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BASE GENÉTICA DE LA RESISTENCIA A ROYA DE LA HOJA DE TRIGO EN GERMOPLASMA REGIONAL

por

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RESUMEN

La roya de la hoja (RH), causada por *Puccinia triticina*, es una de las enfermedades más prevalentes en Uruguay y otros países del Cono Sur. A pesar de que se han identificado un gran número de genes mayores de resistencia, su efectividad suele ser poco durable debido a la aparición de nuevas razas virulentas. La resistencia conferida por genes menores de efecto aditivo, denominada resistencia parcial o resistencia de planta adulta (RPA), determina desarrollo lento de la enfermedad y es considerada durable. El conocimiento de la base genética de la resistencia es fundamental para la generación de variedades con resistencia a RH, dado que es importante mantener diversidad en la base de resistencia en el caso de patógenos altamente variables como *P. triticina*. El primer objetivo fue estudiar el efecto y la interacción de los genes RPA *Lr34*, *Lr68* y *Sr2* en líneas BC_1F_6 derivadas de dos materiales adaptados susceptibles (LE2304 y ORL99192) con le fuente de resistencia Parula. El segundo objetivo fue identificar regiones genómicas asociadas a resistencia por medio de mapeo asociativo (MA), utilizando dos poblaciones derivadas de tres fuentes de RPA (Suz6/Opata, Cep8749/Br35 y BPon/PBred/4/R37/GHL21//KAL/BB/3/KLT´S) y dos variedades nacionales susceptibles (INIA Tero e INIA Torcaza). Los resultados del primer objetivo revelaron que el efecto del gen *Lr68* es mayor que el efecto del gen *Lr34*. *Sr2* no redujo la RH pero potenció el efecto de *Lr34* y/o *Lr68*. La mayor reducción de RH se observó en líneas segregantes portadoras de 2-3 genes de RPA. El estudio de MA, utilizando 5222 SNP, reveló 43 SNPs significativos para resistencia a RH en estado de plántula y 19 para resistencia a campo. Los genes *Lr10*, *Lr16* y *Lr34* probablemente estén presentes en las poblaciones, mientras que el QTL en el cromosoma 7B estaría explicado por el gen *Lr68*. Los SNPs en los cromosomas 4A y 5B son QTLs novedosos asociados a resistencia a RH no reportados anteriormente. La información generada en este trabajo podrá ser utilizada para continuar con los esfuerzos de mejoramiento para obtener resistencia durable a RH en Uruguay.

Palabras clave: Resistencia de planta adulta, resistencia parcial, loci de carácter cuantitativo, marcadores moleculares

GENETIC BASIS OF RESISTANCE TO WHEAT LEAF RUST IN REGIONAL GERMPLASM

SUMMARY

Leaf rust (LR), caused by *Puccinia triticina*, is one of the most prevalent diseases in Uruguay and other countries in the Southern Cone. Although several major resistance genes have been identified, their effectiveness is often not durable due to the emergence of new virulent races. The resistance conferred by genes with additive effect, called partial resistance or adult plant resistance (APR), determines slow development of the disease and is considered durable. The knowledge of the genetic basis of the resistance is essential to obtain resistant varieties, since it is important to maintain diversity in the base of resistance in the case of highly variable pathogens as *P. triticina*. The first objective was to study the effect and interaction of APR genes *Lr34*, *Lr68* and *Sr2* in wheat lines derived from crosses between the resistance line Parula and two susceptible adapted materials (LE2304 y ORL99192). The second objective was to identify genomic regions associated with LR resistance through association mapping (AM), using two populations derived from three sources of APR (Suz6/Opata, Cep8749/Br35 y BPon/PBred) and two susceptible varieties (INIA Tero and INIA Torcaza). The results from the first objective revealed that the effect of *Lr68* was higher than the effect of *Lr34*. *Sr2* did not reduce LR but enhanced the effect of *Lr34* and/or *Lr68*. The highest LR reduction was observed in lines with 2-3 APR genes segregating in the studied populations. The AM study, using 5222 SNPs, revealed 43 significant SNPs for seedling resistance and 19 for field resistance. Genes *Lr10*, *Lr16* and *Lr34* were probably present in our populations, while the QTL on chromosome 7B was probably explained by the effect of *Lr68*. The SNPs on chromosome 4A and 5B appear to be novel QTLs for resistance to LR not previously reported. Information from this study could be used to continue the efforts on wheat breeding for durable resistance to LR in Uruguay.

Keywords: adult plant resistance, parcial resistance, quantitative trait loci, molecular markers

1. INTRODUCCIÓN

El trigo (*Triticum aestivum* L.) es uno de los cultivos más importantes a nivel mundial, ocupando el segundo lugar en producción en el año 2011 (FAOSTAT, 2014). El trigo ha sido y es objeto de interés de numerosos programas de mejoramiento genético en todo el mundo, siendo la resistencia a enfermedades, en especial a roya de la hoja, uno de los caracteres de mayor importancia en este proceso. Dicho aspecto es de gran relevancia para lograr los niveles deseados de rendimiento y calidad, minimizando la necesidad de utilizar fungicidas.

La roya de la hoja del trigo (RH, causada por *Puccinia triticina*), es una de las enfermedades más prevalentes en Uruguay y otros países del Cono Sur (Germán *et al.*, 2007). A pesar que se han identificado más de 70 genes mayores de resistencia (McIntosh *et al*., 2013), estos suelen ser efectivos durante un período corto después de ser liberados para su uso comercial debido a la rápida aparición de razas virulentas del patógeno. Al contrario de lo que ha ocurrido con genes mayores, la resistencia conferida por genes menores de efecto aditivo (herencia cuantitativa) o resistencia parcial, también conocida como resistencia de plata adulta (RPA), determina desarrollo lento de la enfermedad y es considerada durable (Singh *et al*., 2011). Hasta el momento se han reportado cuatro genes de RPA para RH (*Lr34*, *Lr46, Lr67* y *Lr68*, Singh, 2012, Lagudah, 2011, Singh *et al*., 2011) y *Sr2* uno de los cuatro genes de RPA para roya del tallo. La asociación de varios de estos genes con resistencia a otras enfermedades tales como el oídio (causado por *Blumeria graminis* f. sp. *tritici*), la roya estriada (causada por *P. striiformis* f. sp. *tritici*) y/o la roya del tallo (causada por *P. graminis* f. sp. *tritici*), hace que la RPA tenga un valor adicional para el mejoramiento por resistencia (Singh *et al.*, 2011). Existen otros genes que confieren esta resistencia que aún no han sido catalogados, como lo indican 80 locus de carácter cuantitativo (QTL, del inglés "*Quantitative trait locus*") reportados, asociados a RPA a RH en la mayoría de los cromosomas, algunos ubicados en regiones donde se encuentran *Lr34*, *Lr46, Lr67* y *Lr68* (Li *et al*., 2014, Herrera-Foessel *et al.*, 2012, Singh, 2012).

El conocimiento de la base genética de la resistencia es fundamental para la generación de variedades de trigo con resistencia genética a RH, dado que además de incorporar genes que confieren RPA es importante mantener diversidad en la base de resistencia en el caso de patógenos altamente variables como *P. triticina*. Para este propósito se debe conocer la identidad de los genes, lo que muchas veces no es posible basándose únicamente en el fenotipo o el conocimiento de la ascendencia de un cultivar o línea. El uso de marcadores moleculares permite identificar, diagnosticar la presencia y seleccionar por genes que confieren resistencia a RH, complementando y acelerando la obtención de información sobre los genes de resistencia presentes en materiales de trigo. En el caso de RPA a RH, cuya expresión es enmascarada por la presencia de resistencia efectiva conferida por genes mayores, el uso de marcadores moleculares es particularmente ventajoso, más aún cuando la resistencia de plántula es frecuente en el germoplasma del Programa de Mejoramiento Genético de Trigo de INIA (PMGT-INIA) (Germán y Kolmer, 2014, Germán y Kolmer, 2012).

El análisis clásico de QTL y más recientemente el Mapeo Asociativo (MA), son ampliamente utilizados para identificar loci que codifican para caracteres complejos. El MA consiste en la identificación de asociaciones estadísticas significativas entre marcadores y fenotipos de interés, considerándose que cada una de éstas es evidencia de un ligamiento entre el marcador y el locus que codifica para dicho fenotipo (Gupta *et al.,* 2005, Jannink y Walsh, 2002). Utilizando esta metodología se han detectado un número importante de regiones cromosómicas asociadas a resistencia a enfermedades, en especial a las royas del trigo (Bhavani *et al*., 2011, Yu *et al*., 2011, Maccaferri *et al*., 2010).

El objetivo de este trabajo fue contribuir al conocimiento de la base genética de RPA a RH e identificar fuentes de resistencia con esta característica para utilizar en el desarrollo de cultivares resistentes. En el primer artículo, a ser presentado a la revista "*Euphytica*", se determinó, mediante el uso de marcadores moleculares, la presencia/ausencia de los alelos favorables para tres genes de RPA en líneas con distinto comportamiento frente a RH, derivadas de cruzamientos de la fuente de

resistencia Parula con dos materiales adaptados susceptibles. Se estimó el efecto de estos genes de resistencia y su combinación bajo condiciones de producción en Uruguay. Esta sección se presenta según el formato exigido por la citada revista en idioma inglés. En el segundo artículo, a ser presentado ante la revista "*Molecular Breeding*", se identificaron regiones genómicas asociadas a resistencia a RH por medio de genética asociativa, en una colección de genotipos derivados de tres líneas previamente caracterizadas como resistentes en planta adulta y dos variedades adaptadas susceptibles. Esta sección se presenta según el formato exigido por la citada revista en idioma inglés.

1.1. LA ESPECIE *Triticum aestivum* L.

El trigo es una especie autógama y hexaploide (2n = 6x = 42, genomas A, B y D), producto de la hibridación de *Triticum turgidum* (genomas A y B) y *Aegilops tauschii* (genoma D). A su vez, *T. turgidum* es producto de una hibridación previa entre *T. urartu* (genoma A) y *A. speltoides* (genoma B) (Dvorák *et al*., 1993, McFadden y Sears, 1946). Si bien el trigo se comporta como diploide por la supresión de apareamiento de cromosomas homeólogos, la característica poliploide de su genoma ha dificultado el desarrollo de mapas genéticos, así como el estudio de caracteres de herencia compleja, ya que las variables agronómicas se ven influenciadas por genes provenientes de tres genomas distintos. A su vez, el genoma de trigo presenta otras características, como su gran tamaño (16Gb), alto número de regiones repetidas (80%) y un 25 a 30 % de sus genes duplicados (Akhunov *et al*., 2003, Dubcovsky *et al*., 1996), que dificultan el desarrollo de análisis genéticos precisos en esta especie. Posee una gran ventaja ya que, al ser una especie autógama, presenta bajos niveles de heterocigocidad y baja proporción de flujo génico (<1 %) debido a un reducido nivel de polinización cruzada (Gustafson *et al*., 2005).

1.2. EL CULTIVO DE TRIGO

El trigo es uno de los principales cultivos a nivel mundial por su importancia para la alimentación humana. Se ubica en el cuarto lugar en producción mundial (607

Mton) respecto a otros cultivos y ocupa el primer lugar en área sembrada. Los principales países productores son China, India, Estados Unidos y la Federación Rusa y en el hemisferio sur Australia, Argentina y Brasil (FAOSTAT, 2014). En Uruguay el trigo ocupa el segundo lugar luego de la soja en área de siembra, con aproximadamente 0.5 millones de hectáreas promedioanuales durante los últimos 5 años (DIEA, 2013). Es el principal cultivo de invierno, y su producción ha aumentado en los últimos años (producción de 1.200 Mton en 2011/12 frente a 143 Mton en la zafra 2001/02) (DIEA, 2013). El rendimiento promedio para el país en los últimos años fue alrededor de los 3.000 kg/ha, a excepción de las zafras 2001/02 y 2012/13 en las cuales apenas se superaron los 2.000 kg/ha, debido al daño causado por condiciones climáticas adversas y Fusariosis de la espiga.

1.3. MEJORAMIENTO DE TRIGO EN URUGUAY

El mejoramiento genético de trigo en Uruguay comenzó en 1912 en Toledo (Canelones), y continuó en 1913 en la Estación Experimental Bañado de Medina (Cerro Largo), bajo la dirección del Dr. Alberto Boerger. A partir de 1914 se instaló en La Estanzuela (LE, Colonia), y se ha mantenido casi sin interrupciones hasta el presente (Luizzi *et al*., 2014). Durante este largo período se han liberado más de 60 variedades al mercado uruguayo, ocupando actualmente entre 25-30% del área (Martín Quincke, comunicación personal, 15 de enero de 2014).

El Programa de Mejoramiento Genético de Trigo de INIA (PMGT-INIA) actualmente inicia el proceso de mejoramiento con aproximadamente 600 cruzamientos. La generación híbrida F_1 es uniforme, mientras que en la generación F_2 se expresan los mayores niveles de heterocigosis. En esta generación se selecciona por caracteres simples, los cuales poseen en general, altos valores de heredabilidad (por ej.: resistencia a algunas enfermedades, altura de planta, ciclo, entre otras características agronómicas de interés). En generaciones intermedias se continúa con este proceso de selección hasta que en la generación F_6 se comienza a seleccionar por rendimiento y calidad de grano, cuando cada línea derivada de una planta F_5 ya posee alto nivel de homocigosis. A partir del siguiente año, se realizan ensayos con

repeticiones en varias localidades del país. Las líneas con mayor rendimiento, calidad adecuada y sin características indeseables son seleccionadas y evaluadas durante tres años en ensayos de la red de Evaluación Nacional de Cultivares conducida por Convenio INIA-INASE, antes de ser liberadas al mercado. Este proceso de selección y los ciclos de multiplicación de semilla hasta niveles en que un cultivar puede llegar a la producción, insume entre 10 y 15 años (Martín Quincke, comunicación personal, 15 de enero de 2014).

El PMGT-INIA tiene como objetivo obtener cultivares que pueden ser utilizados en diferentes sistemas de producción. Se buscan cultivares de ciclo precoz o intermedio para utilización en la secuencia de siembra trigo-soja, o cultivares de ciclo largo para siembras tempranas o para doble propósito (grano y forraje). Los mayores esfuerzos se dirigen a los objetivos primarios de mejoramiento: identificar cultivares que combinen adecuadamente alto potencial de rendimiento y buena calidad molinera y panadera, pero sin descuidar otros factores que deben estar presentes para que los objetivos primarios se expresen. Una de las limitantes más importantes para obtener buenos niveles de rendimiento es la gran presión de enfermedades que afectan al cultivo de trigo, favorecidas principalmente por el clima húmedo y la ocurrencia de precipitaciones durante la primavera. Las enfermedades más relevantes son mancha amarilla (causada por *Drechslera tritici repentis*), Septoriosis de la hoja (causada por *Septoria tritici*), Fusariosis de la espiga (causada principalmente por *Fusarium graminearum)* y RH.

1.4. ROYA DE LA HOJA DEL TRIGO

Puccinia triticina, agente causal de RH de trigo, es un patógeno biotrófico (puede sobrevivir sólo en tejidos vivos del hospedero). La RH es una de las enfermedades más importantes de trigo en todo el mundo, incluyendo Uruguay (Germán *et al*., 2007, Kolmer, 1996). Una alta proporción de la superficie de trigo está sembrada con cultivares con comportamiento inadecuado frente a RH (54% del área de la zafra 2012/13), lo que está asociado a la aparición del patógeno año a año causando epidemias severas. Son necesarias dos o más aplicaciones de funguicidas

para prevenir las pérdidas de rendimiento de grano en materiales susceptibles, que pueden llegar a un 50 % si no se utiliza control químico (Germán *et al.,* 2007). La intensificación de la agricultura contribuyó al incremento en la importancia de las epidemias causadas por P. *triticina*. Por un lado el incremento del área y concentración de cultivos de trigo determina mayor área donde puede multiplicarse el inóculo y sobrevivir durante el verano. Por otro lado, las mejores prácticas culturales utilizadas para lograr mayores rendimientos determinan mayor disponibilidad de nitrógeno y desarrollo vegetativo, lo que también favorece el desarrollo de RH, incrementando el nivel de infección y el daño (Germán *et al.,* 2009).

Durante 1914-1950, el germoplasma resistente a RH se obtuvo de Argentina y Brasil (Germán *et al.,* 2007). Después de 1950 se seleccionaron fuentes de resistencia a RH de colecciones recibidas del Departamento de Agricultura de EE.UU (USDA) y de la Fundación Rockefeller, luego Centro Internacional de Mejoramiento de Maíz y el Trigo (CIMMYT). La forma tradicional para el control de la RH se ha basado en la obtención de cultivares genéticamente resistentes. Debido a su importancia económica, la resistencia a la RH es una prioridad para el PMGT-INIA. Germán y Kolmer (2014, 2012) indicaron que la resistencia a RH en materiales Uruguayos está basada en la presencia de pocos genes mayores y menores. La mayoría de los genes mayores son infectivos cuando están presentes solos y la resistencia depende de diferentes combinaciones de genes mayores o su combinación con genes menores. Considerando el germoplasma que se ha utilizado en el PMGT-INIA y la selección por resistencia a RH en condiciones de campo, es probable que se haya seleccionado por la presencia de genes menores aun cuando estos no habían sido descritos (Germán *et al*., 2009, Germán *et al*., 2007).

