	109.00	132	0.83	0 – 18.22
	20	53.94	51	1.08	0 – 22.71
7B	1	162.55	320	0.51	0 – 58.03
	9	25.18	111	0.23	0 – 3.89
7D	28	22.07	14	1.70	0 – 20.23
	32	39.36	6	7.87	0 – 13.36
	34	5.88	3	2.94	0 – 5.88
	36	26.13	3	13.07	9.00 – 17.13
TOTAL	36	2,598.77	3020	1.81	0 – 78.49

Table 3. Quantitative trait loci associated with disease severity of leaf rust in the Toropi x IAC13 inbreeding lines population.

QTL[1]	Chr[2]	Marker peak	Position (cM)	LOD value	% Phenotypic variance explained	Donor
QLr.ufrgs-1A	1A	1047843	27.7	2.66	13.47 – IT (Apr)	Toropi
QLr.ufrgs-2A	2A	1094287	174.50	3.82	16.89 – IT (Aug)	Toropi
		1067505	175.09	3.08	14.50 – Severity (Sep)	
		1333613	175.09	3.81	16.78 – CI (Sep)	
		7341185	176.93	3.84		
		1698058	179.43	2.9		
		7351364	181.27	2.9		
QLr.ufrgs-2D	2D	1088274	3.59	2.98	13.66 – Severity (Aug)	IAC13
		3023061	4.18	3.02	32.10 – CI (Aug)	
QLr.ufrgs-3B	3B	2252631	81.8	4.37	45.08 – IT (Aug)	Toropi
		3956124	88.05	4.99	20.33 – CI (Aug) 38.44 – IT (Sep)	
QLr.ufrgs-5A	5A	11914296	0.0005	4.03	25.16 – Severity (Aug)	IAC13
		1218002	6.21	4.025	36.86 – CI (Aug)	
		5010732	6.21	3.54		
		1129930	6.21	3.74		
QLr.ufrgs-6B	6B	990061	6.24	3.07	18.72 – IT (Apr)	IAC13
		1205999	6.24	2.93		
QLr.ufrgs-7D	7D	1178485	8.94	3.57	22.06 – IT (Sep)	IAC13
		gwm437	22.31	3.70		
		2259579	32.89	3.62		

[1]QTL names were designated following the recommended rules (http://wheat.pw.usda.gov/ggpages/wgc/98/Intro.htm). [2]Chr: Chromosome;
*The traits analysed were: Infection type (IT); percentage of disease severity (Severity) and coefficient of infection (CI). The number mentioned after the trait indicate the experiment were the QTL was found: April (Apr), August (Aug) and September (Sep).

Seven leaf rust to adult plant resistance QTL were detected on chromosomes 1A, 2A, 2D, 3B, 5A, 6B and 7D (Figure 3). The QTL *QLr.ufrgs-1A, QLr.ufrgs-2A* and *QLr.ufrgs-3B* were mapped on Toropi background and *QLr.ufrgs-2D, QLr.ufrgs-5A, QLr.ufrgs-6B* and *QLr.ufrgs-7D* are from IAC13. *QLr.ufrgs-2A* and *QLr.ufrgs-3B* may be consistent QTL since they are found in August and September experiments. While *QLr.ufrgs-1A* and *QLr.ufrgs-6B* were detected only in April, *QLr.ufrgs-2D* and *QLr.ufrgs-5A* were detect only in August, and *QLr.ufrgs-7D* only in September.

The first consistent QTL, *QLr.ufrgs-2A*, was mapped in the interval of markers *1094287* and *7351364*, explaining from 14.5 to 16.89% of the phenotypic variation (Table 3). The second consistent QTL, *QLr.ufrgs-3B*, was flanked by markers *2252631* and *3956124*, explained 20.33 to 45.08% of leaf rust variation in adult plants (Table 3). *QLr.ufrgs-3B* was the loci with the major effects on this population.

QLr.ufrgs-1A had the lowest effect, presenting 13.47% of the phenotypic variation. This QTL was significant only to infection type and localizes at 27.7 cM. *QLr.ufrgs-2A* had significant effect to three traits and two experiments being located from 174.5 to 181.27 cM. *QLr.ufrgs-2D* explained from 13.66 to 32.10% of phenotypic variation, it was flanking by *1088274 / 3023061* and located at 3.59 to 4.18 cM. *QLr.ufrgs-3B* was significant to two

traits and localizes on 81.8 to 88.05 cM. *QLr.ufrgs-5A* was flanked by *11914296 / 1129930* and explains from 25.16 to 36.86% of phenotypic variation. *QLr.ufrgs-6B* was mapped at 6.24 cM explaining 18.72% of the phenotypic variation, being flanked by *990061 / 1205999*. A smaller effect QTL, *QLr.ufrgs.7D*, was detected only to infection type trait in September phenotype experiment on chromosome 7D. *QLr.ufrgs.7D* had a LOD of 3.57 to 3.70 and an explained variance of 22%.
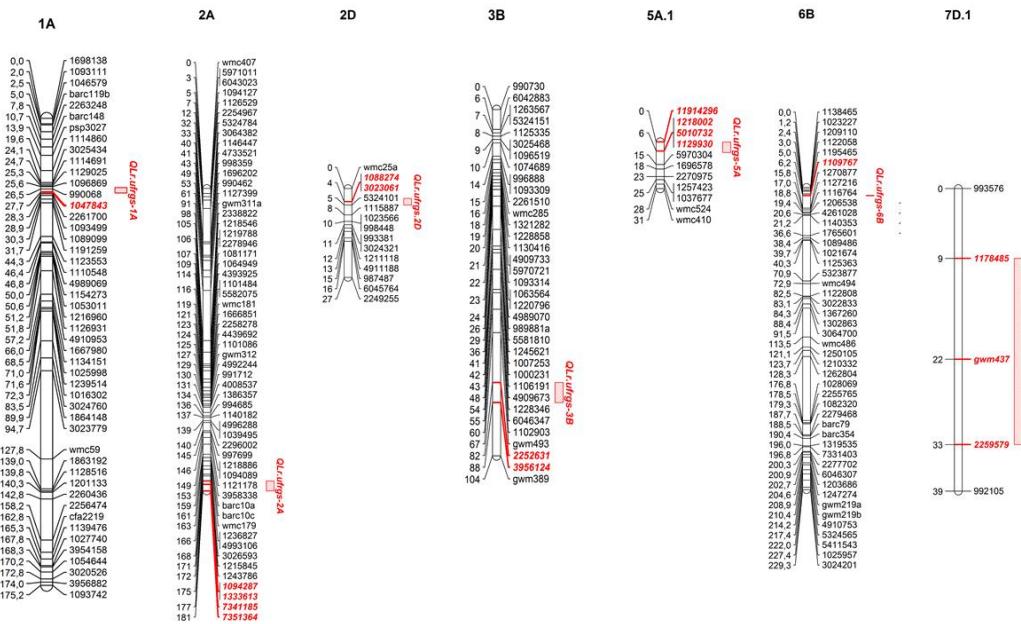
Figure 4. Maps of linkage groups harbouring quantitative trait loci identified in three greenhouse assays for leaf rust resistance in the Toropi x IAC13 inbreeding lines population.

DISCUSSION

Adult plant resistance to leaf rust in the Toropi x IAC13 F_{12} RIL population was found to be controlled by seven loci. Using DArT-Seq, KASP, STS and SSR markers on the population it was possible to exclude the presence of the most important APR genes to wheat leaf rust resistance *Lr34* (*WMAS000003*)*, Lr46* (*ncw7, wmc44* and *cnw1*)*, Lr67* (*cfd23* and *cfd71*) and *Lr68* (*cs7BLNLRR*). These genes are currently the main source of durable resistance on wheat, it makes Toropi a new and important source of durable resistance since its resistance has been not catalogued as yet.

