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ORIGINAL ARTICLE

Characterization of *Lr75*: a partial, broad-spectrum leaf rust resistance gene in wheat

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Abstract

Key message **Here, we describe a strategy to improve broad-spectrum leaf rust resistance by marker-assisted combination of two partial resistance genes. One of them represents a novel partial adult plant resistance gene, named *Lr75*.**

Abstract Leaf rust caused by the fungal pathogen *Puccinia triticina* is a damaging disease of wheat (*Triticum aestivum* L.). The combination of several, additively-acting partial disease resistance genes has been proposed as a suitable strategy to breed wheat cultivars with high levels of durable field resistance. The Swiss winter wheat cultivar 'Forno' continues to show near-immunity to leaf rust since its release in the 1980s. This resistance is conferred by the presence of at least six quantitative trait loci (QTL), one of which is associated with the morphological trait leaf tip necrosis. Here, we used a marker-informed strategy to introgress two 'Forno' QTLs into the leaf rust-susceptible Swiss winter wheat cultivar 'Arina'. The resulting backcross line 'Arina*LrFor*' showed markedly increased leaf rust resistance in multiple locations over several years. One of the introgressed QTLs, *QLr.sfr-1BS*, is located on chromosome 1BS. We developed chromosome 1B-specific microsatellite markers by exploiting the Illumina survey sequences of wheat cv. 'Chinese Spring' and mapped *QLr.sfr-1BS* to a 4.3 cM interval flanked by the SSR markers *gwm604* and *swm271*. *QLr.sfr-1BS* does not share a genetic location with any of the described leaf rust resistance genes present on chromosome 1B. Therefore, *QLr.sfr-1BS* is novel and was designated as *Lr75*. We conclude that marker-assisted combination of partial resistance genes is a feasible strategy to increase broad-spectrum leaf rust resistance. The identification of *Lr75* adds a novel and highly useful gene to the small set of known partial, adult plant leaf rust resistance genes.

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Introduction

Hexaploid bread wheat (*Triticum aestivum* L.) is one of the three most important cereal crops with an annual global production of 713 million tonnes (FAOSTAT 2013 http://faostat3.fao.org). Wheat is attacked by many pathogens of which the fungal rust diseases are the most widespread and devastating. There are three species of wheat rust: leaf or brown rust (*Puccinia triticina*), stripe or yellow rust (*Puccinia striiformis* f. sp. *tritici*) and stem or black rust (*Puccinia graminis* f. sp. *tritici*). Leaf rust is the most common and most widespread rust disease (Bolton et al. 2008; Kolmer 2013). Yield losses caused by leaf rust are characterized by reduced kernel weight and a lower number of kernels per spike (Bolton et al. 2008; Huerta-Espino et al. 2011).

The release of crop varieties with high levels of durable disease resistance represents the most sustainable strategy to reduce production losses caused by fungal diseases. To

date, 74 leaf rust resistance (*Lr*) genes have been described (McIntosh et al. 2013). Most *Lr* genes confer race-specific resistance. Rapid pathogen adaptation often results in the emergence of new virulent leaf rust races and consequently a breakdown of race-specific *Lr* resistance (Huerta-Espino et al. 2011; McIntosh et al. 2013). Hence, there is a need to identify *Lr* genes that show a more durable resistance in the field. A particular type of durable disease resistance is referred to as slow-rusting resistance. It is characterized by a partial resistance phenotype that is often only effective at the adult plant stage but not in seedlings (Caldwell 1968). This type of resistance is therefore also referred to as adult plant resistance (APR). Because of their partial nature it is challenging to combine several APR genes in a single genotype through classical breeding. Marker-assisted selection can serve as a suitable approach to track the presence of APR loci in breeding programs. It has been described that the combination of two or more APR loci with additive effects can result in near immune resistance levels (Singh et al. 2000; Lillemo et al. 2011).