1.4.1. Diversidad del patógeno

La población de *P. triticina* está compuesta por razas que difieren en su combinación de avirulencia/virulencia frente a un grupo de genes mayores presentes individualmente en genotipos de un set diferencial. La población de *P. triticina* es

muy diversa a nivel mundial (Huerta-Espino *et al.*, 2011) y en Uruguay, donde se han identificado más de 100 razas diferentes desde 1991. Cada año se reportan en promedio dos razas que no estaban presentes en años anteriores (Silvia Germán, comunicación personal, 15 de enero de 2014). Los mecanismos más importantes de variación del patógeno son la ocurrencia de mutaciones y la migración de razas que se generan generalmente en países de la misma zona epidemiológica (Argentina, Brasil, Paraguay, y zonas bajas de Bolivia) (Germán *et al*., 2007) o eventualmente en regiones más distantes como el caso documentado de migración intercontinental de la raza MCD-10,20 de *P. triticina* desde México hacia el Cono Sur y EUA (Ordoñez *et al.*, 2010). Las nuevas razas virulentas sobre cultivares inicialmente resistentes incrementan en frecuencia debido a la selección del huésped sobre el patógeno, causando niveles de infección y daño crecientes y resultando en cambios de comportamiento de los cultivares. Debido a la variabilidad del patógeno, la obtención de variedades resistentes con genes mayores y combinaciones de estos, no ha sido una solución permanente para controlar la RH. En Uruguay, la resistencia a RH de los cultivares de trigo suele ser efectiva durante tres o cuatro años luego de su liberación (Germán *et al.*, 2007), lo que ha determinado que históricamente la susceptibilidad a RH haya sido la causa más importante de recambio varietal en el país (Luizzi *et al*., 1983).

1.4.2. Resistencia a roya de la hoja en trigo

La resistencia genética es la medida más económica y ambientalmente amigable para controlar la RH, ya que mediante el uso de cultivares resistentes se logra reducir los daños causados por la enfermedad y frenar su diseminación (Johnson, 1981), disminuyendo el número de aplicación de funguicidas). Esta resistencia implica un valor agregado a un germoplasma de características de adaptación, calidad y alto rendimiento, y no implica un costo adicional para los productores. Se han identificado 74 genes de resistencia a RH (*Lr*, del inglés "*Leaf rust*") en trigo (McIntosh *et al*., 2013). La mayoría de los genes *Lr* identificados se expresan desde el estado de plántula, mientras que otros se expresan de manera óptima en estadios avanzados de desarrollo.

Se han descrito varios tipos de resistencia, los cuales han recibido diferentes nombres dependiendo de: tipo de genes involucrados (mayores o menores), estado fenológico donde se expresan (de plántula o de planta adulta), durabilidad de la resistencia (durable o no durable) y velocidad de infección (enroyamiento lento "*slow rusting*" o rápido). Según Van der Plank (1963) los diferentes tipos de resistencia se agrupan en: i) resistencia conferida por genes mayores expresados al estado de plántula y/o planta adulta, de herencia cualitativa y ii) resistencia conferida por genes menores, con mejor expresión en el estado de planta adulta, de herencia cuantitativa. Los genes que se expresan desde el estado de plántula confieren resistencia cualitativa, que es efectiva a lo largo de todo el ciclo de crecimiento del cultivo frente a razas avirulentas. Los genes mayores siguen la relación "gen por gen" propuesta por Flor (1956), producen reacciones de hipersensibilidad y son muy vulnerables a la selección y al aumento en frecuencia de razas virulentas en las poblaciones del patógeno. La mayoría de los 74 genes de resistencia a RH (*Lr*, del inglés "*Leaf rust*") identificados en trigo (McIntosh *et al*., 2013) tienen estas características.

La resistencia conferida por genes menores es llamada de enroyamiento lento (*slow rusting*, Singh, 2012), resistencia parcial (Parlevliet, 1975) o RPA. La RPA ha sido independiente de los cambios frecuentes que ocurren en la población del patógeno porque ha sido efectiva frente a todas las razas conocidas de *P. triticina* por largo tiempo, por lo que se considera duradera (Herrera-Foessel *et al*., 2012, Herrera-Foessel *et al*., 2011, Singh *et al*., 2011, Hiebert *et al*., 2010). Este tipo de resistencia se caracteriza por menor frecuencia de infección, mayor período de latencia, uredinios de menor tamaño y ausencia de hipersensibilidad (Caldwell, 1968). La resistencia es controlada por genes de efecto pequeño y acción aditiva. Se han descrito cuatro genes de RPA a RH (*Lr34*, *Lr46*, *Lr67* y *Lr68*), varios para roya del tallo (*P. graminis* f. sp. *tritici*), siendo uno de ellos el gen *Sr2*, además de los reportados para roya amarilla (*P. striiformis* f. sp. *tritici*) (Rosewarne *et al*., 2013). Este tipo de genes, presentes por si solos no logran expresar buenos niveles de resistencia, sin embargo la combinación de cuatro o cinco genes de RPA, confiere

niveles de resistencia cercanos a la inmunidad (Singh *et al*., 2011). La expresión de genes menores puede variar en diferentes ambientes, por lo que estudios multi-ambientales detallados son relevantes para determinar qué combinaciones específicas de genes son más estables y apropiadas para reducir la RH en cada ambiente en particular (Herrera-Foessel *et al*., 2012, Lillemo *et al*., 2011, Singh y Huerta-Espino, 2003). Una característica notable de la mayoría de estos genes es que poseen efectos pleiotrópicos o están ligados a resistencia para otros patógenos (Singh *et al*., 2011, Lillemo *et al.*, 2008, Spielmeyer *et al.*, 2005, Singh, 1992a[1], Dyck, 1987), característica que los hace óptimos para aplicar en mejoramiento por resistencia a múltiples enfermedades.

1.4.3. Mejoramiento genético por resistencia a roya de la hoja

Tradicionalmente el mejoramiento por resistencia a RH dirigido a obtener cultivares más resistentes se basó en el uso de genes mayores. Estos genes son fáciles de reconocer mediante el fenotipo y fáciles de manipular a la hora de introducirlos en cultivares con buenos potenciales de rendimiento (Lowe *et al*., 2011). Sin embargo, también es posible utilizar con éxito genes de efecto mayor en combinación con genes de RPA para obtener materiales altamente resistentes, debido a que los genes menores interactúan positivamente con los mayores efectivos que determinan tipo de infección intermedio, resultando en mayor nivel de resistencia expresado (Germán y Kolmer, 1992). Los cultivares que poseen combinación de genes mayores y menores se ven menos afectados cuando aparecen nuevas razas virulentas sobre los genes mayores, que los cultivares que sólo poseen genes mayores (Singh *et al*., 2012). El mejoramiento genético para obtener resistencia durable basado en la presencia de genes de RPA es un proceso más lento y complejo que la obtención de resistencia basada en genes mayores, por varias razones: i) existe un número reducido de genes de RPA debidamente caracterizados para piramidar, ii) son difíciles de seleccionar a campo debido al pequeño efecto en disminuir la RH en forma individual, iii) es necesario esperar a estados avanzados del desarrollo para poder seleccionar en base a

[1] La letra a en Singh, 1992a es a efectos de diferenciar citas en el conjunto de citas de esta tesis

este tipo de resistencia, iv) es necesario acumular 4-5 genes para lograr niveles adecuados de resistencia y v) no se cuenta con marcadores moleculares confiables para la mayoría de los genes de RPA (Singh *et al*., 2012).

1.5. MÉTODOS PARA ESTUDIAR LA BASE GENÉTICA DE LA RESISTENCIA A ROYA DE LA HOJA

El éxito a largo plazo del mejoramiento por resistencia a enfermedades depende de la naturaleza del patógeno y la diversidad de su población, así como de la disponibilidad, diversidad y tipo de resistencia genética presente en el hospedero, y de la metodología utilizada para seleccionar por resistencia. La probabilidad de identificar padres resistentes aumenta con la disponibilidad de una metodología de detección confiable y un entorno favorable para el desarrollo de la enfermedad. Dependiendo de la enfermedad y del tipo de resistencia, la metodología puede requerir pruebas de plántula o planta adulta en invernáculo, pruebas a campo o incluso el uso de marcadores moleculares.

1.5.1. Postulación de genes de resistencia en base a razas del patógeno

El objetivo de la postulación de genes es identificar los genes mayores presentes en materiales de trigo (Singh and Rajaram, 1991). La postulación está basada en la comparación del tipo de infección (TI) producidos en líneas isogénicas con un solo gen de resistencia y los materiales en estudio, utilizando razas de *P. triticina* que difieren en su combinación de avirulencia/virulencia. Se parte del conocimiento de la relación hospedero/patógeno que opera en este sistema, en el que los genes de resistencia en el trigo y los genes de avirulencia en *P. triticina* operan complementariamente, de acuerdo a la teoría "gen por gen" formulada por Flor (1956). Para cada gen que condiciona resistencia en el huésped existe un gen específico que condiciona avirulencia en el patógeno. Dependiendo de la combinación de genes en la planta y en el patógeno, la interacción puede resultar en incompatibilidad (reacción de resistencia, cuando la planta posee el gen de resistencia y el patógeno el gen de avirulencia correspondiente) o de compatibilidad

(reacción de susceptibilidad o infección exitosa del patógeno, cuando la planta no posee el gen de resistencia o el patógeno posee el gen de virulencia complementario). Si un hospedero posee dos o más genes de resistencia, basta la presencia de un solo gen de resistencia para el que el patógeno sea avirulento y se exprese una reacción incompatible. Por lo tanto, un aislamiento de *P. triticina* debe poseer todos los genes de virulencia correspondientes a los genes de resistencia presentes en el trigo para causar una reacción compatible. Utilizando este método es posible estudiar la resistencia en plántula de cientos de líneas anualmente, ya que en invernáculo pueden obtenerse resultados en aproximadamente cuatro semanas (Roelfs *et al*., 1992). Sin embargo, hay varias limitaciones para el uso de esta metodología: no es posible utilizarla cuando no están disponibles razas con la combinación de virulencia que determine reacción compatible o de susceptibilidad ni postular la presencia de genes de RPA, debido a que no existe virulencia específica sobre estos genes (Singh, 1992a), y es difícil postular genes cuando hay combinaciones de más de dos genes presentes en los materiales de trigo que se están evaluando.

1.5.2. Postulación de genes de resistencia en base a marcadores genéticos

Otra alternativa de postulación de genes de resistencia es el uso de marcadores genéticos. En general, no representan a genes en sí mismos, sino que actúan como "marcadores" de los mismos, no afectan el rasgo del fenotipo de interés, ya que se encuentran ligados a él o los genes que lo controlan (Collard *et al*., 2005). Dentro de las características que debe poseer un marcador “ideal” se pueden mencionar: identificación directa o al menos simple, neutralidad, bajo costo, codominancia, abundancia y disponibilidad en la especie de interés. Existen tres tipos de marcadores genéticos: i) morfológicos, controlados generalmente por un locus, ellos mismos son rasgos fenotípicos o propiamente caracteres, ii) bioquímicos, incluyen variantes alélicas de enzimas, llamadas isoenzimas y iii) moleculares, marcadores de ADN que revelan variantes en el ADN en determinados sitios del genoma (Collard *et al*., 2005). Los marcadores morfológicos como los bioquímicos presentan las desventajas de que pueden ser limitados en número, estar influenciados por factores ambientales o por el estado de desarrollo de las plantas y son relativamente poco abundantes. Un

ejemplo de marcador morfológico para resistencia a RH es la necrosis de la punta de la hoja (*Ltn* del inglés *Leaf tip necrosis*). Este carácter se expresa en la hoja bandera y está ligado a los genes de RPA a RH *Lr34*, *Lr46*, *Lr67* y *Lr68* (Herrera-Foessel *et al*., 2014, Herrera-Foessel *et al*., 2012, Rosewarne *et al*., 2006, Singh, 1992b), siendo útiles a la hora de seleccionar directamente por este tipo de resistencia sin tener conocimiento de los genes que están involucrados. Los marcadores moleculares se han convertido en los más usados, debido principalmente a su abundancia y a la ausencia de interacción con factores ambientales y con el estado fenológico de la planta (Winter y Kahl, 1995), además permiten estudiar una gran cantidad de materiales en relativamente poco tiempo. Los marcadores moleculares también pueden ser utilizados en la construcción de mapas de ligamiento, evaluación del nivel de diversidad genética dentro del germoplasma e identificación de cultivares (Collard *et al*., 2005).

1.5.3. Estudios de herencia

El análisis genético convencional se utilizó para determinar el número de genes de resistencia presente en materiales de trigo previamente a la disponibilidad de herramientas moleculares. Para llevar adelante este tipo de análisis se realizan cruzamientos entre la fuente de resistencia y un material susceptible. Las plantas F_1 son llevadas a F_2 o retrocruzadas con el padre susceptible para desarrollar líneas BCF_1 (BC del inglés *backcross*). El BC provee un *background* genético más uniforme en las poblaciones segregantes, lo cual es conveniente para realizar estudios a campo. El número de genes se determina en base a la relación entre el número de familias susceptibles:segregantes:resistentes (cruza simple) o susceptible:segregantes (retrocruza). Una vez identificadas las familias que probablemente segregan para un gen de resistencia mayor, éste se puede postular utilizando distintas razas del patógeno o marcadores moleculares.

1.5.4. Confirmación de la identidad de los genes de resistencia

Para demostrar la identidad de los genes presentes es necesario cruzar el material resistente original o líneas resistentes derivadas de los cruzamientos anteriores con una línea que lleve el gen de resistencia postulado (test de alelismo). La ausencia de plantas susceptibles en la generación F_2 con un alto número de individuos, utilizando una raza avirulenta sobre el gen en estudio, confirma la identidad del gen de resistencia presente. Otra alternativa utilizada para confirmar la identidad de genes de resistencia es el uso de marcadores moleculares perfectos, es decir, aquellos que determinan la presencia o ausencia del gen propiamente dicho. La obtención de marcadores perfectos se logra luego de la obtención de líneas mutantes para el gen en estudio y posterior aplicación de herramientas biotecnológicas de clonación y secuenciación para poder aislar al gen. Los marcadores perfectos son una alternativa más rápida frente al test de alelismo a la hora de confirmar la identidad de genes. Sin embargo, no son muchos los genes *Lr* para los cuales se ha logrado obtener marcadores moleculares perfectos. El único ejemplo dentro de los genes de RPA a RH es el caso del gen *Lr34* (Krattinger *et al*., 2009, Lagudah *et al*., 2009). Actualmente se están realizando muchos esfuerzos para incrementar el número de genes de resistencia a enfermedades clonados y secuenciados, tal es el caso de los genes *Lr46, Lr67, Sr2, Sr50*. (Dodds *et al*., 2014).

1.6. MARCADORES MOLECULARES Y SU APLICACIÓN EN EL MEJORAMIENTO GENÉTICO DE TRIGO

Hasta hace pocos años las herramientas disponibles para genotipar el ADN eran los marcadores del tipo RFLPs (polimorfismo en el largo de los fragmentos de restricción), AFLPs (polimorfismo en el largo de los fragmentos amplificados) y SSR (microsatélites). Estos métodos, requieren la inversión de mucho tiempo y recursos, tanto para su desarrollo como para su aplicación en la evaluación de poblaciones, principalmente cuando es necesario evaluar un gran número de marcadores en diversos individuos (Gupta *et al*., 2008). Son efectivos para identificar genes que codifican características simples, no así para el caso de características

complejas, cuya expresión resulta de la interacción de muchos genes, cada uno de ellos en distinta proporción (Buerstmayr *et al*., 2009, Collard *et al*., 2005). Otro tipo de marcadores desarrollados son los SNP (polimorfismos en una sola base). Estos marcadores han cobrado importancia recientemente debido a su abundancia en el genoma y a las tecnologías desarrolladas para su identificación (Gupta *et al*., 2008). Dentro de las nuevas tecnologías que permiten identificar SNPs se encuentran los métodos de preparación de librerías, la secuenciación y el análisis bioinformático para su identificación. Este tipo de secuenciación a gran escala permite identificar un elevado número de marcadores en el genoma, a un costo más reducido, haciendo posible su utilización como herramienta de apoyo a los programas de mejoramiento de cultivos vegetales. El Genotipado por Secuenciación (GBS, del inglés "*Genotyping by Sequencing*"), se clasifica dentro del grupo de métodos de secuenciación de baja cobertura (Davey *et al*., 2011), que permiten secuenciar un gran número de individuos, aunque con una menor cobertura en cada uno de ellos. Una ventaja muy importante de este método es su sencillez y el bajo costo por muestra (Elshire *et al*., 2011). El GBS se ha aplicado con éxito en especies alógamas como maíz (Elshire *et al*., 2011) y eucaliptus (Grattapaglia, 2012), y también en especies autógamas como cebada (Elshire *et al*., 2011) y trigo (Poland, *et al*. 2012).

Con el objetivo de acortar los tiempos que requiere la liberación de nuevas variedades desarrolladas por metodologías de mejoramiento convencional y aumentar la eficiencia de selección en combinación con métodos de avance generacional (dobles haploides, descendencia por semilla única, generaciones de contraestación), la selección asistida por marcadores (SAM) busca inferir información basándose en el ADN del individuo (Heffner *et al*., 2011). El éxito de la selección mediante características genéticas depende en gran medida del número de genes involucrados en el control genético del carácter, de las relaciones inter-alélicas (dominancia o recesividad) y de la influencia del ambiente (Mackay *et al*., 2009). El interés por la aplicación de estos marcadores dentro de un programa de mejoramiento genético, surge por sus diversas aplicaciones como la posibilidad de identificar factores genéticos útiles en poblaciones, introgresar genes de interés en líneas de

mejoramiento, estudiar la interacción genotipo x ambiente, monitorear la diversidad genética e identificar cultivares y/o germoplasma con un mayor potencial agronómico (Stuber *et al*., 1999). La SAM es particularmente útil en el mejoramiento por resistencia debido a que algunos genes de resistencia son difíciles de identificar basándose sólo en el fenotipo o son enmascarados por otros. Conocer la identidad de los genes presentes en el germoplasma del programa de mejoramiento es básico para poder identificar nuevos genes a introducir de forma de incrementar la diversidad de genes de resistencia expuestos a la población del patógeno, evitando redundancias. Existen hoy en día marcadores moleculares disponibles para la gran mayoría de los genes de resistencia a RH de plántula reportados. La SAM viene siendo exitosamente aplicada para introgresar y piramidar genes de efecto mayor (Tyagi *et al*., 2014, Mago *et al*., 2011, Castro *et al*, 2003). Para llevar adelante un programa de SAM es necesaria la identificación previa de las regiones genómicas asociadas a estos caracteres. Existen diversas estrategias para identificar locus de herencia cuantitativa (QTL, del inglés "*Quantitative Trait Locus"*) asociados a caracteres de interés incluyendo poblaciones bi-parentales y poblaciones amplias. Estas estrategias se basan en buscar una asociación estadística entre un genotipo (marcador molecular) y un fenotipo (Abdurakhmonov y Abdukarimov, 2008, Zhu *et al*., 2008, Collard *et al*., 2005, Doerge, 2002).