Transgressive segregation was observed in all greenhouse assays, with lines pocessing less leaf rust severity than Toropi, as well as more susceptibility than IAC13. This transgressive segregation could be explained by an additive effect of the Toropi and IAC13 loci. Since IAC13 possess *Lr14b, Lr15* (ROSA et al., 2014) and also pocesses others loci to leaf rust resistance as reported on this study. The same transgressive pattern of segregation was also observed to stripe rust by Rosa et al. (2016), which phenotyped a

double-haploid population from cross between Toropi x Thatcher, even this was just noted in New Zealand experiments. Rosa (2012) didn't observe transgressive segregation to leaf rust in Brazilian assays, this may be explained because Thatcher is photoperiod sensitive, what might misidentify the leaf rust scoring. Transgressive segregation was also observed in a cross between Borah and Wampum, suggesting that there are different genes controlling the partial resistance in some wheat cultivars (LEHMAN et al., 2005).

The low rust severity on the April experiment was probably because the evaluation was done at 11 d.a.i. instead of being carried out on 14 as usual. This short period of time until evaluation didn't allow the pathogen to produce pustules in a considerable amount, even in the susceptible parent IAC13. Despite that we were able to found two resistance QTL explaining 13.47 and 18.72% of the phenotypic variation to leaf rust in April assay.

Up to now only Ren et al. (2017) and Chhetri et al. (2016) have published studies mapping leaf rust resistance in hexaploid wheat, and Lan et al. (2017) in durum wheat, using DArT-Seq markers. Chhetri et al. (2016) identified the QTL *QLr.sum-3BS* and the *Lr23* and *Lr67* genes. *QLr.sum-3BS* is a consistent QTL derived by BT-Schomburk Selection (BTSS) that explains from 2.4 to 36.7% of phenotypic variation (CHHETRI et al., 2016). Lan et al. (2017) found the gene *Lr46* and the QTL *QLr.cim.2BC, QLr.cim.5BL* and *QLr.cim.6BL,* in a population developed from a cross of Bairds with the susceptible parent Atred#1. Ren et al. (2017) in turn, also found the *Lr46* and other three minor QTL, *QLr.cim.2BL, QLr.cim.2DS* and *QLr.cim.6AL.* It's worth highlighting that *QLr.cim.2BC* and *QLr.cim.6AL* were derived by the susceptible parents Atred#1 and Avocet-*YrA,* respectively (LAN et al., 2017; REN et al., 2017). On the present work, we found seven APR QTL localized on chromosomes 1A, 2A, 2D, 3B, 5A, 6B and 7D but we cannot suggest yet if any of them should be correspondent to the *Lr* genes previously catalogued.

On mapping APR loci, the D genome was the poorest represented, and no linkage group was identified for chromosome 5D. A recently GWAS study found out that the average distance between markers was also larger in the D genome (2.2 cM) compared with A genome (0.51 cM) and B (0.49 cM) (TURNER et al., 2017). According to Turner et al. (2017), the ability to identify race-nonspecific resistance genes with small effects is limited, particularly in regions of the D genome where marker coverage is so low. Nevertheless, we have identified the *QLr.ufrgs-2D* and *QLr.ufrgs-7D* on this D genome. Additionally, the power of detection is low in small populations (GAO et al., 2016) and probably several minor QTL remained undetected.

According to Da Silva et al. (2012) the APR genes *Trp-1* and *Trp-2* derived by Toropi are located at chromosomes 1AS and 4DS. On the present study, we just found one QTL associated to 1A and none on 4D. One possible explanation is that the D genome is characterized by low genetic diversity, which limits its potential for introducing important

traits associated to the sub-genome (GAO et al., 2016). The low level of polymorphism found in the chromosome 4D may be due to the linkage maps, which had just few markers present with large gaps (GAO et al., 2016).

The Toropi locus *QLr.ufrgs-1A* was mapped on 27.7 cM explaining 13.47% of phenotypic variation. This QTL was significant only on April assay to infection type. The average temperature post inoculation on April experiment was higher (30 ºC) than on August (22 ºC) and September (24 ºC), and according to Rosa et al. (2016) the expression of some *Lr* loci could be altered according to the environment. This particular experiment was slightly different from the others since it presented lower concentration of spores at inoculation (2.13 x 10^5 spores.mL^{-1}) and its evaluation was carried out earlier. Based on that information, *QLr.ufrgs-1A* might be characterized as a minor QTL and its resistance might be also enhanced by high temperatures. These hypotheses need, however, further investigation.

Just a few loci to *Lr* resistance have been associated to 1A and three of them were mapped on regions closed to *QLr.ufrgs-1A*. *QLr.ccsu-1A.2* is derived by Opata 85 and was mapped on 38.3 cM (KUMAR et al., 2013), *QLr.inra-1Aa* mapped on cv. Apache from 0 to 22.3 cM (AZZIMONTI et al., 2014) and *QLr.cim-1AS* located on 22 cM in cv. Sujata (LAN et al., 2015). Despite they all being anchored at the short arm it is very difficult to affirm that these loci are different from *QLr.ufrgs-1A* since several marker technologies were used to mapped them.

QLr.ufrgs-2A is a consistent QTL found in August and September experiments to the three traits evaluated. This locus came by Toropi and was mapped from 174.5 to 181.27 cM at the distal region of chromosome 2AL, ranging from 14.50 to 16.89% of the phenotypic variation. There are more eight QTL reported on this chromosome and none of them have been placed in a region so distal as *QLr.ufrgs-2A*. The closest is a minor locus, *QLr.spa-2A,* which is derived by cv. AC Cadillac and has a marker *rPt-9611* located on 127.8 cM, this locus explains only 6.1% of the phenotypic variation (SINGH et al., 2014).

From those QTL located at the long arm, just *QLr.ifa-2AL* and *QLr.ubo-2A* are major APR QTL and explained 10.2 to 25.7% and 18.6 to 30% of leaf rust reduction, respectively (BUERSTMAYR et al., 2014; MACCAFERRI et al., 2008). *QLr.ifa-2AL* (co-located to *QYr.ifa-2AL*) is derived from cv. Capo and was mapped with *tPt-8937* as peak marker (62.6 cM) (BUERSTMAYR et al., 2014), while *QLr.ubo-2A* come from cv. Lloyd and mapped by the flanking markers *wPT-386 / 310911* (67 to 74 cM) (MACCAFERRI et al., 2008).

Lr38, derived from *Thinopyrum intermedium* (McCALLUM et al., 2012) and *LrTt1*, from *T. timopheevii* (LEONOVA et al., 2010) are the only *Lr* genes reported on the long arm of the 2A chromosome up to now. Toropi does not contain any of these insertions

(http://wheatpedigree.net/sort/renderPedigree/61662). Thus, *QLr.ufrgs-2A* is likely to be a novel APR locus for wheat leaf rust.

The locus *QLr.ufrgs-2D* was mapped on IAC13 background from 3.59 to 4.18 cM at the distal region of the chromosome 2DS. *QLr.ufrgs-2D* shows significant effect to coefficient of infection and leaf rust severity only for the August experiment.

There are 14 QTL mapped on chromosome 2D, being seven of them located on the short arm. The last APR QTL mapped on this region was described by Ren et al. (2017), *QLr.cim-2DS* was flanked by DArT-Seq markers *1020115 / 1242814* at 1 cM and explains from 6.4 to 7.8% of the leaf rust resistance in the cv. Kundan. The DArT-Seq marker *1242814* used by Ren et al. (2017) was also polymorphic on the present study but it didn't show statistical significance.