Wheat breeders at the International Wheat and Maize Improvement Center (CIMMYT) have exploited the strategy of combining slow-rusting genes in wheat disease resistance breeding. The CIMMYT wheat breeding material has therefore been the most important source for the discovery of slow-rusting genes. Cultivars such as 'Frontana', 'Pavon76', 'Parula', 'Trap' or 'Mango' have been released during the past decades using CIMMYT bread wheat germplasm and they show near immune resistance responses to wheat leaf rust by the additive effect of 3–4 slow-rusting genes (Singh and Rajaram 1991; Singh et al. 1998, 2005). The main APR genes identified in these cultivars are *Lr34/Yr18/Sr57/Pm38*, *Lr46/Yr29/Pm39* and *Lr68*. There are also other sources of slow-rusting resistance genes besides CIMMYT germplasm. For example, the gene *Lr67/Yr46/Sr55/Pm46* has been identified in the common wheat accession PI250413 which was collected from Pakistan (Dyck and Samborski 1979; Moore et al. 2015). Some of these genes are mapped at high resolution or cloned such as *Lr34/Yr18/Sr57/Pm38, Lr67/Yr46/Sr55/Pm46* and *Lr68* (Lagudah et al. 2006; Krattinger et al. 2009; Moore et al. 2015). On the other hand, slow-rusting resistance has only been poorly studied in the Central European winter wheat genepool.

The Swiss winter bread wheat cultivar 'Forno' (pedigree: 'NR72837 × Kormoran') was released in Switzerland in 1986. 'Forno' shows near immune levels of leaf rust resistance in the field against all leaf rust isolates tested so far. At least six QTLs contribute to this remarkable leaf rust resistance of 'Forno' (Schnurbusch et al. 2004). Among them is the well-known multi-pathogen resistance gene *Lr34* located on chromosome 7D. *Lr34* encodes for an ATP-binding cassette transporter (Krattinger et al. 2009) and it explained 33–43 % of the phenotypic variance in the QTL study of Schnurbusch et al. (2004). *Lr34* is associated with leaf tip necrosis (LTN), a senescence-like process. LTN is often seen as an unwanted trait in Western European wheat cultivars and *Lr34* has therefore only rarely been used in the Western European wheat breeding programs (Kolmer et al. 2008). A second major QTL for leaf rust resistance, *QLr.sfr-1BS,* was identified on chromosome arm 1BS. *QLr.sfr-1BS* explained 28–32 % of the phenotypic variance and was not linked with LTN. In the 'Arina' × 'Forno' recombinant inbred line (RIL) population generated by Schnurbusch et al. (2004), *QLr.sfr-1BS* was mapped to an interval of 16 cM on chromosome 1BS close to the microsatellite marker *gwm604* (Schnurbusch et al. 2004). This locus interacted with four other QTLs: *Lr34*, two minor QTLs that were contributed by the susceptible parent 'Arina' and *QLr.sfr-7BL*, a minor QTL contributed by 'Forno' that was not linked to LTN. In a different mapping population derived from a cross of 'Forno' with the spelt wheat cv. 'Oberkulmer', Messmer et al. (2000) identified six QTLs for durable leaf rust resistance in cultivar 'Forno'. Three of these QTLs were not detected by Schnurbusch et al. (2004). The strongest QTL detected in the 'Forno' × 'Oberkulmer' population was on chromosome 7BL with a phenotypic variance of 35.8 %. This QTL fell into the same genetic interval as *QLr.sfr-7BL* identified by Schnurbusch et al. (2004). Also, a QTL on chromosome 1BS was identified by Messmer et al. (2000) that explained 10.6 % of the phenotypic variance across four environments. This is most likely be the same QTL as *QLr.sfr-1BS* because it was identified in the same genetic interval as *QLr.sfr-1BS* in the 'Arina' × 'Forno' RIL population.

The objectives of the research presented here were (1) the marker-assisted introgression of the two leaf rust resistance QTLs, *QLr.sfr-1BS* and *QLr.sfr-7BL* into the popular but leaf rust-susceptible Swiss winter wheat cultivar 'Arina' and (2) the mapping of *QLr.sfr-1BS*, a yet uncharacterized, partial leaf rust APR gene that was designated as *Lr75*.

Materials and methods

Plant material

Two different mapping populations were used in this study. The first population consisted of 240 recombinant inbred lines (RIL) generated from a cross of two Swiss winter wheat cultivars, 'Arina' and 'Forno' (Schnurbusch et al. 2004). A second near isogenic line (NIL) population was developed from a cross of 'Arina' and a back-cross line 'Arina*LrFor*' (Arina*3/Forno). The recombinants of the 'Arina' × 'Arina*LrFor*' NIL population were phenotyped

qualitatively as F_3, F_4 and F_5 rows in the field in Reckenholz, Switzerland during 2013, 2014 and 2015.