1.7. MÉTODOS PARA EL ESTUDIO DE ASOCIACIONES ENTRE GENOTIPO Y FENOTIPO

Los estudios de caracteres complejos o estudios de locus de carácter cuantitativo (QTL: región del genoma responsable de la variación de un carácter cuantitativo), se basan en el principio de la detección de una asociación estadística entre el fenotipo y el genotipo, y tienen como objetivo principal identificar el número, la posición y el efecto, de los genes involucrados en un carácter de herencia cuantitativa (Collard *et al*., 2005). Las características de mayor importancia agronómica, como rendimiento, calidad y resistencia a algunas enfermedades, presentan en general herencia compleja o cuantitativa, determinada por numerosos genes de efecto aditivo que contribuyen diferencialmente en la determinación del

fenotipo (Mackay *et al*., 2009). La resistencia a enfermedades generalmente está basada en pocos genes de efecto mayor y varios genes de efecto menor, sobre el carácter estudiado (Kearsey y Farquhar, 1998, Robertson, 1967).

1.7.1. Análisis de QTL en poblaciones biparentales

El análisis clásico de QTL se lleva a cabo en poblaciones de mapeo biparentales que permitan identificar marcadores moleculares que segreguen junto a los QTL de interés, es decir, que se encuentren en mayor proporción en los individuos con el fenotipo de interés (Huang *et al.*, 2011). Existen tres métodos ampliamente utilizados para la detección de QTL: i) análisis de marcador individual, ii) mapeo por intervalo simple (SIM) y iii) mapeo por intervalo compuesto (CIM) (Collard *et al*., 2005). Una de las limitantes de este tipo de análisis es la necesidad de contar con poblaciones biparentales, segregantes y balanceadas, lo cual facilita la detección de diferencias debido al carácter en estudio (Huang *et al*., 2011). El hecho de necesitar una población segregante proveniente de una cruza definida limita la base genética a las diferencias entre los parentales. Por otra parte la tendencia a utilizar progenitores con claras diferencias para los caracteres de estudio como forma de maximizar las posibilidades de detección de componentes genéticos, significa que la base genética es poco representativa del germoplasma en uso en los programas de mejoramiento. Otra limitante es la necesidad de contar con un mapa de ligamiento para la población a analizar (Hori *et al*., 2003). Para su aplicación a un programa de mejoramiento, los QTL conocidos deben ser previamente validados en germoplasma local (Ordas *et al*., 2010, Parisseaux y Bernardo, 2004). Esta metodología sigue siendo ampliamente utilizada en el estudio de resistencia a las royas en trigo (Rosewarne *et al.*, 2013; Singh *et al*., 2013b[2], Ren *et al*., 2012).

1.7.2. Análisis de QTL por mapeo asociativo

El mapeo asociativo (MA), también llamado análisis por desequilibrio de ligamiento (DL), surge como metodología alternativa al análisis de QTL basado en

[2] La letra b en Singh *et al.*, 2013b es a efectos de diferenciar citas en el conjunto de citas de esta tesis

poblaciones biparentales. El MA consiste en la identificación de QTL que codifican para caracteres fenotípicos de interés mediante asociaciones entre los marcadores y dichos caracteres, basados en el DL. El DL es el grado de asociación no aleatoria entre alelos de distintos loci (Zhu *et al*., 2008), es decir la proporción de gametos que no segregan al azar. La técnica fue desarrollada en especies donde el desarrollo de poblaciones segregantes balanceadas presenta dificultades de diverso tipo e inicialmente dirigida a caracteres de efectos relativamente mayores (Gupta *et al*., 2005, Flint-Garcia *et al*., 2003).

En la última década el desarrollo de estudios de genes candidatos y de grandes plataformas genotípicas han promovido al MA como una alternativa viable al análisis de QTL utilizando poblaciones bi-parentales (Zhu *et al*., 2008). Existen numerosos reportes sobre DL para trigo (Chao, 2007, Maccaferri *et al*., 2005). Recientemente, con el desarrollo de nuevas tecnologías para la obtención de altas densidades de marcadores distribuidas a lo largo del genoma, se comenzaron a realizar estudios de asociación conocidos como "*Genome Wide Asociation Mapping*" (GWAS). Esta metodología permite realizar asociaciones marcador–fenotipo simultáneamente en todo el genoma mediante aplicación de modelos mixtos. Sin embargo, en GWAS se desconoce la historia de recombinación por lo que pueden ocurrir asociaciones espurias o falsos positivos debido a diversas causas evolutivas (Jannink *et al*., 2001). La mayor causa de falsos positivos es la correlación genética entre los individuos generada por el relacionamiento filial. Se han desarrollado diferentes estrategias para considerar el relacionamiento genético ya sea a través de estructurar a la población e imponer agrupamientos en el modelo estadístico (Pritchard *et al*., 2000) o a través de la utilización de información de relacionamiento genético entre individuos con el uso de la coancestría en modelos mixtos (Malosetti *et al*., 2007, Yu *et al*., 2006). Una propuesta intermedia es la utilización de componentes principales propuesto por Patterson *et al*. (2006). Más allá de la gran variedad de modelos mixtos que se han propuesto, se destaca que lo más importante no es el método a utilizar, sino que el método utilizado controle de manera eficiente la estructura poblacional (Gutiérrez *et al*., 2011). La utilización de modelos mixtos permite estimaciones adecuadas incluso

cuando el tamaño poblacional es pequeño, siempre y cuando el número de marcadores sea elevado y el tamaño poblacional lo suficientemente grande como para estimar la estructura de la población y el grado de relacionamiento de los individuos (Yu *et al*., 2006). Entre las ventajas que plantea el MA para plantas en comparación al análisis de QTL basado en poblaciones biparentales se encuentran: i) la utilización de una base genética amplia, ii) posibilidad de incluir un *background* genético más representativo de un programa de mejora sin necesidad de la utilización de una población segregante ni que esta sea balanceada, iii) elevada robustez en los resultados (debido a la utilización de individuos con alto número de meiosis en su desarrollo y a la posibilidad de analizar grandes tamaños poblacionales), iv) mayor análisis por locus (en poblaciones biparentales sólo se cuenta con 2 alelos por locus) y v) la posibilidad de utilizar información fenotípica previamente colectada (Kraakman *et al*., 2004). Esta metodología ha sido utilizada en el estudio de la base genética de la resistencia a las royas del trigo (Yu *et al*., 2012, Yu *et al*., 2011, Maccaferri *et al*., 2010, Crossa *et al.*, 2007).

1.7.3. Mapeo asociativo "anidado"

El mapeo asociativo "anidado" o NAM (del inglés "*Nested Association Mapping*") es un nuevo enfoque utilizado para el mapeo de genes que afectan caracteres complejos, en donde el poder estadístico del análisis de QTL en poblaciones biparentales se combina con la alta resolución cromosómica del MA (Buckler *et al*., 2009). El término "anidado" explica el tipo de población que se utiliza, poblaciones multi-familia, donde todos los individuos de la población multi-familia comparten un mismo padre, pero cada sub-población posee un padre alterno diferente. El NAM fue creado con el fin de combinar las ventajas y eliminar las desventajas de los dos métodos tradicionales para la identificación de QTL: análisis de QTL con poblaciones biparentales y MA. El NAM toma ventaja de ambos eventos de recombinación, recientes e históricos, requiere de una menor densidad de marcadores, posee alta riqueza de alelos, alta resolución de mapeo y elevado poder estadístico (Yu *et al.*, 2008).

2. EFFECTS AND INTERACTIONS OF GENES *Lr34*, *Lr68* AND *Sr2* ON WHEAT LEAF RUST ADULT PLANT RESISTANCE IN URUGUAY

2.1. SUMMARY

Achieving durable resistance to leaf rust (LR), caused by *Puccinia triticina*, in wheat has been one of the main objectives of breeding programs. Durability of LR resistance is considered to be associated with adult plant resistance (APR) genes that are quantitatively inherited whose expression is largely influenced by environment. Our objective was to study the effects and interactions of APR genes *Lr34*, *Lr68* and *Sr2* on LR response in Uruguay using two BC_1F_6 populations ('LE2304*2/Parula' and 'ORL99192*2/Parula'). The experimental material was screened for LR in three artificially inoculated environments (La Estanzuela 2012 and 2013 and Young 2012). Linked molecular markers were used to detect the presence of the genes conferring resistance to LR. Gene *Lr34* was fixed in the 'LE2304*2/Parula' population. *Lr68+Sr2* explained 79% of the AUDPC LR reduction (ALRR), while the presence of *Lr68* alone resulted in 51% ALRR. In the 'ORL99192*2/Parula' population, the combined effect of *Lr34+Lr68+Sr2* led to the highest ALRR (73%). The effect of *Lr34* individually explained only 14% ALRR, whereas *Lr68* alone conferred a higher level of resistance (50% ALRR). *Sr2* alone did not reduce LR AUDPC but enhanced the effect of either *Lr34* or *Lr68*. Unlike previous reports the effect of *Lr68* on LR in Uruguay was stronger than the effect of *Lr34*.

Keywords: Durable resistance, Additive resistance, *Puccinia triticina*, Slow rusting, *Triticum aestivum*

2.2. RESUMEN

Lograr resistencia duradera a roya (RH, causada por *Puccinia triticina*) en trigo ha sido un objetivo importante en los programas de mejoramiento. La durabilidad de la resistencia a RH se considera que está asociada a genes de resistencia de planta adulta (RPA). La expresión de los genes RPA puede verse afectada por el ambiente y por el *background* genético. Nuestro objetivo fue estudiar el efecto y la interacción de los genes de APR *Lr34*, *Lr68* y *Sr2* sobre la respuesta a RH en Uruguay utilizando dos poblaciones BC_1F_6 ('LE2304*2/Parula' y 'ORL99192*2/Parula'). La RH se evaluó en tres ambientes inoculados artificialmente (La Estanzuela 2012 y 2013 y Young 2012). Para detectar la presencia de los genes se utilizaron marcadores moleculares ligados. En la población 'LE2304*2/Parula' *Lr34* se presentó fijo. *Lr68+Sr2* explicó una reducción del AUDPC de RH (ALRR) del 79%, mientras que la presencia individual de *Lr68* resultó en un 51% de ALRR. En la población 'ORL99192*2/Parula' el efecto combinado de *Lr34+Lr68+Sr2* condujo a la más alta ALRR (73%). El efecto individual de *Lr34* explicó un ALRR del 14%, mientras que *Lr68* aportó un nivel mayor de resistencia (50% de ALRR). *Sr2* solo no redujo el nivel de RH pero potenció el efecto de *Lr34* y/o *Lr68*. A diferencia de reportes anteriores, el efecto de *Lr68* sobre RH en Uruguay fue mayor que el efecto de *Lr34*.

Palabras claves: Resistencia durable, Resistencia aditiva, *Puccinia triticina*, enrroyamiento lento, *Triticum aestivum*

2.3. INTRODUCTION

Leaf rust (LR, caused by *Puccinia triticina*) resistance genes in wheat (*Triticum aestivum* L.) may express a resistant phenotype to LR infection at different plant developmental stages (McIntosh et al. 1995). Seedling resistance genes, often called major resistance genes, confer resistance at both the seedling and adult plant stages, exhibit phenotypes of major effect, express a hypersensitive reaction (HR), and are often race-specific. Other resistance genes that are commonly detected at post-seedling stages can be further divided into two categories; those with similar characteristics to seedling resistance genes in terms of effect, expression of HR, and race specificity, and those that are quantitatively inherited with partial effects on disease severity (DS), do not express HR, and are race non-specific. These genes are commonly known as adult plant resistance (APR) genes, also called minor genes (Lagudah 2011; Singh et al. 2011). The short life span of deployed major resistance genes due to the emergence and/or increase of virulent pathogen races has been a continuous problem and has caused an increased interest in quantitative partial resistance, here referred to as APR or durable resistance. APR is usually associated with susceptibility at the seedling stage and slow disease development on adult plants (slow rusting). When present singly, APR genes do not confer adequate resistance especially under high disease pressure; however, combinations of four or five genes often result in near immunity (Singh et al. 2011).

Four LR APR genes (*Lr34*, *Lr46*, *Lr67* and *Lr68*) and several stem rust (caused by *P. graminis* f. sp. *tritici*) APR genes, including *Sr2*, have been described. As these genes have been effective for a long period of time, their resistance has been considered durable (Hiebert et al. 2010; Herrera-Foessel et al. 2011; Singh et al. 2011; Herrera-Foessel et al. 2012). The gene *Lr34*, located on chromosome arm 7DS, was first described in the Brazilian cultivar Frontana (Dyck et al. 1966) and was later traced to the Italian variety 'Mentana' (Kolmer et al. 2008). This gene also confers resistance to stripe rust (*Yr18*, caused by *P. striiformis* f. sp. *tritici*; Singh 1992a), stem rust (*Sr57*, Singh et al. 2012), powdery mildew (*Pm38*, caused by *Blumeria graminis* f. sp *tritici*, Spielmeyer et al. 2005; Lillemo et al. 2008) and spot blotch

(*Sb1*, caused by *Cochliobolus sativus*, Singh 2012; Lillemo et al. 2013). *Lr34* is also associated with a premature senescence of the leaf tip in some environments, commonly referred to as leaf tip necrosis (LTN, *Ltn1*) which is enhanced under high LR pressure (Singh 1992b; Krattinger et al. 2009). *Lr34* is the only APR gene cloned, and a putative function for the gene has been suggested, which is different from the major race specific genes (Krattinger et al. 2009).

Another example of a LTN-associated APR gene is *Lr46*, mapped to chromosome 1BL (*Ltn2*; William et al. 2003; Rosewarne et al. 2006), first described by Singh et al. (1998) in cultivar Pavon 76. The pleiotropic action of *Lr46* on response to stripe rust (*Yr29*), stem rust (*Sr58*) and powdery mildew (*Pm39*) also gives this gene the additional value of its broad-spectrum resistance (William et al. 2003; Lillemo et al. 2008; Mago et al. 2011; Singh et al. 2013a[3]). *Lr67* on chromosome arm 4DL, was described in line RL6077 (Hiebert et al. 2010; Herrera-Foessel et al. 2011) but its frequency in both wheat germplasm and deployed varieties is not known. Similarly, *Lr67* is also associated with LTN (*Ltn3*) and confers resistance to multiple diseases, including stripe rust (*Yr46*), stem rust (*Sr55*) and powdery mildew (*Pm46*) (Singh et al. 2013a; Herrera-Foessel et al. 2014). The most recently named LR APR gene is *Lr68* (Herrera-Foessel et al. 2012) located on chromosome 7BL (William et al. 1997), was first described in the CIMMYT spring bread wheat Parula, and traced back to the Brazilian cultivar Frontana.

The stem rust APR gene *Sr2*, located on the short arm of chromosome 3B, was originally transferred from tetraploid Yaroslav emmer (*T. dicoccum*) to the susceptible bread wheat 'Marquis' in the 1920s (McFadden 1930). Traditional breeding to improve resistance with *Sr2* was difficult both because of its recessive nature and the variability on its phenotype, which was influenced by the genetic background and the environment. *Sr2* is closely linked to *Yr30*, a minor APR gene that confers slow rusting to stripe rust, to *Lr27*, a major seedling LR resistance gene complementary to *Lr31* (Singh and McIntosh 1984) and to pseudo-black-chaff

[3] La letra a en Singh et al, 2013a es a efectos de diferenciar citas en el conjunto de citas de esta tesis

(*Pbc1*) (Mago et al. 2011). Even when *Sr2* genomic region has been implicated in resistance to LR (Bariana et al. 2007), it has not been clarified if this effect is only due to *Lr27* or another APR gene may be involved, as suggested by the pleiotropic action of most slow rusting resistance genes.

The line Parula, developed at CIMMYT in 1981, exhibits high levels of APR to LR worldwide (Singh et al. 2011). The genes *Lr34*, *Lr46* and *Lr68* (William et al. 1997; William et al. 2007; Herrera-Foessel et al. 2009) contribute to LR resistance in Parula, which also has *Lr13* (ineffective in Uruguay) and the stem rust resistance APR gene *Sr2* (Singh and Rajaram 1992; Singh et al. 2011). Despite the fact that Parula LR resistance is stable across environments; a clear environmental effect on the expression of specific components of its resistance has been reported (Germán et al. 2010). Further work demonstrated that in Argentina and Uruguay the APR gene *Lr68* had a stronger phenotypic effect than *Lr34* and *Lr46* did not have a significant effect in reducing LR severity, while the phenotypic effect of *Lr68* was smaller than those of *Lr34* and *Lr46* in Mexico (Lillemo et al. 2011). Also Herrera-Foessel et al. (2012) confirmed that the effect of *Lr68* was smaller than the effect of *Lr34* and *Lr46* under Mexican conditions. Therefore, the expression of the APR genes under different environments deserve further study to determine which specific gene combinations might be appropriate to reduce LR in different target environments.

The main objective of the present study was to investigate the relative effects and interactions of *Lr34*, *Lr68* and *Sr2* on leaf rust severity in Uruguay, in two BC_1F_6 populations ('LE2304*2/Parula' and 'ORL99192*2/Parula') using closely linked molecular markers for these genes in order to identify their presence/absence in each genotype.