Only two major QTL have been mapped on 2DS (BUERSTMAYR et al., 2014; XU et al., 2005). *QLrlp.osu-2DS* came from the wheat line CI 13227 (XU et al., 2005). It confers long latent period and localizes about 2.5 cM distant from *xactg.gtg185* and 2.0 cM from *xbarc124* (XU et al., 2005). The phenotypic and genetic variances explained by this major QTL were 42.8% and 54.5%, respectively (XU et al., 2005). Buerstmayr et al. (2014) also found a major APR QTL on chromosome 2D, *QLr.ifa-2DS* explaining 28% of the phenotypic variance and mapped on cv. Capo background at the peak of marker *wPt-6780* (1 cM). Unfortunately, with the present study we cannot conclude if *QLr.ufrgs-2D* corresponds to any of these QTL since they were mapped closed, requiring further analysis to confirm.

Several *Lr* genes are associated to 2DS of which *Lr15, Lr22* (alleles *Lr22a* and *Lr22b*) and *Lr39* have been mapped to telomeric regions of chromosome 2DS, while *Lr2* (alleles *Lr2a, Lr2b* and *Lr2c*) is more proximal (McINTOSH et al., 2017). *Lr39* and *Lr22a* are introgressions from *T. tauschii* (RAUPP et al. 2001) and *Lr15* and *Lr22b* from *T. aestivum* (McINTOSH et al., 2017). It is worth mentioning that *Lr15* is an ASR gene present on cv. IAC13 (ROSA et al., 2014). Its location revealed the closest flanking markers *Xgwm4562* (3.1 cM) and *Xgwm102* (9.3 cM) (DHOLAKIA et al., 2013). The region where *QLr.ufrgs-2D* was placed, from 3.59 to 4.18 cM, coincides with the location of *Lr15.* Thus, it strongly suggests that *Lr15* might be *QLr.ufrgs-2D.* In order to confirm this, specific markers need to be added to the genetic map.

QLr.ufrgs-3B is a consistent QTL identified in August and September experiments to infection type and coefficient of infection, respectively. It was mapped on Toropi background from 81.8 to 88.05 cM. *QLr.ufrgs-3B* explains from 20.33 to 45.08% of the phenotypic variation and based on that it should be characterized as a major APR locus.

Azzimonti et al. (2014) have located two QTL closed to *QLr.ufrgs-3B.* The *QLr.inra-3Bb.1* was mapped on cv. Apache background at the peak of marker *wPt8075* (89.4 cM) explaining from 9.5 to 14.9% of the phenotypic variation (AZZIMONTI et al., 2014). On the

other hand, *QLr.inra-3Bb.2* was mapped on Balance background at a peak of marker *wPt1867* (125.4 cM) explaining from 5.9 to 23.6% of the phenotypic variation (AZZIMONTI et al., 2014). Nevertheless, none of them have presented the phenotypic variation as higher as *QLr.ufrgs-3B.*

Recently, three *Lr* gene have been associated to chromosome 3B, with only *Lr27* conferring seedling resistance (McINTOSH et al., 2017). *Lr74* is an APR gene mapped at chromosome 3BS on BTSS background, flanked by *Xcfb5006-3B* (1.9 cM) and *BS00009992* (2.2 cM) or *Xgwm533-3B* (2.7 cM) (McINTOSH et al., 2017). *Lr77* is also an APR gene mapped at chromosome 3BL from cv. "Santa Fe" background, flanked by the SNPs *IWB32653* (1.15 cM) and *IWB79797* (2.46 cM) (McINTOSH et al., 2017). All this information allow us to hypothesize that *QLr.ufrgs-3B* may be a new source of leaf

The *QLr.ufrgs-5A* was identified on IAC13 background and localizes from 0 to 6.21 cM by the DArT-Seq markers *11914296, 12188002, 5010732* and *1129930*. This locus was associated to leaf rust severity and coefficient of infection on August experiment. *QLr.ufrgs-5A* is a major locus, ranging from 25.16 to 36.86% in the phenotypic variation. The literature only reports five wheat QTL to leaf rust resistance and all being loci of minor effects (CALVO-SALAZAR et al., 2015; ROSEWARNE et al., 2012; SINGH et al., 2009; MESSMER et al., 2000). *QLr.pbi-5AS* identified on cv. Beaver (SINGH et al., 2009) is the closest locus to *QLr.ufrgs-5A.* According to Singh et al. (2009), *QLr.pbi-5AS* localizes at 11 (w*Pt1931*) to 16 cM (*wPt8756*) from the centromere relative to the consensus map of Somers et al. (2004) and explains 11.2% of the phenotypic variation.

In previous studies involving a population derived by Toropi, Rosa (2012) also mapped a QTL on 5A and according with the author it is derived from Toropi. In our study, the locus *QLr.ufrgs-5A* was associated to the susceptible parent, contrasting with the findings reported by Rosa (2012). *QLr.crc-5AL.1* was associated to Toropi conferring partial resistance to leaf rust and co-localizing with a QTL to stripe rust (*QStr.crc-5AL.1*). This QTL was identified on 5AL between *gpw7007* and *cfa2163*, covering an interval of 40.3 cM and explaining from 22.2 to 39% and 12% of phenotypic reduction to leaf and stripe rust, respectively (ROSA, 2012). According to Rosa (2012), *QLr.crc-5AL.1/QStr.crc-5AL.1* may be compared with other partial APR genes that confer multi pathogens resistance or are linked to genes conferring these resistances. Despite the inconsistences among the results involving Toropi populations, both studies need additional information to validate the locus conditioning resistance.

Several QTL studies have identified resistance loci to wheat leaf rust in susceptible genotypes as well. *QLr.cim-5AC* is a locus located at the centromeric region of 5A (36 cM) and is derived by the cv. Avocet-*YrA* (CALVO-SALAZAR et al., 2015), a susceptible line used in several crosses (REN et al., 2017; LAN et al., 2015; ROSEWARNE et al., 2012).

QLr.ufrgs-6B was only significant on April experiment to infection type trait and has been associated to the susceptible parent IAC13. This locus was positioned at 6.4 cM by the peak markers *990061* and *1205999*, explaining 18.72% of the phenotypic variation. There are five catalogued *Lr* genes on chromosome 6B all of them ASR, *Lr3* (alleles *a*, *bg* and *ka*), *Lr9*, *Lr36*, *Lr53* and *Lr61* (McINTOSH et al., 2017). These genes are located from 0 to 6 cM, and there is a probability that one of them corresponds to *QLr.ufrgs-6B.* An exception is the *Lr3* gene, because the *P. triticina* race used in this assay shows virulence to this gene (CHAVES, 2017; personal communication). Specific diagnostic markers to *Lr9*, *Lr36*, *Lr53* and *Lr61* need to be add to this map in order to confirm the presence/absence of these genes on Toropi x IAC13 population.

Additionally, there are more seven QTL associated to 6B but there are some inconsistences among their position on wheat genome (LAN et al., 2017; AZZIMONTI et al., 2014; ROSEWARNE et al., 2012). Because of that we cannot suggest if *QLr.ufrgs-6B* could be one of the loci already reported.

Based on literature there are only two QTL to wheat leaf rust on chromosome 7D which are not the APR *Lr34* gene (LAN et al., 2014; ZHOU et al., 2014). Our work, on the other hand, suggests a novel QTL located on the 7DS region. *QLr.ufrgs-7D* was mapped by the DArT-Seq and SSR markers *1178485*, *gwm437* and *2259579* (8.94 to 32.89 cM). Several studies have placed the *Lr34* locus in this region (ZHANG et al., 2017; SUENAGA et al., 2003), and it always has a significant effect in all tested environments. The marker associated to *Lr34* (*WMAS000003*) used in Toropi x IAC13 population didn't show the resistant allele, stating that this gene is absent on the population. Others works, such as Da Silva et al. (2012) and Barcellos et al. (2000) also suggested the absent of *Lr34* on Toropi x IAC13 background.