Selection of 'Forno' leaf rust QTLs for backcrossing

For re-evaluation of the original phenotypic data of the 'Arina' × 'Forno' RIL population developed by Schnurbusch et al. (2004), we grouped a subset of 117 RILs into different classes based on the availability of unambiguous marker information for the target regions of the three QTLs on chromosomes 1BS, 7DS and 7BL. These groups were made based on the following markers: *gwm604* and *gwm131* for *Lr75*; *cssfr1* and *cssfr2* for *Lr34* (Lagudah et al. 2009) and *ksuD2* and *gbxGb218* for *QLr.sfr-7BL*.

Development of near isogenic lines, 'Arina*Lr75*', 'Arina*QLr.sfr-7BL*' and 'Arina*LrFor*'

In order to introgress both *Lr75* and *QLr.sfr-7BL* into the susceptible cv. 'Arina', 101 BC_2F_5 back-cross lines (Arina*3/Forno) were generated as described by Krattinger et al. (2009). The back-cross lines were screened with the flanking markers of the *Lr75* target region (*barc128–gwm131*). Lines that had the 'Forno' alleles for both markers were further screened with the microsatellite markers *gwm146* and *gwm344* for presence of the *QLr.sfr-7BL* region and for absence of *Lr34* with the diagnostic markers *cssfr1* and *cssfr2* (Lagudah et al. 2009). The resulting backcross line carrying *Lr75* and *QLr.sfr-7BL* was named 'Arina*LrFor*'. We developed two additional near isogenic lines, 'Arina*Lr75*' and 'Arina*QLr.sfr-7BL*', that carry individual gene introgressions. For this, 'Arina*LrFor*' was crossed with 'Arina' and segregating progeny homozygous for either *Lr75* or *QLr.sfr-7BL* were selected based on the markers described above. In addition, a near isogenic line 'Arina*Lr34*' with *Lr34* in the genetic background of susceptible cv. 'Arina' was also generated (Arina*4/Forno). The presence of *Lr34* was confirmed with the *Lr34* diagnostic markers *cssfr1* and *cssfr2* (Lagudah et al. 2009).

Characterization of leaf rust resistance in the field

For field trials in Switzerland, the parental lines, 'Arina', 'Forno, 'Arina*Lr75*', 'Arina*QLr.sfr-7BL*', 'Arina*LrFor*', 'Arina*Lr34*' and the recombinants were sown in randomized 5-row ×1.3 m plots in two replications with 40 seeds per row. Parental lines were replicated after 20 plots and one parental line was sown per plot. The first and the last row in each plot consisted of spreader rows containing a 1:1:1 mixture of highly susceptible wheat lines 'Morocco', 'Bernina' and 'Arina' to facilitate high and uniform pathogen density in the field. All field trials were inoculated with a mixture of 16 Swiss leaf rust isolates as described in Messmer et al. (2000). Inoculation was started by planting artificially infected plants into the spreader rows. Repeated leaf rust observations were made throughout the growing season. The final leaf rust severity on the flag leaves of the parental lines and the population was recorded when the susceptible cv. 'Arina' displayed leaf rust infection levels of 60 % or more.

'Arina', 'Forno', 'Arina*LrFor*' and 'Arin*Lr34*' were also tested in the fields in Cobbitty, Australia in 2014. The lines were planted at the Karalee site of the Plant Breeding Institute of the University of Sydney. Leaf rust-susceptible genotype 'Sonora' was used in spreader rows. Urediniospores of *P. triticina* (Pt) pathotype 76-1, 3, 5, 7, 9, 10, 12 +*Lr37* (Plant Breeding Institute Culture Number = 621) and 10-1, 3, 9, 10, 11, 12 (Plant Breeding Institute Culture Number = 592) were used for infections. The virulence/avirulence formulae of Pt pathotypes which were used to test the parental lines is provided in Online Resource 1. Disease severity to leaf rust on the flag leaves of adult plants in the field were recorded when 'Arina' showed an infection level of 60 %.