2.4. MATERIALS AND METHODS

2.4.1. Plant materials

Two susceptible parents were chosen based on previous high LR field infections in Uruguay: LE2304 (LI 107/C-CH-91-1642), an adapted high grain

yielding genotype selected by the INIA-Uruguay Wheat Breeding Program with high quality and resistance to stem rust, and ORL99192 (PF9099//OR1/GRANITO), a Brazilian genotype selected by the OR-Sementes program with resistance to Fusarium head blight. Parula (FKN/3/2*Frontana//Kenya 350 AD.9C.2/Gabo 55/4/Bluebird/Chanate) was crossed and backcrossed to LE2304 and ORL99192 and 74 BC_1F_6 lines from LE2304*2/Parula (wheat population one, WP1) and 69 BC_1F_6 lines from ORL99192*2/Parula (wheat Population two, WP2), were developed. Preliminary phenotypic evaluation of both populations was conducted at LE during 2011. The lines were selected to represent the full range of observed LR field reactions.

2.4.2. Leaf rust evaluation in the field

Field experiments were conducted at two locations in Uruguay, distant approximately 200 km apart: La Estanzuela (LE: latitude 34.3° S, longitude 57.7° W, elevation 70 masl) during 2012 and 2013 and Young (Y: latitude 32.7° S, longitude 57.6° W, elevation 76 masl) during 2012. The experimental design consisted of an augmented incomplete block design with two replications at each environment with five repeated checks (including the parental lines and the susceptible lines Avocet-S and Thatcher) and the 143 BC_1F_6 lines. Approximately 4-5 g seeds (60-70 plants) of each line were hand-sown in 1 m rows in late July. Spreader rows of LE2304 and ORL99192 were planted perpendicular to all plots to ensure the presence and even distribution of race TFT-10,20 (avirulence/virulence formula *Lr9, 16, 18, 19, 21, 25, 28, 29, 32, 33, 36, 39, 47, 51, 52/1, 2a, 2b, 2c, 3, 3bg, 3ka, 10, 11, 12, 13, 14a, 14b, 15, 17, 20, 23, 24, 26, 27+31, 30, 38, 44, B*) used for artificial inoculation. The race TFT-10,20 is virulent to the three parents at the seedling stage and to *Lr14b*, linked to *Lr68* on chromosome 7BL (Herrera-Foessel et al. 2012) and to *Lr27+31*. This race is also virulent to *Lr13* (present in Parula, Singh and Rajaram, 1992) at the adult plant stage. Inoculum of TFT-10,20 was increased on the susceptible line Little Club as described by Germán and Kolmer (2012). Field inoculations were repeated four times with a suspension of inoculum in lightweight mineral oil Soltrol 170 (Phillips Petroleum Co., Borger, TX), starting about eight weeks after sowing. Four disease

severity (DS) scores were evaluated at 7 to 14 day intervals, using the modified Cobb Scale (Peterson et al. 1948). Area under the disease progress curve (AUDPC) was calculated based on DS values according to Maccaferri et al. (2010).

2.4.3. Leaf rust evaluation in the greenhouse

Seedling tests were conducted to ensure that all lines were susceptible in the seedling stage, so no main effect of resistance genes in field evaluation. Seedling tests in the greenhouse were carried out during 2012 at INIA La Estanzuela Experimental Station, with the same *P. triticina* race used in the field to confirm the susceptibility of all BC_1F_6 lines. Near isogenic lines carrying single seedling resistance genes in Thatcher background were also included in the seedling tests. Fully expanded leaves (8-10 days after planting) were inoculated by spraying urediniospores suspended in Soltrol 170 incubated in a dew chamber overnight and then kept in the greenhouse. Infection types (IT) were recorded 12 days after inoculation using the 0–4 scale as described in Roelfs et al. (1992). IT '3' or higher was considered susceptible.

2.4.4. Molecular analysis

The presence of the APR genes in the parents and 143 BC_1F_6 lines was postulated using the following flanking markers: *csLV34 + LR34PLUSR* for *Lr34* (Lagudah et al. 2009), this is the perfect marker for *Lr34*, located at 0.0cM of the gene. For *Lr68* we used the marker *cs7BLNRR*, located at 0.8cM of the gene (Herrera-Foessel et al. 2012), and the marker *csSr2* located at 1.2cM of the gene *Sr2* (Mago et al. 2010). DNA extractions using the CTAB method, PCR amplifications and identification of marker alleles associated with APR genes by 12% acrylamide (29:1) gel electrophoresis following CIMMYT protocols were used. Bands were detected by silver staining (CIMMYT 2005). As Parula harbors *Lr34*, *Lr68* and *Sr2*, it was considered as a positive control for the expected marker allele sizes.

2.4.5. Statistical analysis

Leaf rust AUDPC values were analysed using the following linear model:

$$Y_{ijklm} = G_i + E_j + GE_{(ij)} + R_{k(j)} + B_{l(jk)} + \beta C + \varepsilon_{ijkl}$$

where, Y: LR AUDPC values, G: effect of *i*-th genotype, E: effect of *j*-th location, GE: interaction between the *i*-th genotype and *j*-th location, R: replication within location, B: incomplete block within location, C: days to heading, used as a covariate, β: regression parameter and ε: experimental error with $N(0, \sigma^2_\varepsilon)$. Days to heading was used as a fixed effect covariate in the model to eliminate the noise in DS caused by differences in maturity between lines as there were sizeable differences in days to heading (from 87 to 118 days) among the lines. Although the effect of GE interaction was significant (results not shown), this was disregarded and included in the experimental residuals since we were interested in the expression of resistance over environment and climate variation present in Uruguay.

The significance of principal and interaction effects of individual genes on LR responses was tested using the following linear model:

$$Y_{ijklmno} = A_i + B_j + C_k + AB_{l(ij)} + AC_{m(ik)} + BC_{n(jk)} + ABC_{o(ijk)} + \varepsilon_{ijklmno}$$

where, Y: adjusted LR AUDPC values, A: principal effect of *Lr34*, B: principal effect of *Lr68*, C: principal effect of *Sr2*, AB: interaction effect of *Lr34* and *Lr68*, AC: interaction effect of *Lr34* and *Sr2*, BC: interaction effect of *Lr68* and *Sr2*, ABC: interaction effect of *Lr34*, *Lr68* and *Sr2* and ε: experimental error with $N(0, \sigma^2_\varepsilon)$. All the effects were considered fixed.

Contrasts were used to compare the average LR AUDPC adjusted values of lines with different gene combinations within each population. The significance threshold level was set as $\alpha \leq 0.05$. Statistical analyses were conducted using the *lme4* package (Bates et al. 2009) in R Software (R Development Core Team, 2010).

2.5. RESULTS

At the seedling stage, Parula, LE2304, ORL99192 and most of the BC_1F_6 lines from WP1 and WP2 were susceptible to *P. triticina* race TFT-10,20. However, four lines showed intermediate responses (IT=2). The susceptible checks (Avocet-S and Thatcher) had high AUDPC values as well as high final LR DS in the three field experiments (Table 1) indicating adequate development of LR epidemics throughout the experiments. The highest score for DS occurred at Y-2012 and the lowest at LE-2013 (Table 1). Parula showed high levels of resistance (low LR AUDPC) with final DS ranging between 3 and 10%. LE2304 had an intermediate level of LR and final severity ranging between 30 and 70%, whereas ORL99192 was the most susceptible parent, with an intermediate to high LR AUDPC and final LR severity between 60 and 100%. The average LR AUDPC of the BC_1F_6 lines derived from WP1 was lower than the average AUDPC of BC_1F_6 lines derived from WP2 (Table 1). LR AUDPC of both BC_1F_6 populations was continuously distributed (showed a wide range of values) (Fig. 1). The frequency distribution of LR AUDPC scores of WP1 lines was skewed towards low LR AUDPC values, whereas in the WP2 the frequency of lines with intermediate to high LR AUDPC was higher (Fig.1).

Table 1 Average leaf rust AUDPC and final disease severity (DS) of the susceptible checks, three parents and two wheat populations: WP1 (LE2304*2/Parula) and WP2 (ORL99192*2/Parula) in La Estanzuela 2012 (LE-2012), Young 2012 (Y-2012) and La Estanzuela 2013 (LE-2013)

	LE-2012		Y-2012		LE-2013	
	DS[§]	AUDPC[¶]	DS	AUDPC	DS	AUDPC
Susceptible checks						
Avocet-S	99	5516	99	6478	99	4527
Thatcher	92	4644	86	4356	90	5090
Parents						
Parula	4	208	5	703	3	45
LE2304	48	1404	68	3659	43	1343
ORL99192	98	3062	88	4954	88	2405
Wheat Populations						
WP1	24	658	37	1991	24	471
WP2	71	1811	72	3464	65	1318

[§]Mean final disease severity
[¶]Mean area under the disease progress curve

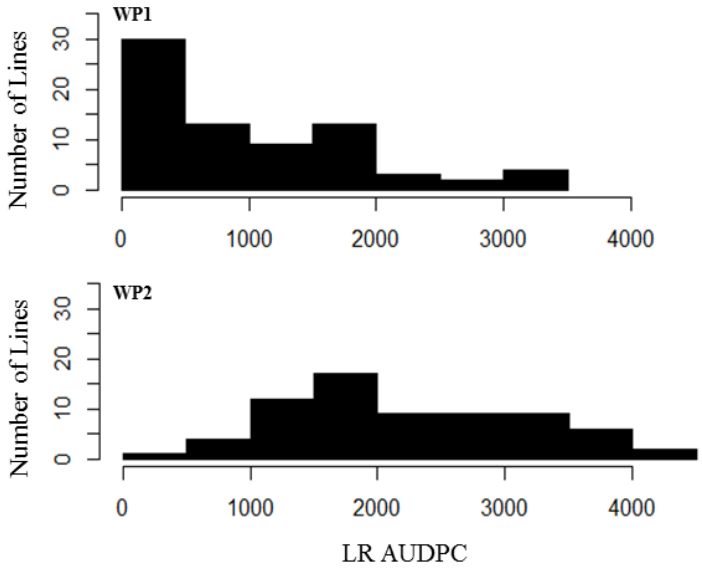

Figure 1 Frequency distributions of leaf rust AUDPC values of 74 BC_1F_6 lines from wheat population one (WP1, LE2304*2/Parula) and 69 BC_1F_6 lines from what population two (WP2, ORL99192*2/Parula). AUDPC: area under the disease progress curve across the three environments.

According to the presence/absence of the molecular markers in each parent, the markers for *Lr68* and *Sr2* were used to screen both populations, while the marker for *Lr34* was used only on the ORL99192*2/Parula population. LE2304 had the favorable allele for *Lr34* and negative alleles for *Lr68* and *Sr2* so the effects of the polymorphic *Lr68* and *Sr2* alleles were studied in the WP1. ORL99192 had negative alleles for *Lr34*, *Lr68* and *Sr2* and therefore the effects of the three genes were studied in the WP2. Four genotypic classes were identified in WP1 (Fig. 2) with one to 30 lines per class while eight genotypic classes were identified in WP2 with three to 19 lines per class (Fig. 3). An ANOVA was carried out to test the principal effects of the single genes and their interactions on the LR AUDPC values. Each of the three genes had significant principal effects in the populations, *Lr68* and *Sr2* in WP1 and *Lr34*, *Lr68* and *Sr2* in WP2 (Table 2). In the WP1, *Lr68+Sr2* combination had non-significant interaction effect on LR AUDPC, whereas in WP2 the interaction effects of *Lr34+Sr2* and *Lr68+Sr2* were significant.

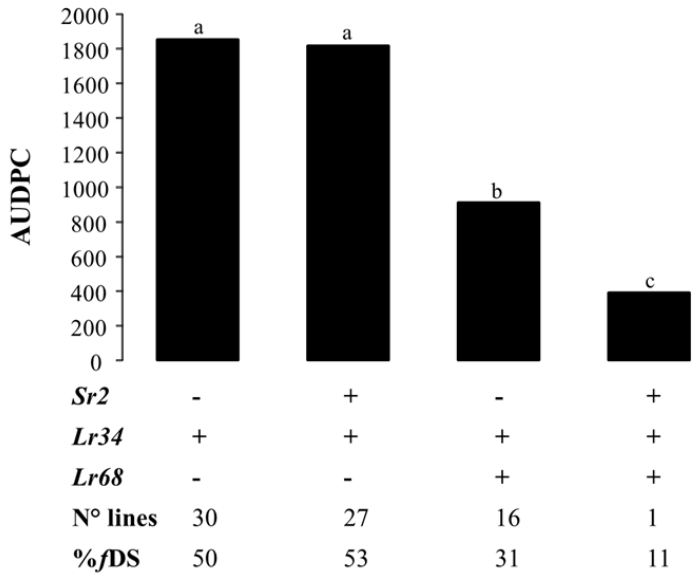

Figure 2 Mean leaf rust AUDPC of 74 BC_1F_6 lines from wheat population one (WP1, LE2304*2/Parula) and number of lines with different gene combinations. AUDPC: area under the disease progress curve. %*f*DS: final disease severity. Bars with different letters are significantly different at $P \leq 0.05$. +: positive allele, -: negative allele. The positive allele for *Lr34* was fixed in this population.

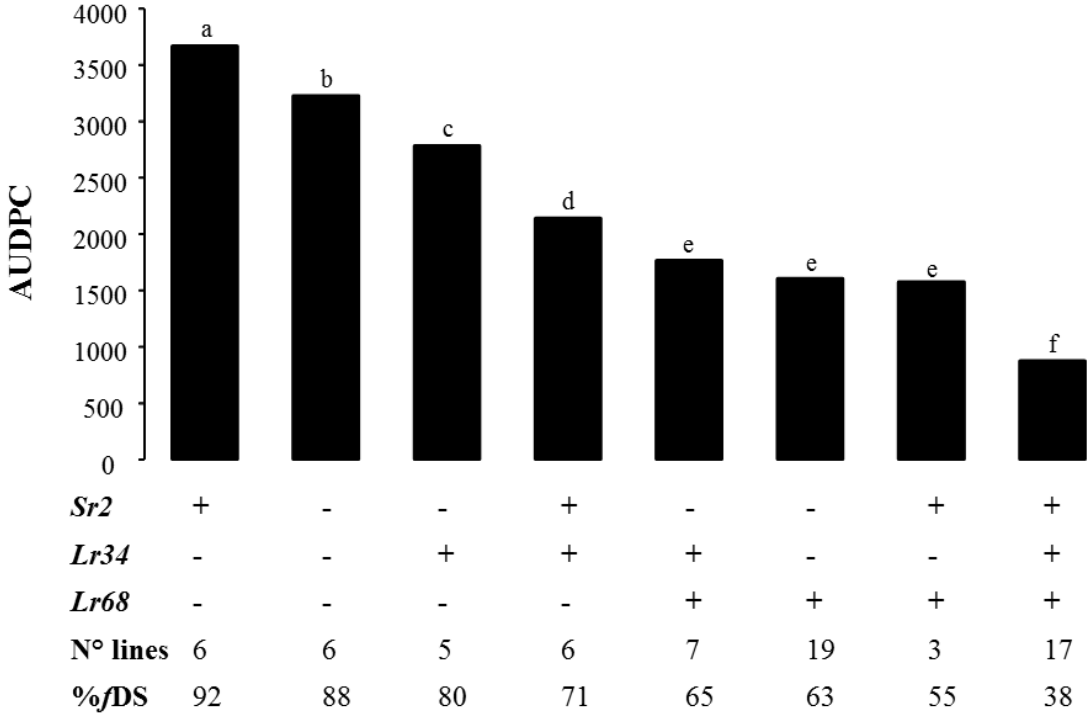

Figure 3 Mean leaf rust AUDPC of 69 BC_1F_6 lines from wheat population two (WP2, ORL99192*2/Parula) and number of lines with different gene combinations. AUDPC: area under the disease progress curve. %*f*DS: final disease severity. Bars with different letters are significantly different at $P \leq 0.05$. +: positive allele, -: negative allele

Table 2 Analysis of variance for principal and interaction effects of *Lr34*, *Lr68* and *Sr2* in wheat population one (WP1, LE2304*2/Parula) and wheat population two (WP2, ORL99192*2/Parula)

	WP1				WP2			
	Df	Mean sq	Pr(>F)		Df	Mean sq	Pr(>F)	
Lr34					1	2697284	0.0028	**
Lr68	1	24383811	5.47E-12	***	1	36803007	< 2e-16	***
Sr2	1	2468037	0.0071	**	1	1012247	0.0033	**
Lr34:Lr68§					1	363016	0.2572	
Lr34:Sr2					1	2168032	0.0069	**
Lr68:Sr2	1	198019	0.4578		1	48093	0.0487	*
Lr34:Lr68:Sr2					1	271081	0.3267	
Residuals	70	355277			61	277519		

†Df, degrees of freedom
§ The symbol : indicates interaction
*** P<0.001 ** P<0.01 * P<0.05

To estimate the effects of individual LR APR genes as well as their interactions on LR development, the average LR AUDPC of each class carrying different gene combinations from each BC_1F_6 population was compared with those of the corresponding 'no genes' class (class with no positive allele markers detected). In WP1, in which *Lr34* was fixed, *Sr2* alone had no effect on LR response, the presence of *Lr68* alone caused an LR AUDPC reduction (ALRR) of 51% and the combination of *Lr68*+*Sr2* resulted in an ALRR of 79% (Fig. 2). The BC_1F_6 lines from WP2 carrying only *Sr2* had a significantly higher LR AUDPC than the 'no genes' class whereas all the remaining classes carrying either single genes or gene combinations had lower LR AUDPC than the 'no genes' class (Fig. 3). The presence of *Lr34* alone caused an ALRR of 14% whereas *Lr68* alone had a higher ALRR of 50%. LR on lines with *Lr68* alone did not differ from lines with *Lr68* combined with either *Lr34* or *Sr2*. The significant interaction effect of *Sr2* and *Lr34* led to higher levels of resistance in lines with this gene combination (34% ALRR) than lines carrying the

genes independently. The interaction effect of *Sr2* and *Lr68* were not as clearly expressed, since similar ALRR were observed for lines with *Lr68* singly (50%) and lines with the *Lr68+Sr2* combination (51%). The highest ALRR (73%) was observed in six WP2 derived BC_1F_6 lines with *Lr34+Lr68+Sr2* combination.