The locus *QLr.ufrgs-7D* should be considered as an important source to adult leaf rust resistance since just two other loci have been mapped on this chromosome. *QLr.hebau-7DS* was also mapped at 7DS in a similar position to *Lr34*, showing a relatively weak effect (from 4.4 to 6%) (ZHOU et al., 2014). Genotyping with the marker *cssfr5* (LAGUDAH et al., 2009) suggested that *QLr.hebau-7DS* is different from *Lr34* (ZHOU et al., 2014). *QLr.cim-7DS* is a minor APR QTL for slow rusting derived by Francolin#1 and it is flanked by the markers *wPt-744857* and *Xgwm295* (2 cM) (LAN et al., 2014). According to Lan et al. (2014) the locus *QLr.cim-7DS*, despite to be mapped close to *Lr34*, did not correspond to this gene.

CONCLUSIONS

This study identified seven resistance loci alleles, but further genetic tests are required to confirm whether the QTL found here correspond to any published genes. This finding highlights the complex genetic architecture of resistant genotypes possessing APR. Additionally, we suggest that Toropi does not possesses the *Lr34*, *Lr46*, *Lr67* and *Lr68* on its background.

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5 ARTIGO 3

Aluminium tolerance of wheat population Toropi x IAC13 under hydroponic assay[5]

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[5] Artigo formatado conforme as normas do periódico *Theoretical Applied Genetics*

Aluminium tolerance of wheat population Toropi x IAC13 under hydroponic assay

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ABSTRACT: Soil aluminium toxicity is a major yield constraint to wheat. Few wheat populations presented levels of variation in tolerance that is not explained by the known Al tolerance loci. The objectives of this work were to screen a 76 F_{14} recombinant population of Toropi x IAC13 and associate these results to *TaALMT1*, *TaMATE1* genes and other possible loci. The population was genotyped using Diversity Array Technology (DArT)-Seq and Single Sequence Repeats (SSR) markers and phenotyped in hydroponic assays under controlled growth chamber with low-pH and at a concentration of 370 µM of Al^{3+}. The SSR marker *wmc331*, linked to *TaALMT1*, revealed 36 lines with the favourable allele. The upstream transposon insertions to *TaMATE1B* was detected only on 14 lines and just eight RILs possessed the two genes. Although used on other studies, *wmc331* was not fully applicable on the population evaluated. On the other hand, the transposon insertion to *TaMATE1B* was very useful to classified RILs. The data indicate the necessity to improve the pre-existent genetic map further, with the addition of new markers in order to discriminate *TaALMT1* alleles.

Key-words: Al^{3+}; *TaALMT1*; *TaMATE1B*; Toropi; IAC13;

INTRODUCTION

Aluminium (Al) toxicity affects around 15% of Earth's soils (BOT et al., 2000) and is the main yield restriction on acidic and arable land (KOCHIAN et al., 2015). Acid soil is one of the major limitations to wheat (*Triticum aestivum* L.) production in Brazil. Al is toxic to most part of the winter cereals when its concentration increases and pH reaches levels below 5.0. One of the main concerns about acid soil is the increased mobility of Al ions and their propensity to procedure highly stable complexes with phosphorus (SHARMA et al., 2016).

Al can bind to the cell walls of the roots and as consequence impedes meristematic elongation in sensitive species (MA et al., 2004). It also causes root stunting, which is the main reason for lower crop performance in acid soils. High levels of Al on soil results in changes of Ca^{2+} homeostasis and signalling, induction of reactive oxygen species, disruption of cytoskeletal dynamics and mitochondrial dysfunction. Thus, as a consequence, occurs inhibition of root growth and diminishing of crop yields due the inhibition of water and nutrient uptake (GARCIA-OLIVEIRA et al., 2015).

To deal with the Al toxicity plants have evolved some strategies to detoxify it. The most well-characterized mechanism is Al-dependent root exudation of organic acid (OAs) into the rhizosphere. Where the OAs chelate Al^{3+} ions forming nontoxic compounds that do not penetrate the roots. There were some genes responsible for aluminium tolerance identified in wheat germplasm, with emphasis to those excluding OAs, especially malate and citrate. The genic family of Al-activated malate transporter (*ALMT*) and the multidrug and toxic compound extrusion (*MATE*) are responsible for the plasma membrane malate and citrate efflux (KOCHIAN et al., 2015).

The *TaALMT1* gene encodes a transmembrane protein constitutively expressed on wheat root apices of tolerant cultivars (SASAKI et al., 2004). Up to now it is known that there are nine alleles reported to this gene in several genotypes worldwide (AGUILERA et al., 2016; SASAKI et al., 2006). According to Pereira et al. (2015) there are seven promoter alleles (Types I to VII) on this gene present in the Brazilian genotypes and they are correlated to the Al tolerance/sensitivity. Promoters contained the allele types V to VI present higher levels of gene expression, showing good levels of Al tolerance, whereas the ones with lower number of repeats, I and II, present lower gene expression and they are more sensitive to Al^{3+} (SASAKI et al., 2006).

Differences in tolerance and sensitivity conferred by *TaMATE1B* can be notable by a transposon insertion (presence or absence) at upstream region correlated with the level of gene expression to Al response (AGUILERA et al., 2016; PEREIRA et al., 2015). According to Tovkach et al. (2013) the transposon insertion is responsible for increments and changes of the location of expression to the roots tips, causing increase of citrate efflux and consequently Al^{3+} tolerance.

Analyses of hundreds of Brazilian wheat genotypes revealed that lines possessing *TaALMT1* promoters V or VI, associated to the presence of the transposon-like insertion in *TaMATE1B* promoter presented higher relative root length (PEREIRA et al., 2015). Working with 388 wheat genotypes from different parts of the world, Aguilera et al. (2016) observed that the Brazilian genotypes had the best performance in acid soil. In fact, the Brazilian genotypes have been considered good sources to Al tolerance (AGUILERA et al., 2016; PEREIRA et al., 2015) and they are used in different breeding programmes around the

world. The most used lines include BH1146, Carazinho, Frontana, IAC 5 – Maringá, Toropi and Trintecinco (GARCIA-OLIVEIRA et al., 2015). The origin of this tolerance is mainly from the line PG1, which comes from a selection of Polyssú, an ancient wheat cultivar.

The Brazilian cultivar Toropi is another important ally to wheat breeders, nevertheless still underexplored. In diverse assays Toropi has been proved its contribution to Al^{3+} tolerance (AGUILERA et al., 2016; PEREIRA et al., 2015; BOFF, 2006) and adult plant resistance (APR) to leaf (ROSA et al., 2016; CASASSOLA et al., 2015), stripe (ROSA et al., 2016) and stem rust (*unpublished data*). According to Da Silva et al. (2012) and Boff (2006) reports, it is believed that the loci to Al tolerance and rust resistance are located in the region of the chromosome 4D. Some studies have suggested that Toropi can deal with high levels of Al^{3+} in hydroponic tests ranging from 30 µM (RYAN et al., 2009) to 222 µM (BOFF, 2006). Also, it has been demonstrated that Toropi possesses the allele VI to *TaALMT1* and the presence of the insertion in *TaMATE1B* promoter (PEREIRA et al., 2015). Previously studies suggest that Toropi tolerance is conditioned for more than two genes (BOFF, 2006). The mapping of the loci is still unidentified and they probably act together to confer good levels of Al tolerance, which might be useful to wheat breeding programs.