Characterization of leaf rust resistance at the seedling stage

The parents 'Arina', 'Forno', 'Arina*Lr75*', 'Arina*QLr.sfr-7BL*' and 'Arina*LrFor*' along with 'Arina*Lr34*' were characterized at the seedling stage in the greenhouse with the seven Swiss isolates 91,047, 96,002, 95,219, 93,012, 96,209, 95,001 and 90,035 that were also used in the isolate mixture for field infections. For each line, 20–25 seeds were sown in two replicates in soil (Rasenerde [20 % org. matter, pH ($CaCl_2$) 6.5, 1.4 g/L salt content (KCl), filler (DIN EN 12580)], ökohum GmbH, Herrenhof, Switzerland) in pots with a diameter of 13 cm. After treatment with growth inhibitor (Cycocel® Extra (4 mL/L), Omya AG, AGRO, Oftringen, Switzerland) and fertilizer (Wuxal® Profi (2–3 mL/L), Maag Garden, Syngenta, Düsseldorf, Germany), they were grown for 10 days under diurnal conditions (16 h light/20 °C, 8 h dark/16 °C, 70 % humidity). At the two-to-three-leaf stage (approx. after 10 days) the plants were inoculated with urediniospores suspended in oil (3 M™ Fluorinert™ FC-43, 3 M Electronics, Zwijndrecht, Belgium). After inoculation, the plants were allowed to air-dry for 30 min before they were placed in darkness for 24 h at 16 °C with 95 % humidity. Afterwards, the plants were transferred to growth chambers providing 16 h light/20 °C, 8 h dark/16 °C, 70 % humidity. The disease was assessed 10 days after inoculation (dai) using the 0–4 infection type scoring system described by Roelfs et al. (1992).

Measurement of pustule density of urediniospores

Pustule density of the uredinial infection was measured on field-infected flag leaves of 'Arina', 'Forno', 'Arina*LrFor*' and 'Arina*Lr34*' when the plants were between the Zadoks growth stages 55–69 i.e. from half ear emergence to complete anthesis (Zadoks et al. 1974). For each line, leaves from two different plots were sampled and nine flag leaves per plot were randomly selected. For each leaf, a surface of 4 cm in the middle of the leaf was marked. The marked area was photographed at 3 different time points (84, 87 and 93 days after inoculating the spreader rows) with a Nikon camera using a macro objective lens. Subsequently, the images were analyzed with the image analysis software ImageJ 1.48 (Abràmoff et al. 2004). The area of interest (4 cm × width of the leaf) was calculated using the polygon selection tool and the pustules were counted using the oval selection tool. Not fully visible pustules at the edge of the leaf or the marked area were ignored. The average pustule density (number of pustules per area) was calculated for each of the four lines at different time points. Differences in pustule density among lines and time points were assessed using ANOVA. The distribution of the residual was tested with the Shapiro test and pustule density data were cube root transformed to fit normal distribution of the residuals. Then, a full model that included the line, time, plots effect and their potential interactions was used. As only marginally significant ($p = 0.051$), the plot effect was dropped from the full ANOVA. Only the line, time effects and their interaction were kept for further analysis. Pairwise p value between lines and time points were computed with the Tukey's HSD (Honestly Significant Difference) test. All analyses were performed using R v.3.1.3 (R Core Team 2016).

Marker analysis and genetic linkage mapping

The parents and BC_3F_2 recombinants developed from the cross of 'Arina' × 'Arina*LrFor*' were grown in the greenhouse and leaf tissue was harvested from the seedlings (8–10 days old). DNA was extracted with a CTAB (cetyl trimethyl ammonium bromide) protocol as described by Stein et al. (2001). The quantity and concentration of DNA was measured using a NanoDrop spectrophotometer (Witec AG, Lucerne, Switzerland). The final concentration was standardized to 650 ng/µL. Dilutions of 13 and 65 ng/µL were used in PCR reaction for 6 % LiCOR gel (LiCOR DNA Sequencer 4200) and agarose gel electrophoresis, respectively. The simple sequence repeat identification tool, SSRIT (http://www.archive.gramene.org/db/markers/ssrtool) was used to identify the repeat motifs and SSR primers were designed using the software program Primer3 (v. 0.4.0). The PCR products of SSR primers were resolved on 6 % LiCOR gel. SSR markers were named as 'swm' (swiss wheat microsatellites). Primer sequences along with their repeat motifs are given in Online Resource 2. The genetic linkage map was constructed on a subset of F_2-derived F_3 lines (lines with missing phenotypic data were excluded) by calculating the recombination frequency between the markers. MapChart 2.3 (Voorrips 2002) was used to draw the linkage map.