2.6. DISCUSSION

The uniform and intermediate to high levels of LR infection with even distribution observed in all experiments yielded phenotypic data that allowed differentiating the effects of *Lr34*, *Lr68* and *Sr2* under wheat growing conditions in Uruguay. The continuous distribution of LR AUDPC values observed for both populations (Fig. 1) confirmed that the high resistance in Parula is conferred by additive genes (Herrera-Foessel et al. 2012). Although four lines, two of WP1 and two of WP2, showed an intermediate score (IT=2) at seedling stage, the low IT was not associated to any particular gene class. This was probably due to a misclassification and was disregarded.

Among the APR genes present in Parula (*Lr34*, *Lr46*, *Lr68* and *Sr2*, Singh et al. 2011), we were unable to study the effect of *Lr46* on LR response because the molecular markers *csLV46G22* and *csLV46* (Lagudah E. pers comm) used to detect the presence of this gene were not polymorphic between the parents of the two studied populations. Additional work on *Lr46* using other populations or genetic stocks will be necessary to assess the contribution of this gene to LR resistance in Uruguay. All the expected gene combinations classes based on the marker alleles present in the parents were found in the BC_1F_6 lines from both populations: four classes in WP1 which was segregating for *Lr68* and *Sr2*, and eight classes in WP2, which was segregating for *Lr34*, *Lr68* and *Sr2* (Fig. 3). However, some of the classes were represented by low numbers of lines. As only one line lacking the marker alleles of both *Lr68* and *Sr2* was identified in WP1, both the LR AUDPC data of this class as well as the corresponding ALRR estimations caused by these genes in this population were probably not accurately estimated. However, the relative performance of lines from classes *Lr68*, *Sr2* and *Lr68+Sr2* were precisely estimated

since these classes comprised from 16 to 30 lines. At least three lines per gene combination class were found for WP2. The distribution of lines resulted in at least three lines in the different gene combination classes of WP2, which probably resulted in a good estimation of their LR phenotype.

The lower average LR AUDPC of the WP1 compared to WP2 may be explained by the presence of the *Lr34* allele in LE2304 and all BC_1F_6 derived lines. The effect of *Lr34* could only be assessed in the WP2 as it segregated (Table 2, Fig. 3). The presence of *Lr34* was associated with significant ALRR (Table 2, Fig. 3), and moderate levels of resistance, which is in agreement with previous reports (Dyck et al. 1966; Singh et al. 2011).

The effect of *Lr68* in reducing LR AUDPC was similar and significant in both populations (Table 2). It is well documented that *Lr68* is closely linked to *Lr14*b, a race-specific resistance gene, ineffective to most races worldwide. As the race used in this study was also virulent to Tc*Lr14b* (IT=3+), we assumed that the effect in the reduction of LR severity associated to the marker allele used for *Lr68* was due to *Lr68 per se* and not to *Lr14b*. *Lr68* expressed mostly a principal effect and only the presence of *Sr2* appeared to modify its expression in WP2. The effect of *Lr68* was higher than the effect of *Lr34* in WP2 (Fig. 3), confirming the earlier report of Lillemo et al. (2011), who found that *Lr68* conferred a greater reduction in LR than *Lr34* in Uruguay and Argentina. However, these results contrast with previous reports that consistently concluded that *Lr34* was the APR gene with the highest effect on LR (Dyck et al. 1966, Singh and Rajaram 1992; Herrera-Foessel et al. 2012). We considered that this apparent disagreement can be explained by different relative expressions of *Lr34* and *Lr68* in different environments. In Uruguay (33 to 36°S, at low altitude) and Cd. Obregon, Mexico (27°N, at low altitude) spring wheat is planted in winter to avoid high temperatures during grain filling stage. It is well documented that *Lr34* expresses higher levels of resistance at low temperature (Pretorius et al. 1994; Singh and Huerta-Espino 2003). Herrera-Foessel et al. (2012) also reported that *Lr68* is also more effective at low temperatures in growing conditions in Cd. Obregón, Mexico. The different effects of *Lr34* and *Lr68* in each

geographical region can possibly be attributed to different temperature regimes or other environmental differences occurring between the Southern Cone and México (day length, temperature range, etc.). Analysis of climatic data from the test environments could help to understand the relationship between environmental conditions and the relative expression of APR genes.

We studied the effect of stem rust resistance gene *Sr2* on LR to detect any possible effect of this gene in reducing LR in Uruguay. Although *Sr2* is linked to the seedling gene *Lr27* (Mago et al. 2011) and Bariana et al. (2007) reported a that *Lr27* may have been responsible for reducing LR severity, we disregarded a possible effect of this LR resistance gene because the *P. triticina* race used in this study was virulent to *Lr27+Lr31* (IT=3) at the seedling stage and all BC_1F_6 lines (data not shown). *Sr2* could have a similar resistance mechanism as *Sr57*/*Lr34*, *Sr58*/*Lr46*, *Sr55*/*Lr67*, however the actual implication of *Sr2* locus or another LR resistance gene that might be pleiotropic or closely linked cannot be ruled out until this genomic region is further studied

In both wheat populations, a similar or even higher LR AUDPC was observed on lines from classes with *Sr2* alone when compared with the 'no genes' class (Fig. 2 and Fig. 3) which indicates that this gene has no effect or even slightly increases LR severity when present alone. Although the 'no genes' class in WP1 was too small, therefore affecting the contrast, the trend was similar in both populations, suggesting that the confounding effect was probably low. However, when *Sr2* was present in combination with LR APR genes, in most cases it contributed to a higher reduction of LR AUDPC than expected by the presence of these *Lr* genes (lines with *Lr68+Sr2* from WP1, and lines with *Lr34+Sr2* and *Lr34+Lr68+Sr2* from WP2, Fig. 2 and Fig. 3). Lines with *Lr68* and *Sr2* from WP2 had the same LR severity as lines with *Lr68* alone while *Sr2* expressed interaction effects with *Lr34* and *Lr68* in WP2 (Table 2). Therefore the interaction effect of *Sr2* with LR APR genes appears to vary with different genetic backgrounds. In both populations, lines combining all the resistance genes were most resistant and had similar LR AUDPC to the resistant parent,

confirming the additive nature of the APR genes, and that multiple APR genes are required to attain high levels of resistance (Singh et al. 2011).

The principal effect of *Lr68* in combination with slow rusting genes *Lr34* and *Sr2* highlighted the value of *Lr68* in breeding for effective and durable resistance to LR in wheat in Uruguay. *Lr34* pleiotropically determines LTN (Singh, 1992b; Krattinger et al. 2009) and expresses higher levels of resistance under cool temperatures (Pretorius et al. 1994). *Lr34* has been sequenced and its protein product is different from that produced by major genes (Krattinger et al. 2009), indicating that this APR gene has a different mechanism of action. *Lr68* is also associated with LTN and expresses more strongly under lower temperature (Herrera-Foessel et al. 2012), suggesting that both genes may share a common defense mechanism. The ongoing work to clone *Lr68* and elucidate its molecular function will ultimately answer this question. The multiple or broad disease resistance spectrum conferred by slow rusting gene *Lr34* implies an added value for breeding. The effect of *Lr68* on other diseases needs to be studied further.

This study confirmed the stronger effect of *Lr68* than *Lr34*, in reducing LR infection levels in field tests in Uruguay based on their expression on two local elite genetic backgrounds and, highlighted the relevance of combining *Lr34*, *Lr68* and *Sr2* to increase LR resistance. *Lr68* and *Lr34* are key genes to be used in breeding programs although they should be combined with additional APR genes to attain highly effective levels of LR resistance. These APR genes occur at low frequency in Uruguayan elite germplasm and are mostly present individually (Silva et al. 2013b[4]). Increasing the frequency of these genes and pyramiding through phenotyping or marker-assisted selection in wheat breeding lines in Uruguay will be valuable for increasing the levels of overall LR resistance in future cultivars.

[4] La letra b en Silva et al. 2013b es a efectos de diferenciar citas en el conjunto de citas de esta tesis

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3. GENOME-WIDE ASSOCIATION MAPPING OF RESISTANCE TO WHEAT LEAF RUST IN URUGUAY

3.1. SUMMARY

Leaf rust (LR) is one of the most important diseases that affect wheat production worldwide. Chemical control of this disease is not the most economical or environmentally sound solution, making the development of durable resistant varieties a priority for breeding programs. However, the availability of new resistance sources for LR is a limiting factor. The objective of this work was to detect genomic regions associated with seedling and field resistance to LR in Uruguay. Two different populations with a total of 186 spring wheat lines derived from three resistant lines and two Uruguayan susceptible cultivars were used. Phenotypes were the field LR AUDPC recorded in three environments in Uruguay in 2012 and 2013 crop seasons and seedling infection types to three *P. triticina* races. The wheat lines were genotyped using GBS and a total of 5222 SNP markers were used for GWAS analysis. Additionally we used markers for characterization of *Lr34* and *Lr68* APR genes. We identified 43 significant SNP markers for seedling resistance and 19 for field resistance to LR, on chromosome 1A, 1B, 1D, 2B, 2D, 3A, 4A, 5B, 6B, 7A, 7B and 7D. We confirmed that genes *Lr10*, *Lr16* and *Lr34* were present in our populations. The QTL detected for ARP in chromosome 7B might be explained by the presence of *Lr68*. QTLs on chromosome 4A and 5B appears to be novel associated to LR resistance. Information from this study could be used in breeding wheat cultivars with durable resistance to LR in Uruguay.

Keywords: APR, *Puccinia triticina*, durable resistance, partial resistance, QTL, Genotyping by sequencing

3.2. RESUMEN

La roya de la hoja (RH) es una de las enfermedades más importantes que afecta a la producción de trigo en todo el mundo. El control químico de esta enfermedad no es la mejor solución económica ni ecológica, siendo el desarrollo de variedades resistentes una prioridad para los programas de mejoramiento. Sin embargo, la disponibilidad de nuevas fuentes de resistencia es un factor limitante. El objetivo de este trabajo fue detectar regiones genómicas asociadas a resistencia de plántula y de campo frente a RH en Uruguay. Se utilizaron dos poblaciones con un total de 186 líneas derivadas de tres fuentes de resistencia y dos cultivares uruguayos susceptibles. Los fenotipos fueron el AUDPC a campo en tres ambientes (Uruguay 2012 y 2013) y tipo de infección en plántula a tres razas de *P. triticina*. Las líneas fueron genotipadas mediante GBS y un total de 5222 marcadores SNP se utilizaron para el análisis GWAS. Además se utilizaron marcadores para los genes de RPA *Lr34* y *Lr68*. Se identificaron 43 SNP significativos para resistencia en plántula y 19 para resistencia a campo, en los cromosomas 1A, 1B, 1D, 2B, 2D, 3A, 4A, 5B, 6B, 7A, 7B y 7D. Se confirmó la presencia de los genes *Lr10*, *Lr16* y *Lr34*. El QTL en 7B estaría explicado por el gen *Lr68*. Los QTLs en 4A y 5B serían nuevos QTLs de resistencia a RH. Esta información podrá ser utilizada en el desarrollo de nuevos cultivares de trigo para lograr resistencia duradera a RH en Uruguay.

Palabras claves: RPA, *Puccinia triticina*, Resistencia durable, Resistencia parcial, QTL, Genotipado por secuenciación

3.3. INTRODUCTION

Wheat leaf rust (LR), caused by *Puccinia triticina* Eriks., is the most common disease of wheat (*Triticum aestivum* L.) worldwide, including Uruguay (Germán et al. 2007; Bolton et al. 2008). In South America, wheat is planted annually on approximately 8.5 mha in Argentina, Brazil, Chile, Paraguay, and Uruguay (FAOSTAT 2014). A favorable environment and a high proportion of the wheat area sown with LR susceptible or moderately susceptible cultivars allow *P. triticina* to cause severe epidemics in large areas. In Uruguay, two or more fungicide applications are required to prevent grain yield losses that can be as high as 50% on susceptible cultivars (Germán et al. 2007). *Puccinia triticina* populations worldwide are highly diverse (Huerta-Espino et al. 2011). In Uruguay, many races are present annually and their relative frequencies may change significantly from year to year (Germán et al. 2007). The cultivation of LR resistant wheat varieties is considered the most economical, efficient and environmentally safe method for controlling wheat LR. Therefore, in order to achieve effective and durable LR resistance, it is essential to develop wheat cultivars with combinations of effective resistance genes and to identify new resistance sources in wheat germplasm (Singh et al. 2005; Singh et al. 2011).

Resistance to LR may be based on major resistance genes, multiple minor genes or a combination of both types of resistance genes. To date, 74 LR resistance genes (*Lr* genes) are cataloged in wheat (McIntosh et al. 2013), most of which are race-specific. Major race-specific resistance genes (R-genes) have often been used by wheat breeders because of their high level of effectiveness throughout the entire growth cycle of the crop. A major problem has been their short-lived effectiveness due to the fast emergence of virulent races of the pathogen that overcome the resistance (Singh 2012). Other resistance genes are commonly detected at adult plant stages and can be further divided into two categories: those with similar characteristics to R-genes in terms of effect, expression of hypersensitive reaction (HR), and race specificity (APR-R genes), and those that are quantitatively inherited, do not express HR, and are race non-specific, also called minor genes (ARP-PR)

(Lagudah 2011; Singh et al. 2011). APR-PR genes have conferred partial but durable resistance for a long period of time (Singh et al. 2011). These genes confer a slow rusting type of resistance despite a compatible host reaction and are effective to all races of the pathogen (Singh et al. 2005). Although only a few APR-PR genes for LR have been cataloged (*Lr34*, *Lr46*, *Lr67*, *Lr68* and *Lr74*), (Singh et al. 1998; Herrera-Foessel et al. 2011; Singh et al. 2011; Herrera-Foessel et al. 2012) at least 80 QTLs associated with durable resistance have been described to date (Herrera-Foessel et al. 2012; Li et al. 2014), illustrating the diversity for this type of resistance genes in wheat germplasm. A notable characteristic of these genes is that they often have pleiotropic effects on multiple rust diseases (Rosewarne et al. 2012, Singh 2012). Individual APR-PR genes do not confer adequate levels of resistance, but combinations of 4-5 genes confer near immunity (Singh et al. 2011). Fine mapping of slow rusting APR-PR genes remains a challenge as their often small individual effects pose difficulties in phenotyping.

Marker-based approaches are valuable methodologies to identify genes/quantitative trait loci (QTL) controlling plant response to diseases. The standard approach to determine the number and the chromosomal location of resistance loci is to use bi-parental mapping populations to relate phenotypic information to genotypic data obtained from molecular markers. Alternatively, association mapping (AM) or linkage disequilibrium (LD) mapping is based on genotype-phenotype relationships explored in germplasm collections or natural populations (Flint-Garcia et al. 2003; Gupta et al. 2005). The underlying principle of AM approach is that LD tends to be maintained over many generations between loci that are genetically linked (Zhu et al. 2008). One approach for AM is a whole-genome scan, often called genome-wide association study (GWAS), which identifies genomic regions associated with the trait of interest throughout the genome. A new approach used for mapping genes affecting complex traits is the use of nested association mapping populations (NAM). The term nested explains the type of population, multi-family populations where all individuals of the multi-family population share the same father, but each sub-population has a different alternate

father (Buckler et al. 2009). NAM populations combine the statistical power of bi-parental QTL analysis with the high chromosomal resolution of AM (Yu et al. 2008).The advent of cost-effective whole genome profiling utilizing next-generation sequencing technologies (e.g. genotyping by sequencing, GBS); (Elshire et al. 2011; Poland et al. 2012) and the improvement in statistical methods have contributed to increase the interest in identifying useful and/or novel alleles using large numbers of SNP markers for GWAS, (Gupta et al. 2005; Zhu et al. 2008). One problem in the GWAS analysis is how to handle the information about the major genes present in the population under study. When GWAS involves major genes that have large effects, the markers for such genes should have fixed effects in the mixed model (Bernardo 2014). GWAS has been used successfully to identify marker-trait associations in wheat for many traits (Sajjad et al. 2012), end-use quality traits (Breseghello and Sorrells 2006; Zheng et al. 2009), yield and yield component traits (Neumann et al. 2010; Maccaferri et al. 2011; E.A. Edae, P. E. Byrne 2014) and disease resistance (Yu et al. 2011; Kollers et al. 2013; Letta et al. 2014), including wheat LR resistance (Crossa et al. 2007; Maccaferri et al. 2010)

The objective of this study was to identify genomic regions associated to seedling and field wheat LR resistance expressed in Uruguay in two populations derived from three resistant lines, using GWAS.

3.4. MATERIALS AND METHODS

3.4.1. Plant materials

Two BC_1F_6 populations were derived from three sources of resistance: Suz6/Opata (Mexican line), Cep8749/Br35 (Brazilian line) and BPon/PBred/4/R37/GHL21//KAL/BB/3/KLT´S (BPon/PBred, Argentinian line), and two Uruguayan susceptible parents: INIA Tero (I. Tero, LI107/C-CH-91-1642) and INIA Torcaza (I. Torcaza, E.Federal/4/Trigal800/3/K.Impacto/Agatha//K.Impacto/Pat24). The sources of resistance were selected based on field resistance levels expressed in field nurseries.

Suz6/Opata and BPon/PBred were selected from the regional project "Vivero Regional de Royas" funded by CIMMYT, INIA Uruguay, INIA España and PROCISUR (2006-2009). Cep8749/Br35 was selected from a USDA/FONTAGRO funded project "Identification and utilization of durable genetic resistance to rust diseases in wheat" (Agreement No 58-4001-9-0F162, 2000-2003). The information on the presence of known APR genes in the parental lines was not available when the populations were developed. The three resistant donors were crossed to each of the susceptible parents and the F_1 was backcrossed to the same susceptible parent. Wheat population one (WP1), derived from the susceptible parental line I. Tero, consisted of 94 wheat lines (four parental lines and 90 BC_1F_6 lines) with 29 to 31 lines per subpopulation. Wheat population two (WP2), derived from the susceptible parental line I. Torcaza, consisted of 95 wheat lines (four parental lines and 91 BC_1F_6 lines) with 30 to 31 lines per subpopulation. The lines were not completely random, but selected to balance different LR field reactions and to avoid losing QTL by minor allele frequencies.