Variation in Al tolerance is observable in many crops and it can be under simple or complex genetic control (FROESE & CARTER, 2016; BOFF, 2006; MA et al., 2004). Several studies identified different regions associated to Al tolerance in wheat. Using genome wide association Froese and Carter (2016) uncovered a total of 55 loci to acidic field tolerance in two populations of winter lines. Population A containing 459 lines and Population B with 401 lines (FROESE & CARTER, 2016). Additionally, they used the molecular marker *wmc331* linked to *ALMT1*, revealing only eight individuals in the two populations with the favourable allele. Raman et al. (2005) identified a high LOD score QTL linked with SSR marker *wcm331* that explained up to 75% of total phenotypic variance for Al tolerance scored on the basis of root growth in a doubled-haploid Diamondbird x Janz population.

The main objectives of this work were to screen Toropi x IAC13 F_{14} population in hydroponic assays conducted in controlled growth chamber and associate this results to *TaALMT1*, *TaMATE1* genes and other possible tolerance loci.

METHODS

Plant material

In all assays it was used a population of 76 Recombinant Inbreed Lines (RILs) in F_{14} derived from a cross between Toropi and IAC13-Lorena. Toropi (Frontana

1971.37/QuadarnaAA//Petiblanco 8) is a Brazilian cultivar developed in 1965 that shows high levels of aluminum tolerance (AGUILERA et al., 2016; PEREIRA et al., 2015). IAC13-Lorena (Ciano67/IAS51) was identified as showing moderate tolerance to Al^{3+} (http://wheatpedigree.net/sort/show/35705) and tolerance (CAMARGO et al., 1987; CAMARGO & OLIVEIRA, 1981; Bol. Tec. IAC 163, 1977). We also used the cultivar Anahuac (II-12300//LERMA-ROJO-64/SIETE-CERROS-66/3/NORTENO-67) as control, considering that it shows high sensitive to Al^{3+} (AGUILERA et al., 2016; GARCIA-OLIVEIRA et al., 2014; CAMARGO et al., 1987).

Aluminium toxicity screening

It was used an adaptation of the protocol reported by NAVA et al. (2016), where the aluminium response of each RIL and the parents were determined based on the regrowth rate of the main root after the exposure to Al^{3+}. The seeds from the population were germinated on a moist filter paper for 72h in a growth chamber at 17 ºC and 12/12h photoperiod. When the pre-germinated seeds were about 5 mm-long radicles, they are mounted on nylon-mesh floats on plastic vessels filled with continuously aerated solution with pH adjusted to 4.0 (free of Al^{3+}) for 24h. Soon after, they were transferred to an aluminium solution 370 µM (10 ppm) of aluminium chloride ($AlCl_3$) for 48h and finally transferred back to the solution free of Al for other 72h. The evaluation of each seedling was carried out through the measurement in centimetre of the point of the thickening (callosity) of the main root **(Figure 1)** using a digital paquimeter. The solution free of Al was: $Ca(NO_3)_2$ – 4 mM, $MgSO_4$ – 2 mM, KNO_3 – 4 mM, $(NH_4)_2SO_4$ – 0.435 mM, KH_2PO_4 - 0.5 mM, $MnSO_4$ – 2.0 µM, $CuSO_4$ – 0.3 µM, $ZnSO_4$ – 0.3 µM, NaCl – 30 µM, Na_2MoO_4 – 0.1 µM and H_3BO_3 - 10 µM. The aluminium solution treatment was composed by 10% of the solution above plus 370 µM of $AlCl_3$.

ANAHUAC   IAC13   TOROPI
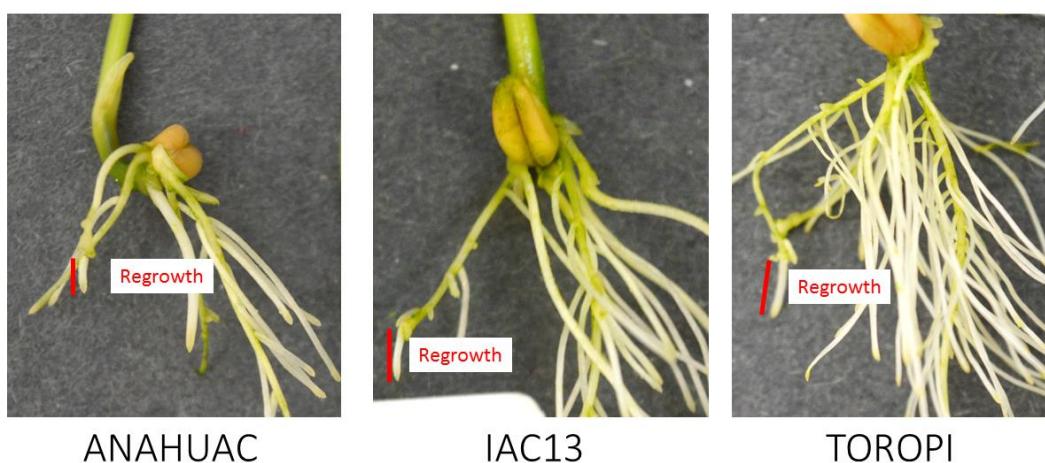
Figure 1. Anahuac, IAC13 and Toropi main root regrowth.

Since all lines could not be screened simultaneously because of the large size of the population, the root regrowth experiment was conducted considering an unbalanced incomplete block design, where the 76 RILs were divided in eight subsets. Each subset was conducted in different buckets ranging from 6 to 8 per subset, consisting of at least 17 and up to 63 seedlings per line, totalizing 2733 seedlings analyzed (**Table S1**). To verify the consistency of growth conditions among the distinct subsets, Toropi and Anahuac were used in all subsets and buckets, having been analyzed a total of 197 and 205 seedlings, respectively (**Table S1**). The parental IAC13 was analyzed in three different subsets (subset 6, 7 and 8), with a total of 99 seedlings (**Table S1**).

The subsequent phenotypic analysis were developed to each subset individually. To create the RILs range of classification, it was considered that each subset was statistically different. Thus each one had its own range, which was based on the respective values to Toropi and Anahuac. To screen for aluminium response a modified method from Portaluppi et al. (2010) was used to group individuals where: sensitive (S), were the RILs with values lower than Anahuac response plus 50%; moderately sensitive (MS), RILs presenting values ranging from 50% to 75% of Anahuac´s value; moderately tolerant (MT), RILs with values ranging from 50 to 75% Toropi mean; and tolerant (T), RILs above 75% of Toropi values.

At the end of each of the eight subsets assays of hydroponic regrowth, all the seedling roots of the RILs, Toropi, IAC13 and Anahuac were cut and collected in paper bags to assess their dry matter. Roots were dried in an oven with air circulation at 60 ºC during three days and weighed on the analytical balance. It resulted in the weight of the root dry matter of regrowth assays.

In order to assess the total root dry matter of the parents plus Anahuac in hydroponic solution, two new subsets of experiment were carried out. On the first subset, the genotypes were grown in a complete nutrient solution only, without Al^{3+} for five days. On the second, they were maintained only in a 10% of the complete solution plus 370 Al^{3+} µM also during five days. At the end of the five days in each subset, the roots were collected and dried as described above producing the weight of dry matter in solution free of Al^{3+} and in solution containing Al^{3+}.

Detection of the allelic variability in the TaALMT1 and TaMATE1B promoters

DNA of all 76 RILs and parental genotypes had their DNA extracted from seedlings using a CTAB buffer method (LEFORT & DOUGLAS, 1999) and the samples were quantified on nanodrop (Nanodrop Technologies, Oxfordshire, U.K.). To detect the presence of the transposon insertion in the *TaMATE1B* promoter we used primers described by Garcia-Oliveira et al. (2014). The PCR reactions were carried out in a final

volume of 20 µl containing 1X PCR buffer with 1.5mM of $MgCl_2$, 0.3 mM each dNTPs, 0.3 mM each primer, and 0.5 U FastStart *Taq* DNA Polymerase (Roche). The PCR amplification programs were run using the following parameters: denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30s, 60 °C for 30s, and 72 °C for 2 min, and a final extension for 7 min at 72 °C. PCR fragments were run on a 1.5% agarose gel, stained with ethidium bromide. To confirm the absence of the transposon insertion in the *TaMATE1B* promoter, we used positive in each set of amplifications as well as we performed PCR replicates using the same DNA.