3.4.2. Pathogen materials

Three *P. triticina* races were used to study seedling and field resistance: TFT-10,20 (avirulence/virulence formula : *Lr9, 16, 18, 19, 21, 25, 28, 29, 32, 33, 36, 39, 42, 47, 51, 52/Lr1, 2a, 2b, 2c, 3, 3bg, 3ka, 10, 11, 12, 13, 14a, 14b, 15, 17, 20, 22b, 23, 24, 26, 27+31, 30, 37*), MDR-10,20 (avirulence/virulence formula: *Lr2a, 2b, 2c, 9, 16, 17, 19, 21, 25, 26, 29, 32, 33, 36, 37, 39, 42, 47, 51, 52/Lr1, 3, 3bg, 3ka, 10, 11, 12, 13, 14a, 14b, 15, 18, 20, 22b, 23, 24, 27+31, 28, 30*) and TPR-20,39 (avirulence/virulence formula: *Lr10, 16, 17, 19, 21, 25, 27+31, 29, 32, 33, 36, 37, 42, 47, 51, 52/Lr1, 2a, 2b, 2c, 3, 3bg, 3ka, 9, 11, 12, 13, 14a, 14b, 15, 18, 20, 22b, 23, 24, 26, 28, 30, 39).* Avirulence/virulence for *Lr12, 13, 22b* and *Lr37* was determined in the greenhouse on adult plants. Inoculum was increased on the susceptible wheat line Little Club as described by Germán and Kolmer (2014). The races used were probably avirulent to *Lr22a* and *Lr35,* since Thatcher lines with these genes have been resistant in the field (Germán pers comm).

3.4.3. Seedling resistance phenotyping

Seedling tests were carried out during 2012 in the greenhouse at INIA La Estanzuela Experimental Station. The experimental design was incomplete blocks with two replications. The parental lines, all BC_1F_6 lines and the same near isogenic lines used to characterize avirulence/virulence of the races were tested. Fully expanded leaves (8-10 days after planting) were inoculated by spraying urediniospores suspended in Soltrol 170 (Phillips Petroleum Co., Borger, TX), incubated in a dew chamber overnight and then kept in the greenhouse at approximately 20ºC. Infection types (IT) were recorded 12 days after inoculation using the 0-4 scale described for Long and Kolmer (1989) according to the following convention: 0 = immunity, no visible infection, ; = diffuse presence of hypersensitive flecks, no uredinia, 1 = small uredinia surrounded by necrosis, 2 = small or medium uredinia surrounded by chlorosis, X = mesothetic response, with all kind of uredinia present together, 3 = numerous uredinia of moderate size without necrosis or chlorosis, 4 = large uredinia. Larger or smaller uredinia were indicated with the + and - symbols. Infection types from 0 to 2 and X were considered as avirulent (resistant response of the plant) while ITs 3 and 4 were considered as virulent.

3.4.4. Field resistance phenotyping

Field experiments were conducted at three environments: La Estanzuela (LE: latitude 34.3° S, longitude 57.7° W, elevation 70 masl) during 2012/13 (LE-2012) and 2013/14 (LE-2013) and Young (Y: latitude 32.7° S, longitude 57.6° W, elevation 76 masl) during 2012/13 (Y-2012). The experimental design was an augmented incomplete block with two replications with seven repeated checks, including the susceptible lines Avocet-S, Thatcher and the five parental lines. Approximately 4-5 g seeds (60-70 plants) of each line were hand-sown in 1 m rows in late July. In order to an even LR distribution, spreader rows of the susceptible parents were planted perpendicular to the plots. Field inoculations of races TFT-10,20, MDR-10,20 and TPR-20,39 were repeated three times with a suspension of urediniospores in lightweight mineral oil starting c.a. eight weeks after sowing. Disease severity (DS)

was evaluated from anthesis to dough stage, scored in values ranging from 0-100 based on a visual assessment using the modified Cobb Scale (Peterson et al. 1948). Severity scores for each assessment were combined with an infection response (IR) following Roelfs et al. (1992). Four DS + IR scores were taken at 7 to 14 day intervals, and were used to calculate the infection coefficient (IC) = DS x IR, where DS was multiplied with a coefficient according to R=0.2, MR=0.4, M=0.6, MS=0.8 and S=1.0. The IC was used to calculate the area under the disease progress curve (AUDPC) following Maccaferri et al. (2010).

3.4.5. Genotyping and SNP identification

Since APR-PR genes *Lr34* and *Lr68* have a significant effect in reducing LR AUDPC in Uruguay (Silva et al. in review), we characterized the lines for the presence/absence of these alleles. Later, we included these genes in the mixed model for GWAS either as fixed covariates, or by including the marker alleles as SNPs markers in the GWAS screening. Genotyping was done using the flanking markers *csLV34* + *LR34PLUSR* (Lagudah et al. 2006; Lagudah et al. 2009) for *Lr34* and *cs7BLNRR* (Herrera-Foessel et al. 2012) for *Lr68.* DNA extractions using the CTAB method, PCR amplifications and identification of marker alleles associated with APR genes by 12 % acrylamide (29:1) gels electrophoresis followed CIMMYT protocols. Bands were detected using silver staining (CIMMYT Protocols, 2005). The Mexican resistant line Parula, which carries both, *Lr34* and *Lr68* (Herrera-Foessel et al. 2012), was also included as a control for the expected marker allele band sizes.

The SNPs markers were obtained using the genotyping by sequencing approach (GBS, Elshire et al. 2011). Library construction followed the PstI-MspI GBS protocol described by Poland et al. (2012). The sequencing was performed on an Illumina HiSeq 2000. The sequences analysis, imputation of missing data and SNP identification were done according to Lado et al. (2013). All markers with a minor allele frequency lower than 5% were excluded. For the sequence alignments the SNP tags were BLASTed against the sequence database available from the Synthetic x Opata map by Poland et al. (2012) (available at

http://www.wheatgenetics.org/index.php/download/viewcategory/10-synop) using blast from packages NCBI-BLAST+ (Altschul et al. 1990) setting the parameters maximum target and number of threads at 1 and percent of identity at 95%. To study the pattern of LD among markers we used the pairwise r^2 estimates of LD using the computer program Haploview (Barrett et al. 2005).

3.4.6. Statistical analysis

3.4.6.1. Phenotypic means

The seedling resistance phenotypic values used for GWAS were obtained expressing the ITs as a numeric scale as follows: avirulent phenotypes: 0 = 0, ; = 0, 1 = 1, 2 = 2, X = 1.5, virulent phenotypes : 3 = 3, 4 = 4, + = +0.25, - = -0.25, according to Maccaferri et al. (2010). Phenotypic scores from seedling tests to the three P. *triticina* races and field resistance AUDPC values were analyzed using the following mixed model:

$$y_{ijkl} = \mu + R_i + \underline{\beta}_{j(i)} + G_k + GE_l + H_m + \underline{\varepsilon}_{ijklm}$$

where y_{ijkl} is the response variable (i.e ITs and LR AUDPC values), μ is the overall mean, R_i is the effect of the i-th block, $\beta_{j(i)}$ is the effect of the j-th incomplete-block within the i-th complete block and considered as a random effect with $\beta_{j(i)} \sim N(0, \sigma^2_\beta)$, G_k is the effect of the k-th genotype, GE_l is the genotype x environment interaction effect, H_m is the effect of days to heading, and ε_{ijklm} is the experimental error with εijkl ~ N(0, σ2ε). The best linear unbiased estimators (BLUE) for seedling ITs and LR AUDPC in each field environment were used for GWAS analysis. This analysis was performed in R software (R Development Core Team 2008) using the lme4 package (Bates et al. 2009). Pearson correlations between pairs of environments were calculated using R statistical software (R Development Core Team 2010).

3.4.6.2. Population Structure

SNP data from BC_1F_6 lines from the two populations and the five parental lines were used to estimate the additive relationship matrix of genotypes using the function A.mat of the package rrBLUP (Endelman 2011) in R statistical software (R Development Core Team 2010). The additive matrix was analyzed by principal component analysis (PCA) using the stats package in R (R Development Core Team 2010). In addition, the population structure was investigated using the program STRUCTURE (Pritchard et al. 2000). Models with a putative number of subpopulations (K) from 1 to 10 without admixture and with noncorrelated allele frequencies were considered. To infer the K value the ΔK method (Evanno et al. 2005) was used.

3.4.6.3. Association analysis

We used the kinship linear mixed model approach for GWAS analysis as described by Yu et al. (2006). The vector of phenotypes was modeled as:

$$y = X\beta + Zu + \varepsilon$$

where y is the phenotypic vector, X is the molecular marker matrix, β is the unknown vector of allele effects to be estimated, Z is the model matrix of random effects, u is the vector of random background polygenic effects with N (0, $A\sigma^2_u$), where A is the additive relationship matrix, and ε is the residual errors with N (0, $I\sigma^2_\varepsilon$). Two different GWAS models were used to test the significance of marker-trait associations: i) K model: mixed model including the genotypes as random effects with the variance-covariance matrix given by a function of the A.mat matrix and ii) K + *Lr* model: same mixed model as i) but including the APR genes *Lr34* and *Lr68* as fixed effect covariates. Both GWAS models were performed separately for each wheat population, each field environment and each *P. triticina* race. GWAS analyses were performed using R statistical software (R Development Core Team 2010) with

the rrBLUP package (Endelman 2014). A false discovery rate (FDR) with α level equal to 0.05 (Storey 2002; Storey et al. 2004) was used.

3.5. RESULTS

3.5.1. Seedling resistance

High IT on the susceptible check Thatcher and expression of differences in seedling IT were observed for all races, which were virulent to at least one of the five parents (Table 1). Race TFT-10,20 was virulent for I. Tero and Suz6/Opata, race MDR-10,20 was virulent for I. Torcaza and Suz6/Opata, and race TPR-20,39 was virulent for Cep8749/Br35 and BPon/PBred. Race TFT-10,20 had the highest average IT to the 90 BC1F6 lines of WP1 (mean IT=3.0) followed by MDR-10,20 (mean IT=2.2), while TRP-20,39 had the lowest value (mean IT=1.4) (Fig. 1). No lines with IT=4 to races MDR-10,20 and TRP-20,39 were found in WP1. For the 91 BC1F6 lines in WP2, MDR-10,20 had the highest average IT (mean IT=3.0), followed by TFT-10,20 (mean IT=1.9) and finally TPR-20,39 (mean IT=0.9) (Fig. 1). Most lines in WP1 had high IT to race TFT-10,20, virulent to I. Tero, while most lines in WP2 had high IT to race MDR-10,20, virulent to I. Torcaza. Most lines in both populations had low IT to race TPR-20,39, which had IT=0 to both adapted recurrent parents. Correlations between the races were very low, ranging from 0.09 for TFT-10,20 and TPR-20,39 to 0.16 between MDR-10,20 and TPR-20,39, while the correlation between TFT-10,20 and MFP-10,20 was 0.13.

Table 1. Presence of molecular marker alleles for APR genes *Lr34* and *Lr68*, leaf rust area under the disease progress curve (AUDPC) and field disease severity (DS) in three environments, and seedling infection types (IT) to three *Puccinia triticina* races, of five parental lines. INIA Tero: susceptible to LR, INIA Torcaza: susceptible to LR, Suz6/Opata, Cep8749/Br35 and BPon/PBred: resistant to LR. Environments: La Estanzuela 2012 (LE-2012), Young 2012 (Y-2012), La Estanzuela 2013 (LE-2013). *P. triticina* races: TFT-10,20, MDR-10,20 and TPR-20,39

	APR genes		AUDPC[a]				IT to *P. triticina* races[b]		
Parental Lines	*Lr34*	*Lr68*	LE-2012	Y-2012	LE-2013	DS[c]	TFT-10,20	MDR-10,20	TPR-20,39
INIA Tero	+[d]	-	2772	6034	2202	80	**3+**	2	0
INIA Torcaza	-	-	1328	3566	1752	60	2	**3+**	0
Suz6/Opata	+	+	620	1507	482	20	**3+**	**3**	2-
Cep8749/Br35	-	-	1132	2082	810	40	1-	1-	**4**
BPon/PBred	-	-	881	2512	1322	30	1-	1-	**4**
Susceptible Checks									
Avocet-S			6269	6492	5974	95			
Thatcher			4875	5502	5259	90	**3+**	**3+**	**3+**

[a] Average of four independent scores of infection coefficient, according to Maccaferri et al. (2010)
[b] ITs according to Long and Kolmer (1989)
[c] Average final disease severity of the three environments
[d] Presence (+) or absence (-) of the molecular marker alleles *csLV34 + LR34PLUSR* for *Lr34* (Lagudah et al. 2006, Lagudah et al. 2009) and *cs7BLNRR* for *Lr68* (Herrera-Foessel et al. 2012)

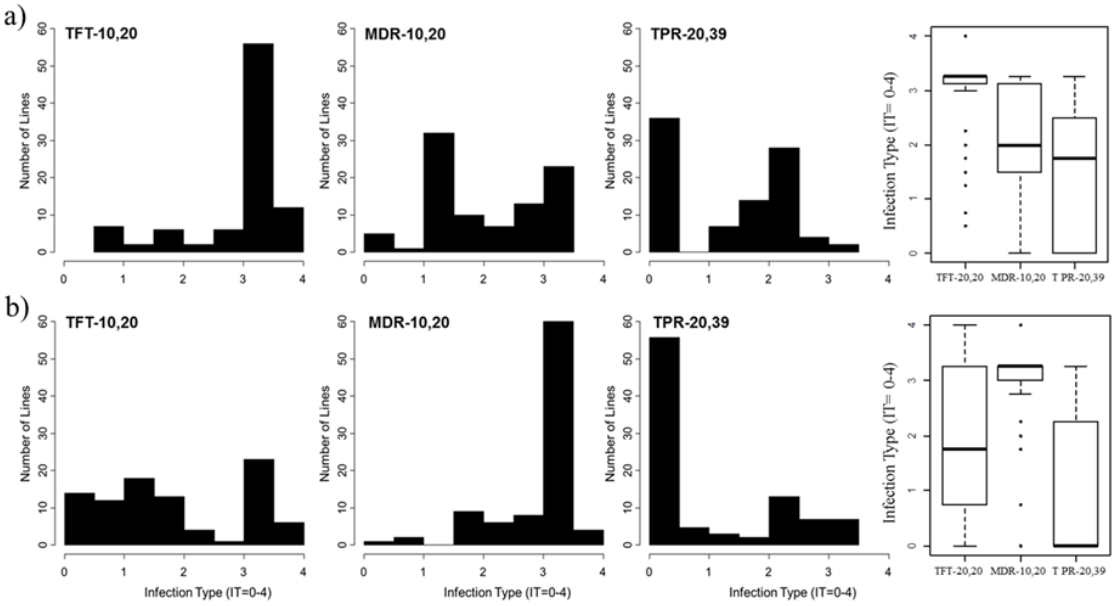

Figure 1. Frequency distribution and boxplot of ITs of a) WP1 and b) WP2 challenged with three *Puccinia triticina* races: TFT-10,20, MDR-10,20 and TPR-20,39. Box edges represent the upper and lower quantile with median value shown as a bold line inside the box. Whiskers represent 1.5 times the quantile of the data. Wheat lines falling outside the range of the whiskers are shown as open dots.

3.5.2. Field resistance

In the three field environments the susceptible checks Avocet-S and Thatcher had high LR AUDPC and final DS scores, indicating adequate development of LR to detect differences in field resistance (Table 1). Environment, days to heading, genotype and genotype x environment effects were significant on LR AUDPC (data not shown). Correlations between LR AUDPC of different field experiments were high, ranging from 0.72 for Y-2012 and LE-2013, to 0.82 for LE-2012 and LE-2013, while the correlation between LE-2012 and Y-2012 was 0.81. According to the final IC, the highest LR infection occurred at Y-2012, while lower and similar disease development occurred at LE-2012 and LE-2013 (Fig. 2). The resistant parental lines, Suz6/Opata, Cep8749/Br35 and BPon/PBred showed intermediate levels of resistance, with final DS scores below 50% (Table 1). I. Tero and I. Torcaza had

higher LR than the resistant parental lines in the three field environments (Table 1), but lower levels than the susceptible checks. WP1 had higher frequency of lines with high AUDPC in all environments than WP2 (Fig. 2).

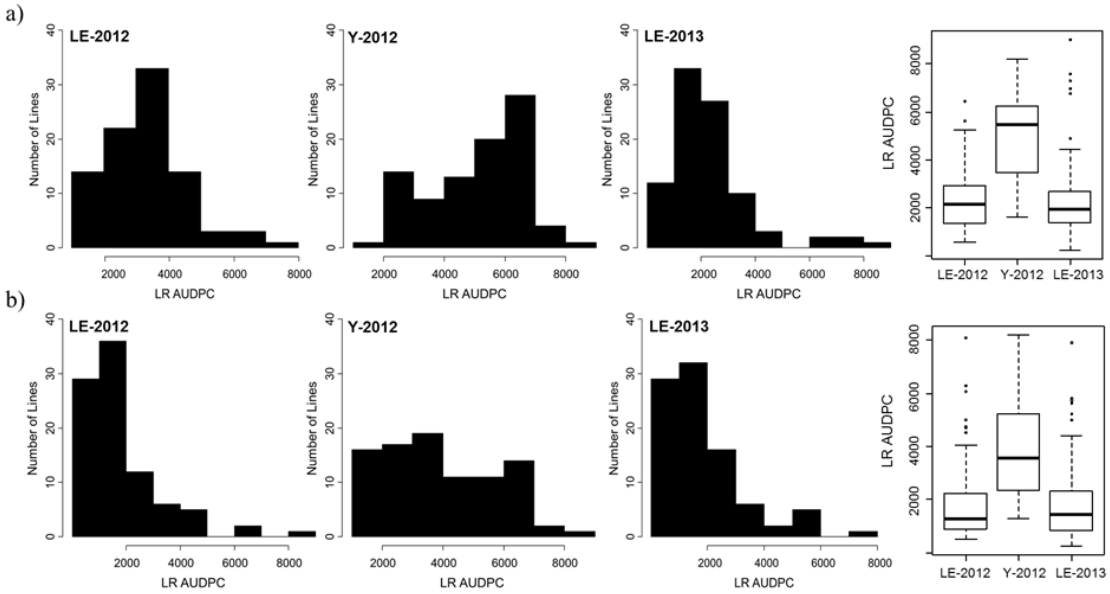

Figure 2. Frequency distribution and boxplot for LR AUDPC values in La Estanzuela 2012 (LE-2012), Young 2012 (Y-2012) and La Estanzuela 2013 (LE-2013). a) WP1and b) WP2. Box edges represent the upper and lower quantile with the median value shown as a bold line inside the box. Whiskers represent 1.5 times the quantile of the data. Wheat lines falling outside the range of the whiskers are shown as open dots.

3.5.3. Genotyping and SNP identification

The molecular markers for *Lr34* and *Lr68* were used to screen both BC_1F_6 populations according to the presence/absence of the molecular marker alleles in each parent (Table 1). Resistance gene *Lr34* was segregating in two WP1 subpopulations (I. Tero*2//Cep8749/Br35 and I. Tero*2// BPon/PBred) and *Lr68* only in one subpopulation (I. Tero//Suz6/Opata). Resistance genes *Lr34* and *Lr68* were segregating only in one WP2 subpopulation (I. Torcaza*2//Suz6/Opata) (Table 1). GBS-SNPs were identified among sequence tag pairs by allowing one to three

mismatches between tags. One library for both wheat populations was analyzed producing a total of 72,000 SNPs. When comparing sequences using BLAST against the Poland et al. (2012). Of all the SNPs, 7.3% (5222) found high-quality matches. Although a good coverage was observed, the D genome had fewer SNPs (7%, 361 SNPs) than the A (35%, 1843 SNPs) and B (58%, 3018 SNPs) genomes. The chromosome with the highest marker density was 3B (618 SNPs) and that with the lowest density was 4D (13 SNPs). The final genotypic matrix consisted on 5222 SNPs with chromosome and recombination bin position for each SNP marker.

3.5.4. Population Structure

The additive relationship matrix to perform the PCA was estimated with the complete set of SNPs markers (5222 SNPs). The first two principal components explained 93% of variation. The BC_1F_6 lines from WP1 and WP2 were separated by the first component of PCA (Fig.3), and then GWAS analysis was implemented for the populations separately. Population structure analyzed with the program STRUCTURE found an optimum number of two groups (K=2), one group represented by WP1 and the other group represented by WP2 (data not shown). In both cases, PCA or STRUCTURE, the BC_1F_6 lines were shifted towards the susceptible parents, I. Tero and I. Torcaza. No relevant population structure was detected within each population; therefore no corrections for this factor were used in the mixed model for running GWAS analysis and only kinship relationship was used.

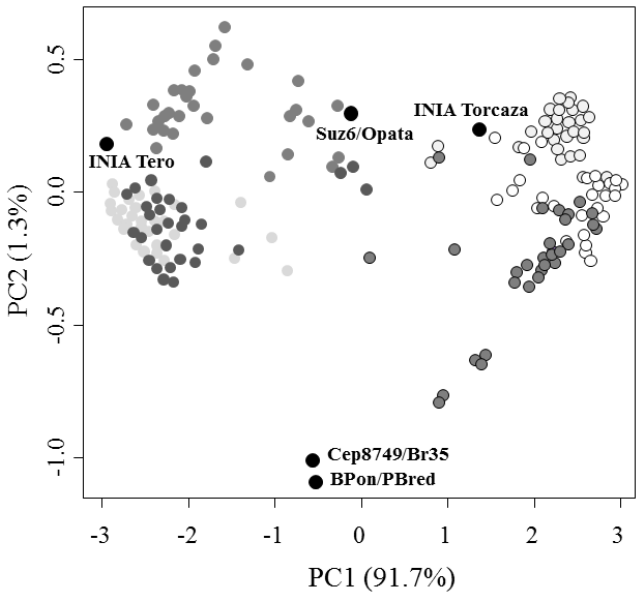

Figure 3. Principal component analysis (PCA) of WP1+WP2, including the five parental lines (*black circles*), conducted with 5222 SNP markers. Each data point represents a genotype. Lines from WP1 are represented by circles without line edge and lines from WP2 with line edge. Lines from different crosses were indicated by different combinations of line edge and colors. I. Tero*2//Suz6/Opata (●), I. Tero*2//Cep8749/Br35 (●), I. Tero*2//BPon/PBred (●), I. Torcaza*2//Suz6/Opata (○), I. Torcaza*2//Cep8794/Br35 (○) and I. Torcaza*2//BPon/PBred (●).

3.5.5. Association analysis

Genome-wide association analysis with K model of WP1 and WP2 detected a total of 43 significant marker-trait associations for seedling resistance to at least one LR race and GWAS analysis with K and K + *Lr* models detected 19 significant marker-trait associations for field resistance in at least one of the three environments. These significant SNPs were located on chromosomes 1A, 1B, 1D, 2B, 2D, 3A, 4A, 5B, 6B, 7A, 7B and 7D (Table 2 and 3). We detected more significant marker-trait associations for seedling resistance in WP2 (31 significant SNPs) compared to WP1 (12 significant SNPs) (Table 2). However, we detected more significant marker-trait associations for field resistance in WP1 (13 significant SNPs) than in WP2 (6 significant SNPs) (Table 3). Seedling resistance was studied only with the K model.

For WP1 we found significant marker-trait associations only for race TFT-10,20. Nine significant SNPs with positive effect on LR IT were donated by Cep8749/Br35 or BPon/PBred, and three with negative effect were donated by the susceptible parent I. Tero (Table 2). Seedling GWAS for WP2 detected, four, 17 and 10 significant marker-trait associations for races TFT-10,20, MDR-10,20 and TPR-20,39, respectively. Only four SNPs had negative effect on LR IT in this population, two of them for MDR-10,20 donated by I. Torcaza and the other two for TPR-20,39 donated by Cep8749/Br35 or BPon/PBred (Table 2). These parents contributed all SNP alleles with positive effects. Suz6/Opata did not contribute any significant SNP associated with seedling resistance in any of the two populations. The same SNPs on chromosome 5B (GBS12036 and GBS12037) were significant in WP1 and WP2 tested with race TFT-10,20. SNP GBS86541 on chromosome 2B was significant to race TFT-10,20 in WP1 and to race MDR-10,20 in WP2. Other SNPs were significant in either WP1 or WP2 (Table 2).

Table 2. Summary of significant markers associated with QTL for seedling resistance to leaf rust detected in wheat population 1 (WP1) and WP2, challenged with three *Puccinia triticina* races (TFT-10,20, MDR-10,20 and TPR-20,39), showing genomic location (Chromosome and Bin), allele frequency (AF), p-value and possible parental line donor

GWAS K model								
Pop.	Marker	*Chr.*	Bin[a]	AF	TFT-10,20	MDR-10,20	TPR-20,39	Parental line donor
					p-value			
WP1	GBS86541	2B	239-270	0.077	0.0016	-	-	Cep8749/Br35 or BPon/PBred
WP1	GBS90766	3A	118-149	0.836	**0.0167**	-	-	I. Tero
WP1	GBS61217	3A	124-128	0.125	0.0219	-	-	Cep8749/Br35 or BPon/PBred
WP1	GBS79403	4A	92-94	0.073	0.0064	-	-	Cep8749/Br35 or BPon/PBred
WP1	GBS64791	5B	32-53	0.08	0.0002	-	-	Cep8749/Br35 or BPon/PBred
WP1	GBS81469	5B	60-67	0.761	**0.0026**	-	-	I. Tero
WP1	GBS51940	5B	63-84	0.156	0.0012	-	-	Cep8749/Br35 or BPon/PBred
WP1	GBS83166	5B	83-84	0.185	0.0058	-	-	Cep8749/Br35 or BPon/PBred
WP1	GBS12036	5B	97-99	0.081	0.0001	-	-	Cep8749/Br35 or BPon/PBred
WP1	GBS12037	5B	97-99	0.081	0.0001	-	-	Cep8749/Br35 or BPon/PBred
WP1	GBS106647	5B	105-113	0.776	**0.0018**	-	-	I. Tero
WP1	GBS21709	7A	273-280	0.05	0.0025	-	-	Cep8749/Br35 or BPon/PBred
WP2	GBS44077	1A	25-54	0.75	-	-	0.0006	I. Torcaza
WP2	GBS73770	1A	43-86	0.758	-	-	4.97E-08	I. Torcaza
WP2	GBS97618	1A	49-66	0.693	-	-	1.46E-18	I. Torcaza
WP2	GBS7618	1A	52-66	0.657	-	-	8.58E-11	I. Torcaza

WP2	GBS78507	1A	64-70	0.684	-	-	1.75E-09	I. Torcaza
WP2	GBS71468	1A	65-74	**0.148**	-	-	0.0117	Cep8749/Br35 or BPon/PBred
WP2	GBS71467	1A	65-74	**0.146**		-	0.0117	Cep8749/Br35 or BPon/PBred
WP2	GBS94107	1A	66-75	0.792	-	-	4.74E-08	I. Torcaza
WP2	GBS62556	1A	65-79	0.774	-	-	0.0047	I. Torcaza
WP2	GBS83675	1A	66-83	0.889	-	-	0.0095	I. Torcaza
WP2	GBS107343	1A	82-99	0.053	-	0.0058	-	Cep8749/Br35 or BPon/PBred
WP2	GBS107344	1A	82-99	0.053	-	0.0058	-	Cep8749/Br35 or BPon/PBred
WP2	GBS107345	1A	82-99	0.053	-	0.0058	-	Cep8749/Br35 or BPon/PBred
WP2	GBS99276	1B	121-155	0.074	-	0.0204	-	Cep8749/Br35 or BPon/PBred
WP2	GBS99277	1B	121-155	0.074	-	0.0204	-	Cep8749/Br35 or BPon/PBred
WP2	GBS73308	1B	136-154	0.053	-	0.0003	-	BPon/PBred
WP2	GBS7399	1D	197-213	0.075	-	0.0012	-	Cep8749/Br35 or BPon/PBred
WP2	GBS89378	2B	41-52	0.582	0.0071	-	-	I. Torcaza
WP2	GBS86541	2B	239-270	0.11	-	0.0037	-	Cep8749/Br35 or BPon/PBred
WP2	GBS100376	2D	0-16	**0.906**	-	0.0006	-	I. Torcaza
WP2	GBS48333	2D	2-50	0.694	0.0113	-	-	I. Torcaza, Cep8749/Br35 or BPon/PBred
WP2	GBS99142	3A	175-180	0.202	-	0.0221	-	Cep8749/Br35 or BPon/PBred
WP2	GBS77049	4A	21-46	0.123	-	0.0163	-	Cep8749/Br35 or BPon/PBred
WP2	GBS104326	4A	69-75	0.121	-	0.0054	-	Cep8749/Br35 or BPon/PBred
WP2	GBS70262	4A	105-144	0.181	-	0.0226	-	Cep8749/Br35 or BPon/PBred
WP2	GBS17748	5B	63-83	**0.85**	-	0.0145	-	I. Torcaza
WP2	GBS12036	5B	97-99	0.357	0.0078	-	-	Cep8749/Br35 or BPon/PBred
WP2	GBS12037	5B	97-99	0.357	0.0078	-	-	Cep8749/Br35 or BPon/PBred
WP2	GBS74136	7A	46-65	0.107	-	0.0223	-	Cep8749/Br35 or BPon/PBred

WP2	GBS28930	7D	242-258	0.054	-	0.0203	-	Cep8749/Br35 or BPon/PBred
WP2	GBS28931	7D	242-258	0.054	-	0.0203	-	Cep8749/Br35 or BPon/PBred

[a] Recombination Bin for the SNP markers (Poland et al. 2012). Bold indicates a negative effect

The GWAS analysis for field resistance was done first using the K model. In WP1 we found QTLs on chromosomes 5B and 7B only for the environment Y-2012 (Table 3, Fig. 4a). All the SNPs located on chromosome 7B were donated by the resistant parental line Suz6/Opata. When the second GWAS model (K + *Lr* model) was used all the significant SNPs on chromosome 7B disappeared, the SNP on chromosome 5B was still significant at Y-2012 and more significant SNPs on the same chromosome were found. New significant SNPs on chromosome 4A were detected either at LE-2012, LE-2013 or at the three environments (Table 3, Fig. 4). All the significant SNPs detected with the K + *Lr* model in WP1 might have come from Cep8749/Br35 or BPon/PBred resistant parents. Only one SNP located on chromosome 4A (GBS41840) was significant across the three environments. The SNP located on chromosome 5B (GBS51940) was the only one that was significant for both GWAS models (Table 3). For WP2, no significant markers for LR field resistance were detected using the K model (Table 3). When the K + *Lr* model was used we found significant marker-trait associations on chromosomes 2D, 4A, 5B and 6B at LE-2012. Only two SNPs, one on chromosome 4A (GBS45077) and one on chromosome 6B (GBS61864), donated by I. Torcaza. The SNP GBS67197 from Suz6/Opata on chromosome 4A was the only one with negative allele substitution effect (ASE) to LR in this population. No significant SNPs in common were detected for both populations using the different GWAS models (Table 3).

Table 3. Summary of significant markers associated with QTL for field resistance to leaf rust (LR) detected in wheat population 1 (WP1) and WP2, in three environments (LE-2012, Y-2012 and LE-2013), showing genomic location (Chromosome and Bin), allele frequency (AF), p-value, allele substitution effect (ASE) and possible parental line donor

GWAS K model									
Pop.	Marker	*Chr.*	Bin[a]	AF	LE-2012	Y-2012	LE-2013	ASE[b]	Parental line donor
					p-value				
WP1	GBS51940	5B	63-84	0.157	-	0.0125	-	1643	Cep8749/Br35 or BPon/PBred
WP1	GBS19261	7B	0-24	0.112	-	0.0092	-	2346	Suz6/Opata
WP1	GBS109034	7B	0-26	0.176	-	0.0056	-	2494	Suz6/Opata
WP1	GBS82247	7B	1-23	0.124	-	0.0016	-	2381	Suz6/Opata
WP1	GBS88335	7B	32-36	0.163	-	0.0096	-	1872	Suz6/Opata
GWAS K + *Lr* model									
WP1	GBS79403	4A	92-94	0.073	-	0.0058	-	2220	Cep8749/Br35 or BPon/PBred
WP1	GBS41840	4A	131-142	0.080	0.0041	0.0180	0.0019	1206[c]	Cep8749/Br35 or BPon/PBred
WP1	GBS41841	4A	131-142	0.080	-		0.0019	1110	Cep8749/Br35 or BPon/PBred
WP1	GBS64791	5B	32-53	0.085	-	0.0133	-	2150	Cep8749/Br35 or BPon/PBred
WP1	GBS51940	5B	63-84	0.157	-	0.0035	-	1643	Cep8749/Br35 or BPon/PBred
WP1	GBS83166	5B	83-84	0.189	-	0.0094	-	1468	Cep8749/Br35 or BPon/PBred
WP1	GBS12036	5B	97-99	0.081	-	0.0089	.-	1934	Cep8749/Br35 or BPon/PBred
WP1	GBS12037	5B	97-99	0.081	-	0.0089	-	1951	Cep8749/Br35 or BPon/PBred
WP2	GBS48333	2D	2-50	0.666	0.0057	-	-	1118	Cep8749/Br35 or BPon/PBred

WP2	GBS45077	4A	20-40	0.837	0.0004	-	-	1297	I. Torcaza, Cep8749/Br35 or BPon/PBred
WP2	GBS67197	4A	70-76	0.206	0.0065	-	-	**1101**	Suz6/Opata
WP2	GBS15155	5B	187-218	0.833	0.0131	-	-	686	Cep8749/Br35 or BPon/PBred
WP2	GBS15156	5B	187-218	0.833	0.0131	-	-	686	Cep8749/Br35 or BPon/PBred
WP2	GBS61864	6B	32-36	0.833	0.0019	-	-	793	I. Torcaza, Cep8749/Br35 or BPon/PBred

[a] Recombination Bin for the SNP markers (Poland et al. 2012)

[b] Difference between the LR AUDPC of BC_1F_6 lines with "0" allele minus the LR AUDPC of BC_1F_6 lines with "1" allele, average of three environments. Bold indicates a negative association between LR resistance and the positive SNP allele and non-bold indicates a positive association between LR resistance and the positive SNP allele at each locus

[c] Average ASE in the three field environments

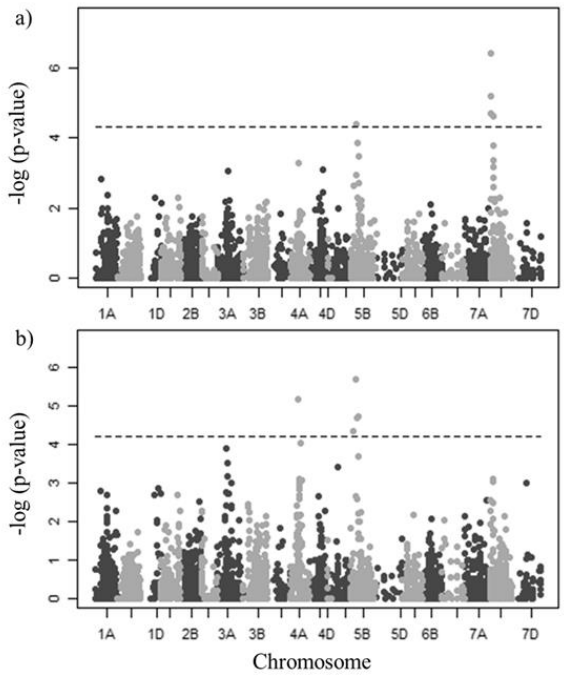

Figure 4. Genome-wide association study (GWAS) for LR AUDPC of WP1 derived from the parental line INIA Tero at Y-2012. LR AUDPC was calculated based on four independent scores of infection coefficient, according to Maccaferri et al. 2010. a) GWAS *K* model and b) GWAS *K+Lr* model; x axis shows the SNPs along each chromosome, y axis is the −log (p-value) for the association. The significance threshold was determined with a FDR α level of 0.05 (*dashed line*). Dotes above the dashed line indicate SNPs that were significant.