We also genotyped the microsatellite (SSR) marker *wmc331* (Forward primer - CCTGTTGCATACTTGACCTTTTT and Reverse primer – GGAGTTCAATCTTTCATCACCAT; GUPTA et al., 2002), which was identified to be linked to *TaALMT1* promoter and previously mapped on chromosome 4DL (FROESE & CARTER, 2016; RAMAN et al., 2005).

Statistical analysis of aluminium experiment

Analysis of variance (ANOVA) and correlation of regrowth data were calculated using the linear mixed model (REML) procedure, with lines as fixed effect and experiments/bucket as random effect. A Log 10 transformation was chosen to adjust the root regrowth data to achieve near normality. The statistical analysis of the root regrowth experiments was done on non-transform and the Log 10 transformed datasets. Predicted means for the RIL population were extracted from the REML analyses for each phenotypic dataset. All analyses were performed using the software GenStat (GenStat 16th Edition, Rothamsted Experimental Station, Harpenden, UK).

Linkage map

We used a previous genetic map for Toropi x IAC13 population developed with Diversity array technology (DArT)-Seq and microsatellites markers used in plant adult leaf rust resistance experiments (Silva et al. *in prep;* Charter 3). We also added the *TaMATE1B* promoters results on the previous map.

QTL analysis

QTL calculations were carried out with R/qtl package version 1.40-8 (BROMAN et al., 2009). Genome wide QTL searches were conducted for non-transform and the Log10 transformed datasets, as well as for predicted mean separately. Single marker regression (SMR), standard interval mapping (IM), Harley-Knott regression (HK) and composite interval mapping (CIM) were performed with all regrowth data set. Significance thresholds of 10 and 5% LOD were obtained using 1000 permutation tests for each phenotypic dataset.

RESULTS

Aluminium toxicity screening

The parental lines Toropi and IAC13 showed general mean primary regrowth of 6.67 and 2.45 cm, respectively, while the high sensitive pattern Anahuac presented just 1.66 cm regrowth (**Table S1.**). The mean primary regrowth of parental lines plus Anahuac to each experiment are showed in a box-plot graph on **Figure 2**. There were a great range of variation among subsets, with Toropi presenting higher variation on subset 4, while Anahuac seems to present variation on all subsets. Toropi also presents some outliers on subsets 1, 2, 3, 5 and 6. Anahuac presented outlier only on subset 2, and IAC13 on 6. IAC13 was used only on subset 6, 7 and 8, having the last one presented slight difference in comparison to the previous two.

Toropi and Anahuac showed maximum values on subset 4, each one with 10.59 and 2.14 cm mean of regrowth, respectively. Conversely, minimum values were verified on subset 1, with Toropi presenting 3.92 cm and Anahuac 0.80 cm (Table 1).

Table 1. Descriptive analysis for Toropi, Anahuac and IAC13 and the range of values to classify aluminium response of recombinant inbreed lines in each hydroponic subset.

| Subsets	Mean			Median			Stand. deviation			Range of classification			
	Trp.	Anh.	IAC	Trp.	Anh.	IAC	Trp.	Anh.	IAC	S	MS	MT	T
1	3.92	0.80	-	3.36	0.72	-	1.92	0.33	-	<1.20	1.21>1.95	1.96>2.94	>2.95
2	6.35	1.66	-	5.89	1.49	-	1.73	0.61	-	<2.49	2.50>3.16	3.17>4.76	>4.77
3	5.65	1.80	-	5.15	1.74	-	1.92	0.57	-	<2.69	2.70>2.81	2.82>4.24	>4.25
4	10.59	2.14	-	10.77	2.07	-	3.47	0.56	-	<3.21	3.22>5.29	5.30>7.94	>7.95
5	8.18	1.80	-	7.22	1.76	-	3.19	0.42	-	<2.70	2.71>4.08	4.09>6.12	>6.13
6	6.70	1.48	2.68	6.60	1.44	2.53	0.92	0.48	0.47	<2.21	2.22>3.34	3.35>5.01	>5.02
7	5.85	1.63	2.73	6.00	1.67	2.74	0.85	0.40	0.41	<2.45	2.46>2.92	2.93>4.38	>4.39
8	5.52	1.34	1.94	5.49	1.4	1.93	0.75	0.25	0.34	<2.01	2.02>2.75	2.76>4.13	>4.14
Total	6.67	1.66	2.45	6.20	1.56	2.44	2.60	0.56	0.54	-			

*Stand. deviation= standard deviation; Trp= Toropi; Anh= Anahuac; IAC= IAC13; S= sensitive; MS= moderately sensitive; MT= moderately tolerant; T= tolerant.

The higher mean of regrowth was observed on lines 792, 684 and 682, presenting 10.72, 10.37 and 10.01 cm, respectively (**Table S1**). From 76 RILs analysed seven exhibited regrowth larger than Toropi, 14 RILs were lower than IAC13 and just one lower than Anahuac. **Figure 3** shows the distribution of RILs into classes, in which 10 lines were classified as sensitive (S), 18 as moderately sensitive (MS), 25 moderately tolerant (MT), 19 as tolerant (T) and four lines presented divergent response among the experiment subsets (667; 693; 697 and 783; showed on **Table S1**). The sensitive lines were identified in all experiments, except to the subset 1. The tolerant lines were identified on subsets 1, 3, 4, 5 and 6.
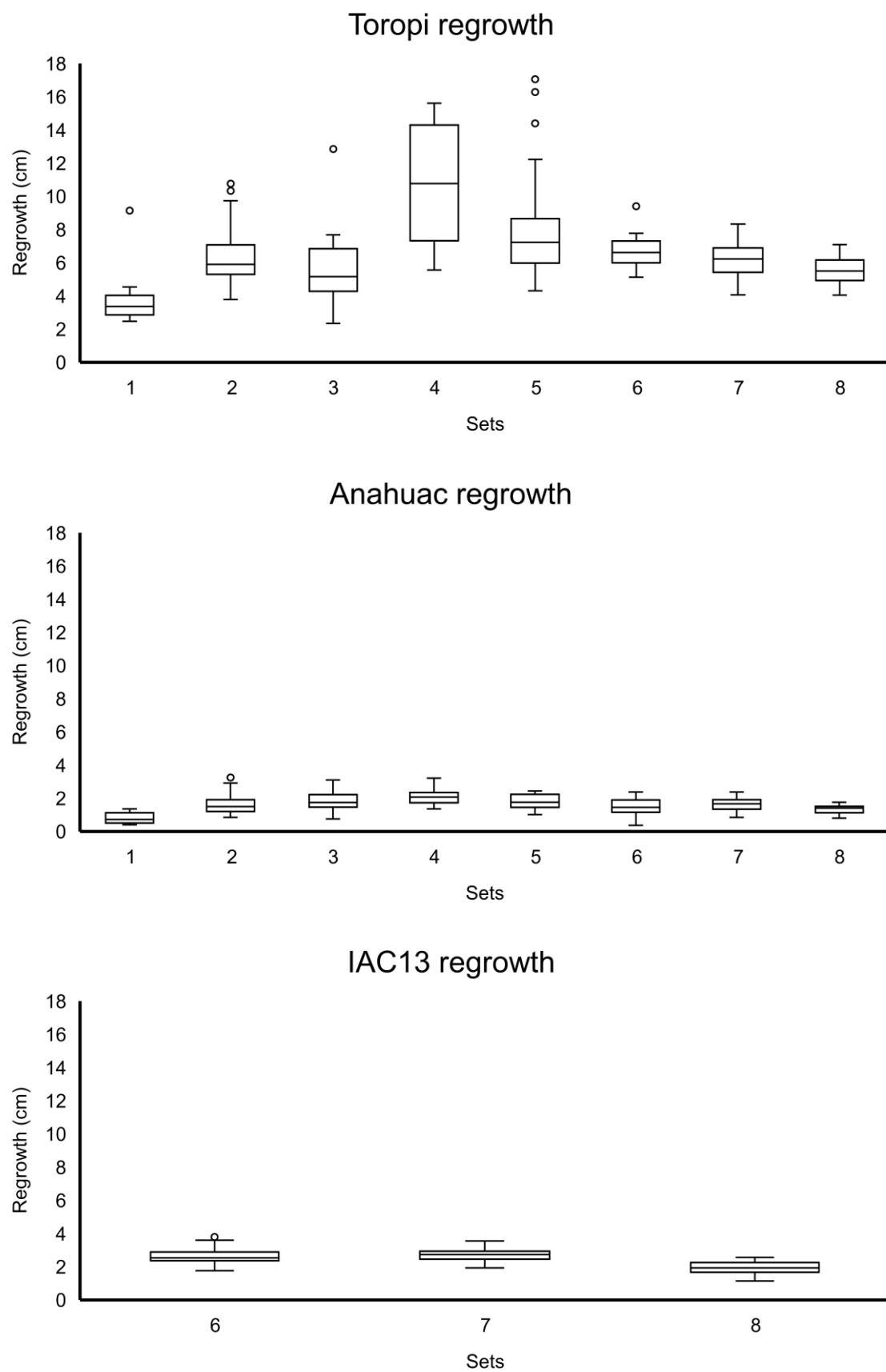
Figure 2. Box plot graph of regrowth data for each experiment subset to Toropi, Anahuac and IAC13.

The mean of roots dry matter weight of Anahuac, IAC13 and Toropi is presented on Figure 4. For the regrowth experiments the ANOVA ($P \leq 0.005$) indicated statistical difference among the lines. Toropi presented the best values, although it didn't show statistical difference to IAC13. Anahuac had the lower weight of the three wheat cultivars. Seedlings grown only in solution free of Al^{3+} presented higher production of root dry matter, following the ones grown on regrowth assay containing aluminium. The lower root weight was verified on seedlings cultivated in nutrition hydroponic solution containing only 370 µM of Al^{3+} (5 days). The means of regrowth dry matter to each RIL is presented in on Table S1.
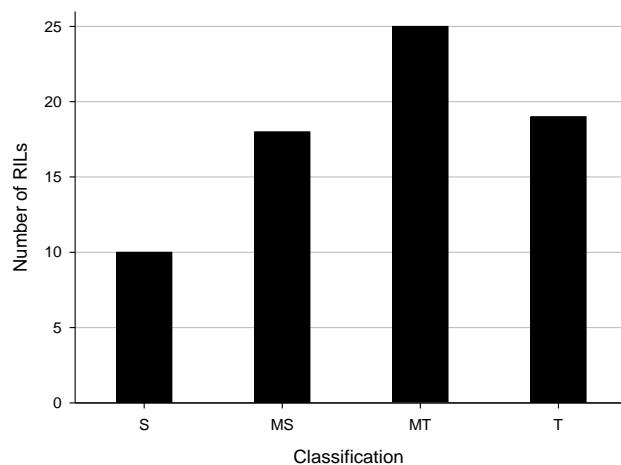
Figure 3. Distribution of number of RILs based on their responses to aluminium toxicity on each class to all subsets. This frequency of distribution was based by Portaluppi et al. (2010) with modifications. *S= sensitive; MS= moderately sensitive; MT= moderately tolerant; T= tolerant. (Four RILs are not integrated to the graph because they presented different classification on the subsets of experiments)
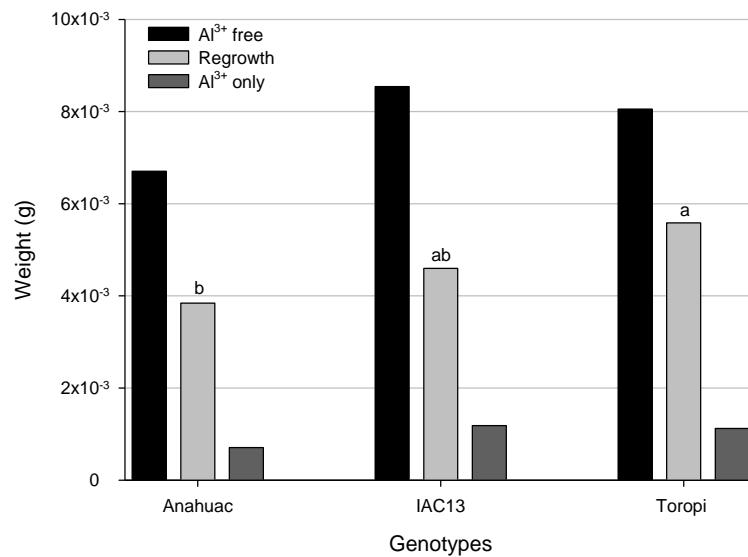
Figure 4. Roots dry matter weight of Anahuac, IAC13 and Toropi on hydroponic subsets: solution free of Al^{3+} (5 days); solutions Al^{3+} free (1 day) + 370 µM of Al^{3+} (2 days) + Al^{3+} free (3 day); and 370 µM of Al^{3+} (5 days).

Genotypic analysis and QTL detection

The marker associated to the upstream insertion of *TaMATE1B* gene revealed 14 RILs possessing it and 62 did not. From lines possessing the insertion, five were classified as tolerant, five as moderately tolerant, two moderately sensitive, one sensitive and one MS/MT. It was also confirmed that Toropi possesses the upstream insertion of *TaMATE1B* while IAC13 and Anahuac do not.

For SSR marker *wmc331*, which is linked to *TaALMT1* promoter, the genotype screening showed 36 RILs possessing the Toropi allele, 31 possessing IAC13 allele and seven did not amplified (**Table S1**). The size of target band (base pairs - bp) to this marker revealed 130 bp for IAC13 and 128 bp for Toropi. This marker only showed the presence of *TaALMT1* promoter based in a comparison with the size of alleles of Toropi and IAC13. The *wmc331* does not permit the discrimination among the types of *TaALMT1* promoter alleles.

Eight RILs were identified possessing the insertion to *TaMATE1B* gene and the Toropi parental allele to *TaALMT1* promoter, in addition to the line 792, which presented the highest mean of root regrowth. The line 709 was the single one containing *TaMATE1B* classified as sensitive, and it didn't amplify *wmc331*. Additionally, we were no able to find QTL with significative effects on these population to Al.

DISCUSSION

Wheat aluminium tolerance is frequently screened under field assays. However, inconsistent phytotoxicity and differences of pH value among the plots can induce large environmental variations (MA et al., 2005). Therefore, field experiments sometimes do not provide useful results to phenotypic screening. Assays using hydroponic culture has been used now as an alternative method for evaluating Al tolerance since it provides a better control of nutrient solution and pH. This method also allows non-destructive measurements in large wheat populations. In this study, the mean regrowth of primary root was used to measure Al tolerance of a F_{14} RIL mapping population derived from Toropi x IAC13.