We also run the K model with the addition of the presence/absence of the marker alleles for *Lr34* and *Lr68* as SNPs markers in the genotypic matrix. We found that both markers were significant for field resistance in both populations (Fig. 5). We did not find high correlation values between *Lr34* and most of the 55 SNPs on chromosome 7D ($0.00< r^2 < 0.52$), only four SNPs markers had an r^2 value above 0.45 (data not shown). The same analysis between *Lr68* and the significant SNPs markers on chromosome 7B in WP1 detected using the K model showed higher levels of LD ($0.42 < r^2 < 0.55$).

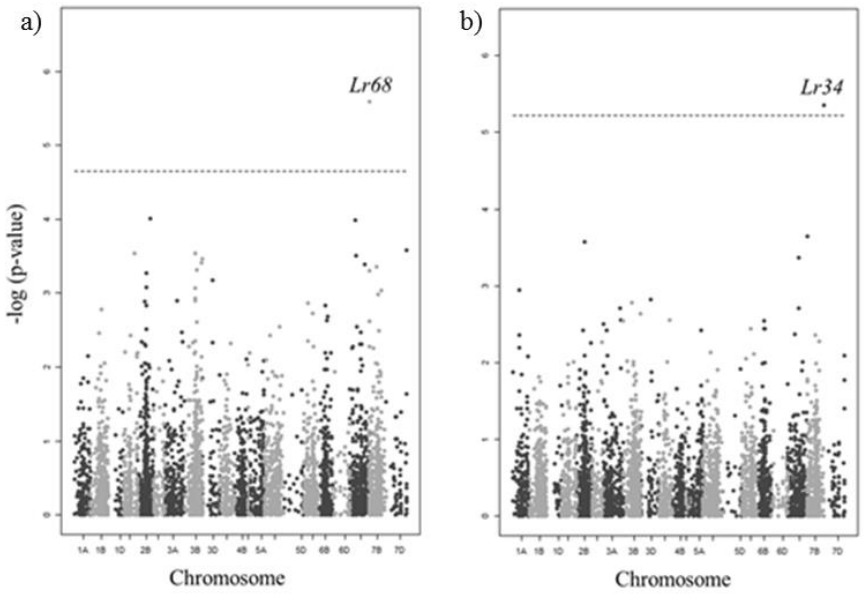

Figure 5. GWAS K model for LR AUDPC from the three environments using the total wheat lines from WP1+WP2. In order to run the GWAS model we added presence/absence for *Lr68* marker (a) or presence/absence for *Lr34* marker (b), to the SNP matrix. x axis shows the SNPs along each chromosome, y axis is the −log (p-value) for the association. The significance threshold was determined with a FDR α level of 0.05 (dashed line). Dots above the dashed line indicate SNPs that were significant.

3.6. DISCUSSION

Introducing effective and more durable resistance in new competitive cultivars represents one of the mayor challenges in breeding for LR resistance in wheat. Using more than two effective R-genes reduces the probability of rapid adaptation of the pathogen population to this resistance, while combinations of 4-5 APR genes may confer high levels of durable resistance (Singh et al. 2011). Even though combinations of both strategies may also be used, it is highly desirable to have a diverse genetic basis of resistance to avoid germplasm vulnerability to the pathogen. Such an approach requires knowing the genes present in the germplasm and which may be introduced to provide diversity. At the same time, it is important to identify new resistance genes or genes combination and introduce them in

commercial cultivars to maintain or increase the levels of resistance and its durability. New available tools for highthrouput genotyping such as GBS allow to more efficiently incorporate previously reported genes through MAS and to identify new sources of resistance.

Leaf rust infection was even and high in all field environments (high LR infection of susceptible checks), which allowed the detection of statistical associations. Although we used three different races to inoculate field experiments, it is possible that other races from the natural *P. triticina* population might also be present in these experiments. The predominant races in the natural population in 2012 and 2013 were TFT-10,20 and MFP-20 (Germán, pers. comm). LR response of BC_1F_6 lines was similar in different environments, as indicated by high correlation between LR AUDPC detected within the GWAS panels used in this study ($r^2 > 0.72$). However, genotype x environment interaction of phenotypic values was significant and therefore the GWAS analysis was performed for each environment individually. With this approach it was possible to detect all the significant SNPs at each environment as well as those expressed in two or all environments under study, which are more meaningful for LR resistance breeding in Uruguay.

Genotyping by sequencing (GBS) is a low-cost and flexible platform for whole-genome profiling. The abundance of molecular markers generated by GBS is excellent for a range of applications and different selection strategies in applied breeding (Poland et al. 2013). However, the potential usefulness of SNPs markers obtained by GBS for GWAS cannot be fully exploited yet, due to the lack of a publicly available reference genome in wheat. Therefore, although we can find genomic regions where SNP markers are located it is not possible to conclude whether these are the same genes or QTLs previously reported in the same position in other studies. This method also allows rapid marker discovery, which can then be used through applied bioinformatics approaches for gene pyramiding in the routine marker selection scheme of breeding programs (Poland et al. 2013). The recent report of a new chromosome-based draft sequence of the hexaploid bread wheat genome will allow the use of new approaches using the GBS data (IWGSC 2014).

In this study, we used small populations (less than 100 individuals). This fact might lead to an overestimation of the effects of QTLs and an underestimation of the number and interactions among QTLs (Melchinger et al. 1998). However, comparison between different population sizes indicates that major QTL can be detected even with relatively small populations (Vales et al. 2005). Even when smaller population sizes could affect the estimation of all QTL and their effect, good phenotypic data allows the detection of the most important ones (Vales et al. 2005). We tested the utility of this kind of markers for GWAS studies using AM of days to heading, a highly heritable trait. We found the same two significant SNPs markers in the three environments for both populations (data not shown) confirming that GWAS using SNPs derived from GBS is a feasible approach to study the genetics of days to heading and most probably other characters, including LR resistance.

We detected significant marker-trait associations for seedling resistance in both populations (Table 2). For WP1 we found significant SNPs markers only for race TFT-10,20. The significant SNP on chromosome 2B (GBS86541) from Cep8749/Br35 or BPon/PBred could be explained by the R-gene *Lr16* (Dyck and Kerber 1971), which is commonly present in regional cultivars and CIMMYT lines (Huerta-Espino et al. 2011; Vanzetti et al. 2011; Germán and Kolmer 2012; Germán and Kolmer 2014). Several SNPs on chromosome 5B donated by the same resistant parents had significant effects on seedling resistance. *Lr52* is also located on chromosome 5B (Hiebert et al. 2005). However, this gene was not present in the parents since it was effective (low IT 1;=) to all races used in the seedling studies. Therefore, QTL on chromosome 5B was explained by an unknown R-gene. Two SNPs from this chromosome were also significant in WP2 to the same race.

Higher numbers of significant SNPs markers were found in WP2, partly due to seedling resistance of I. Torcaza to two of the three races used to screen the populations. We found a significant QTL for race TPR-20,39, on chromosome 1A contributed by I. Torcaza. This QTL was probably explained by the R-gene *Lr10* (Dyck and Kerber 1971), since this gene was present in I. Torcaza (Germán and Kolmer 2012; Germán and Kolmer 2014) and TPR-20,39 is the only avirulent race

for *Lr10* used in this study. Similarly to WP1, the significant marker-trait associations for race TFT-10,20 in this population may be explained by *Lr16* and an unknown R-gene on chromosomes 2B and 5B, respectively. QTL on chromosome 1B to race MDR-10,20, could be explained by *Lr33* (Dyck et al. 1987), however this gene was not present in the parents since it was effective (low IT 2) to all races used in the seedling studies. The significant SNPs GBS48333 on chromosome 2D contributed by any of the parents except Suz6/Opata for race TFT-10,20 could be explained by gene *Lr2* (Dyck and Samborski 1974), however this gene was not present in I. Torcaza (Germán and Kolmer 2012; Germán and Kolmer 2014). The other significant SNPs on chromosome 2D for race MDR-10,20 contributed by I. Torcaza, had negative ASE and was not *Lr2* since MDR-10,20 was virulent for I. Torcaza and avirulent for *Lr2* alelles. We were not able to assign a possible *Lr* gene to the others significant SNPs markers on chromosomes 1D, 3A, 4A, 7A and 7D due to either the virulence pattern of the race or the very low probability of previously reported genes being present in our plant material. These could be different seedling resistance genes, although this result should be confirmed, due to the limitation of the genotyping methodology previously discussed. The utility of these genomic regions is limited for breeding for resistance to LR since these conferred seedling resistance to one race and did not confer field resistance.

We detected significant QTLs for field resistance in both populations (Table 3). Most of these SNPs markers were found on chromosomes where genes or QTL influencing resistance to LR were previously reported (Crossa et al. 2007; Li et al. 2014), providing an independent validation of this study. The three *P. triticina* races used are virulent to major LR APR-R genes *Lr12, Lr13, Lr22b* and *Lr37,* therefore these should not be detected in the association analysis. Other reported APR R-genes (*Lr22a* and *Lr35*) are probably absent in our materials, therefore we could concluded that the resistance was conferred by other genes.

For WP1, the QTL present on chromosome 7B from the resistant parent Suz6/Opata, detected with the K model for Y-2012 (Fig. 4a) is probably the APR gene *Lr68* which is frequent in CIMMYT germplasm (Singh et al. 2011; Herrera-

Foessel et al. 2012). Previous reports indicate that this gene is the most effective in Uruguay, rather than *Lr34* (Lillemo et al. 2011; Silva et al. 2013a[5]). The ASE was the highest of all QTLs detected in all environments (Table 3). Further evidence of presence of this gene was demonstrated when the marker *cs7BLNRR* for *Lr68* (Herrera-Foessel et al. 2012) was used as a fixed covariate in the K + *Lr* model, the QTL on chromosome 7B was already accounted for (Fig. 4b). Additionally we found that *Lr68* was significant in all environments when the presence/absence of *Lr68* CAPS marker on chromosome 7B was added to the genotypic matrix (Fig. 5a). High LD values ($0.42 < r^2 < 0.55$) were found between *Lr68* and four significant SNPs on chromosome 7B for K model, where many QTL involved in ARP for LR have been reported (Li et al. 2014).

The QTL on chromosome 4A including GBS41840 and GBS41841, donated by the resistant parent Cep8749/Br35 or BPon/PBred, was only detected in WP1 when the effect of *Lr34* and *Lr68* were removed by using the K + *Lr* model since they might be masking other possible QTL with minor effects (Bernardo 2014). This QTL was significant in all environments, with an intermediate ASE on LR (Table 3), and was not detected at the seedling stage for any race, therefore probably confers APR. Since no other QTL for APR-PR has been reported on chromosome 4A (Li et al. 2014), we are reporting a new source of ARP-RP to LR. More studies are required to confirm our results and obtain more precisely information for this QTL.

The QTL on chromosome 5B detected in WP1, probably donated by the resistant parent Cep8749/Br35 or BPon/PBred, was expressed in seedlings to race TFT-10,20 and under field conditions at Y-2012 for both GWAS models. This was not conferred by the R-gene *Lr52* (Hiebert et al. 2005), effective in Uruguay to races used in this study and other frequent races present in 2012 and 2013 (Germán per comm). Anyway this QTL was conferred by a mayor R-gene since the SNPs markers were significant at the seedling stage. Study more in deep this genomic region is required to confirm our result.

[5] La letra a en Silva et al. 2013a es a efectos de diferenciar citas en el conjunto de citas de esta tesis

No significant associations involving chromosome 7D were found in WP1 even when the *Lr34* marker, located on chromosome 7D, was segregating in WP1. *Lr34* significantly reduced LR AUDPC in Uruguay in previous reports (Lillemo et al. 2011; Silva et al. 2013a). An evidence of the expression of *Lr34* without detection by the GWAS analysis was that the marker for this gene was significant when the K model was run adding the presence/absence of the marker *csLV34 + LR34PLUSR* (Lagudah et al. 2006; Lagudah et al. 2009) as a SNPs in the genotypic matrix (Fig. 5b). Additionally LD values for *Lr34* and all the SNPs markers on chromosome 7D ranged between $0.00 < r^2 < 0.52$, with only four of the 55 SNPs markers above 0.45. The low marker coverage on chromosome 7D and low populations sizes used in this study, are probably responsible for no significant SNP association detected in this region.

For WP2 we did not find any significant QTL for field resistance using the K model. When the K + *Lr* model was used, removing the possible effects of *Lr34* and *Lr68* and probably unmasking other possible QTL (Bernardo 2014), significant marker-trait associations appeared on chromosomes 2D, 4A, 5B and 6B only at LE-2012. These associations were not detected at the seedling stage; therefore this resistance is expressed in adult plants. Significant markers on chromosomes 4A and 5B were different from those detected in WP1. Even when the marker on 4A is different from the marker in WP1, the same considerations could be made. The SNPs for 5B and 6B cannot be differentiated from previously reported QTLs (Li et al. 2014) due to the lack of precise position information. Additionally, the same results as WP1 were obtained when running the GWAS K model adding the markers for *L34* or *Lr68* to the genotypic matrix.

We confirmed that genes *Lr10*, *Lr16* and *Lr34*, previously reported to be present in Uruguayan germplasm, were also present in our populations. We highlight the important effect of *Lr68* in reducing LR infection in Uruguay and underline the role of the QTLs on chromosome 4A and 5B putatively harboring possible new genomic regions associated to field and seedling LR resistance. These QTLs are relevant for the PMGT at INIA-Uruguay, thus MAS could be implemented to

pyramid these genomic regions for resistance to LR in background that carry other resistance genes. Additionally, lines carrying desirable resistance alleles at both QTLs could be identified within adapted breeding germplasm and used in crosses. In summary, we provide information about known and possible novel resistance genes, some of which could be used for breeding efforts to reduce the vulnerability to LR in Uruguay.

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4. DISCUSION Y CONCLUSIONES GENERALES

La RH del trigo es una de las enfermedades más importantes del cultivo en Uruguay y en la región (Argentina, Brasil, Chile, Paraguay) por causar epidemias generalizadas cada año (Germán *et al.,* 2007). La investigación y el mejoramiento genético en INIA a lo largo del último siglo han ayudado a reducir las pérdidas provocadas por la presencia de este patógeno. Sin embargo, razas con nuevas combinaciones de virulencia continúan emergiendo y causando pérdidas significativas, siendo los niveles altos de susceptibilidad a esta enfermedad una de las principales causas del reemplazo de variedades en nuestro país (Germán *et al.*, 2007). El uso de combinaciones de genes de resistencia mayores, varios genes de resistencia menores o ambos, ofrece una oportunidad única para lograr niveles de resistencia durables, sin la necesidad de utilizar fungicidas y así mejorar los ingresos de los agricultores al tiempo de proteger el medio ambiente. La resistencia a RH en cultivares del PMGT y cultivares comerciales utilizados en Uruguay conferida por combinación de genes de efecto mayor, en general ha sido poco durable, (Germán y Kolmer 2014; Gemán y Kolmer, 2012) debiéndose monitorear constantemente los cambios de comportamiento de los cultivares comerciales, asociados a cambios de virulencia en el patógeno.

En los últimos veinte años, el desarrollo de herramientas biotecnológicas ha brindado la oportunidad para profundizar en el conocimiento de resistencia a RH, permitiendo acelerar el desarrollo de germoplasma con resistencia mediante la acumulación de genes de resistencia. Por otro lado, el desarrollo tecnológico en el área de la secuenciación ha permitido la puesta a punto de técnicas de genotipado masivo, asociadas a disminución de costos y simplificación de la metodología de obtención de marcadores moleculares. Recientemente se ha propuesto a la metodología de genotipado por secuenciación como una herramienta alternativa, de lata eficiencia y bajo costo, para la simultanea identificación de marcadores SNPs y genotipado de individuos en trigo (Poland *et al.,* 2012). La necesidad de desarrollar nuevas metodologías para la selección de características complejas junto a los avances en el área del genotipado, han llevado al desarrollo de nuevas metodologías

de selección como la selección asistida por marcadores (SAM) y la selección genómica. La SAM ha sido particularmente exitosa en el caso de resistencia de tipo cualitativo y de herencia sencilla para las cuales se cuenta con alto número de loci de resistencia mapeados y marcadores informativos, como lo es el caso de la resistencia a RH (Maccaferri *et al.,* 2010; Crossa *et al.,* 2007). Con el objetivo de avanzar en el conocimiento de la base genética de la resistencia a RH de trigo en Uruguay, nos propusimos contribuir al conocimiento del efecto de los genes de RPA a RH, *Lr34*, *Lr68* y *Sr2*, en nuestras condiciones y estudiar la base genética de la resistencia a RH en fuentes de resistencia con esta característica, utilizando un contexto genético elite derivado del PMGT de INIA.

Los resultados revelaron que el efecto del gen *Lr68* fue mayor que el efecto del *Lr34* bajo las condiciones de Uruguay al contrario que lo reportado para otros ambientes, demostrando la importancia de incorporar este gen en el germoplasma local con el fin de obtener buenos niveles de resistencia durable a RH. A su vez, el gen *Sr2* no disminuyó la RH o la aumentó, pero incrementó el efecto del gen *Lr68* y *Lr34*. La combinación de los genes *Lr68*, *Lr34* y *Sr2* fue la que más redujo el desarrollo de RH.

Se identificaron regiones genómicas asociadas a resistencia a RH tanto en el estado de plántula como a campo, en etapas avanzadas del desarrollo. Se confirmó que los genes *Lr10*, *Lr16* y *Lr34* estaban presentes en nuestras poblaciones. A su vez, el QTL para RPA en el cromosoma 7B parece estar explicado por el efecto del gen *Lr68*, el gen de RPA más efectivo en Uruguay. Los QTLs en el cromosoma 4A y 5B parecerían ser nuevos QTLs asociados a resistencia a RH. Esta información podrá ser utilizada para continuar con los esfuerzos de mejoramiento de trigo con el fin de obtener resistencia durable a RH en Uruguay.

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