The inhibition of primary root growth is recognized as the major effect of Al toxicity (HOUDE & DIALLO, 2008). A higher rate of primary root regrowth suggests that the plant possesses a more efficient mechanism of tolerance to Al^{3+} (NAVA et al., 2016). Toropi showed significant primary root regrowth in all subsets tested on this study. The concentration of 370 µM ($AlCl_3$) is usually much higher than usually used to assess aluminium tolerance in wheat. In all subsets Toropi showed larger mean regrowth than IAC13 or Anahuac despite the difference among set of experiments, an evidence that Toropi is highly tolerant to Al^{3+} under hydroponic assays.

The results obtained from this experiment showed RILs with mean of regrowth higher than the parental Toropi and lines presenting regrowth lower than IAC13. This suggests a transgressive segregation among the population, indicating that IAC13 could also presents minor loci that can provide some levels of tolerance to Al^{3+} as reported by CAMARGO et al. (1987) and CAMARGO & OLIVEIRA (1981). These authors also tested 10 ppm of Al, on this case using $Al_2(SO_4)_3$ as source, although they considered the whole length of the roots instead their regrowth (CAMARGO et al. 1987; CAMARGO & OLIVEIRA, 1981). The transgressive segregation mentioned above may be caused by the association of *TaMATE1B* and *TaALMT1* present in Toropi genome (AGUILERA et al., 2016) as well as those unknown loci of IAC13.

Regrowth exhibited by the seedling roots demonstrated the great capacity of the root meristem to avoid toxic effects of Al^{3+}. Indeed this kind of phenotypic assay measures the ability of the roots to recover development after aluminium removal (NAVA et al., 2016). Froese & Carter (2016) reported that some wheat populations have shown a variation in tolerance under acid field that is not being explained by the known major Al tolerance loci. This might indicate the existence of minor loci acting together to the major ones.

The dried weight of the roots growing in a free Al^{3+} solution suggested that IAC13 seedlings had an excellent production of dry matter, presenting no statistical difference from

Toropi. Contrasting, when they were tested on regrowth assays, Toropi has shown the best capacity of recovery, presenting the higher dry weight.

TaALMT1 has been considered a major contributor to Al tolerance in a number of wheat cultivars (ZHOU et al., 2007; SASAKI et al., 2004). Several markers associated to *TaALTM1*, such as *ALMT1-CAP*, *SSR3a*, and *SSR3b* were developed from the gene-coding region for marker-assisted selection of this gene (RAMAN et al., 2006). Even though, these markers might be only effective in some crosses but not others (ZHOU et al. 2007). Froese & Carter (2016) found that the positive allele of marker *wmc331*, despite useful, was not widely present in the unique germplasm of the study, although this may be because this marker was not fully diagnostic to *TaALMT1*.

The marker *wmc331*, used on Toropi x IAC13 population showed different allele sizes to parental lines. But this information alone doesn't permit to identify the type of promoter allele to *TaALMT1*, which range from I to VII, based on the number of repeats. Since it is already known that Toropi processes the type VI (PEREIRA et al., 2015), we cannot conclude that all lines processing the same Toropi allele to *wmc331* also present the promoter type VI. The genotypic screening suggests that the marker *wmc331* didn't show a good relation to discern the types of alleles. If we had the types I to III, the lines possessing it would have a sensitive phenotype. Additionally, if the lines would have the types IV to VII, they probably would be tolerant. Thus, the marker was just used in order to separate the lines showing the alleles correspondent to Toropi or IAC13, not allowing us to infer clearer assumptions about it. Based on that, it is necessary to add more markers which allow to discriminate the allele type of each RIL.

Additionally, according to Sasaki et al. (2006) and Raman et al. (2005), there is also a possibility that some other aspects may be involved in the control of malate efflux, aside to the levels of *ALMT1* expression. Several metabolic pathways might be activated in wheat under Al. A transcriptome profiling analysis of wheat under Al toxicity suggested the existence of 83 candidate genes related to Al stress and 25 to Al tolerance (HOUDE & DIALLO, 2008).

A transposable element-like sequence is inserted close to the start of the *TaMATE1B* coding region, specifically in wheat genotypes that release citrate (TOVKACH et al., 2013). This insertion, allow us to identify the presence/absence of this gene. In Toropi x IAC13 population, the insertion to *TaMATE1B* was found in a low frequency, only 18% of the RILs possessing it. Despite the low number, it seems evident the role of *TaMATE1B* in this population, since only one of the lines carrying it was classified as sensitive. According to Garcia-Oliveira et al. (2014) the *TaMATE1* and its homologues transcript levels seem to be fairly stable under Al treatments.

The marker used to identify the insertion to *TaMATE1B* has shown completely applicability to the population used in this work. According to Pereira et al. (2015) SSR markers analysis indicated a great genetic variability among different Al^{3+} tolerant sources and among genotypes with the *TaALMT1* and *TaMATE1B* tolerant alleles. Lines differing in phenotypic response, but with the same *TaALMT1* and *TaMATE1B* alleles, could show different resistance to Al (PEREIRA et al., 2015). Our work also indicates that Toropi x IAC13 population need the addition of new markers for a better distinction of alleles to *TaALMT1*. Only then it will be possible to conducted a more accurate analysis of the whole population and Toropi inheritance.

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6 CONSIDERAÇÕES FINAIS

O trigo é uma das culturas de maior genoma conhecido na agricultura moderna, possuindo em torno de 17 Gb, o que por si só dá indícios do tempo consumido para o trabalho detalhado com esta espécie. Um outro fator que eleva a dificuldade em se realizar estudos genéticos em trigo é a presença de três genomas, conhecidos como A, B e D. Verificou-se que o genoma B de trigo é uma rica fonte de genes de resistência à ferrugem da folha. Esse padrão foi verificado tanto em estudos de mapeamento utilizando marcadores microssatélites, como utilizando marcadores associados a modernas tecnologias de sequenciamento. O genoma D, por outro lado, apresentou uma baixa taxa de polimorfismo em praticamente todos os trabalhos revisados. Devido ao seu baixo polimorfismo, frequentemente numerosos marcadores têm sido associados a mapas pré-existentes com a finalidade de melhorar a sua qualidade. Da mesma forma, o genoma A apresentou loci de resistência em número similar ao apresentado pelo genoma D. O levantamento de numeroso loci de resistência demostrou que o avanço nas tecnologias de mapeamento e sequenciamento genômico permitiu um crescimento significativo de estudos para ferrugem da folha do trigo, confirmando a presença de loci para resistência em todos os 21 cromossomos do trigo hexalóide.

A partir da utilização da população biparetal de Toropi x IAC13, confirmou-se que Toropi apresenta excelente respostas para ferrugem da folha em planta adulta e ao alumínio em níveis tóxicos. O *screening* genotípico revelou que Toropi possui no mínimo três genes de resistência de planta adulta à ferrugem da folha, com um deles provavelmente atuando em respostas a altas temperaturas. Esses indícios demonstram a complexa natureza destes genes de resistência, visto que estudos anteriores relataram apenas dois genes atuando na resistência não-específica de planta adulta.

No que se diz respeito a tolerância ao alumínio tóxico, Toropi comportou-se como tolerante em todos os ensaios. Confirmou-se ainda, que o mesmo possui a inserção do promotor de *TaMATE1B* e possivelmente possui *TaALMT1*, embora não se pode confirmar o tipo de alelo promotor associado a este último gene. Para uma melhor discriminação das linhas endogâmicas é necessário a adição de novos marcadores ao mapa pré-existente, já que não possível associar nenhum QTL a população em estudo.

Constatou-se ainda que devido ao reduzido tamanho da população, as análises de detecção dos loci de interesse foram limitadas. Sugere-se ainda que determinadas RILs possam ter sido aleatoriamente selecionadas durante o processo de avanço das gerações, privando o potencial da população para detecção dos QTL, tanto para a ferrugem da folha quanto para a tolerância ao alumínio.