To further saturate the 8 cM target region between *wmc230* and *gwm18* with additional markers, we exploited the flow-sorted Illumina survey sequences of chromosome 1BS of wheat cv. 'Chinese Spring' (http://wheat-urgi.versailles.inra.fr/Seq-Repository), the 1BS physical map generated by Raats et al. (2013) and information obtained by comparative genetics from a 1BS reference zipper based on synteny information of *Brachypodium*, rice and sorghum. The 1BS reference zipper was constructed in a similar manner as described by Breen et al. (2013). The gene-containing 1BS wheat Illumina sequences were physically anchored to BAC end sequences of wheat chromosome 1BS. These Illumina sequence contigs were further anchored to the reference zipper. The sequences of the flanking markers *wmc230* and *gwm18* were aligned against the integrated model of Illumina sequence contigs and reference zipper and the target region was defined. Then, we searched the Illumina sequences for microsatellite motifs within this target region and designed primers flanking the repeat motifs.

Deletion bin mapping

Chromosome 1B specificity of the SSR markers was confirmed by the absence of amplification in nulli-tetrasomic lines of cultivar 'Chinese Spring'. Further, to determine the bin localization of all SSR markers, a set of 11 deletion lines for chromosome 1B was used along with two ditelosomic lines of wheat cv. 'Chinese Spring'. Six deletion lines for the short arm (1BS4-sat-0.52, 1BS18-sat-0.50, 1BS2-sat-1.06, 1BS9-0.84, 1BS10-0.50 and 1BS1-0.35) and five deletion lines for the long arm (1BL11-0.23, 1BL6-0.32, 1BL1-0.47, 1BL2-0.69 and 1BL3-0.85) were used (Fig. 4b). Two ditelosomic lines DT1BS where the 1BL arm is missing and DT1BL where the 1BS arm is absent were also used. The fraction length (FL) value of each deletion line depicts the length of the remaining chromosome arm from the centromere after deletion relative to the length of the complete arm (Endo and Gill 1996). All the cytogenetic stocks were kindly provided by J. Raupp, Wheat Genetic Resource Centre, Department of Plant Pathology, Kansas State University, USA.

Results

Selection of 'Forno' leaf rust QTLs for backcrossing

Schnurbusch et al. (2004) identified *QLr.sfr-1BS* (subsequently referred to as *Lr75*) as the strongest leaf rust resistance QTL that was not associated with LTN in the 'Arina' × 'Forno' RIL population. *Lr75* interacted with the minor QTL, *QLr.sfr-7BL*. Based on this information from the QTL study by Schnurbusch et al. (2004) we selected these two loci as candidates for backcrossing into the leaf rust-susceptible cultivar 'Arina'. To validate our selection, we first re-evaluated the original phenotypic data of the 'Arina' × 'Forno' RIL population in order to estimate the phenotypic effect of this gene combination. For this, we grouped RIL lines based on marker information and compared the phenotypes of different groups. *Lr34* was also included for comparison.

The RIL group that only contained *Lr34* showed the strongest leaf rust resistance provided by a single QTL. The AUDPC value for the group with *Lr75* alone (238.0) was lower than for the group with no resistance QTL (388.6) although the difference was not significant ($p = 0.16$). No significant difference in AUDPC values was observed between the group with only *QLr.sfr-7BL* and the group with no QTL. However, the combination of *Lr75* and *QLr.sfr-7BL* resulted in leaf rust resistance levels comparable to *Lr34,* confirming the original finding that *Lr75* and *QLr.sfr-7BL* are additive (Fig. 1). All gene combinations with *Lr34* were associated with LTN whereas the ones without *Lr34* were not. Based on the re-evaluation of these original RIL data we expected that the combination of *Lr75* and *QLr.sfr-7BL* would result in partial leaf rust resistance levels similar to *Lr34* but without LTN. *Lr75* and *QLr.sfr-7BL* were therefore co-introduced into the genetic background of 'Arina' through marker-assisted backcrossing.

Evaluation of near isogenic lines 'Arina*Lr75*', 'Arina*QLr.sfr-7BL*' and 'Arina*LrFor*' for field resistance

In order to introgress both *Lr75* and *QLr.sfr-7BL* into the susceptible cv. 'Arina', 101 BC_2F_5 back-cross lines

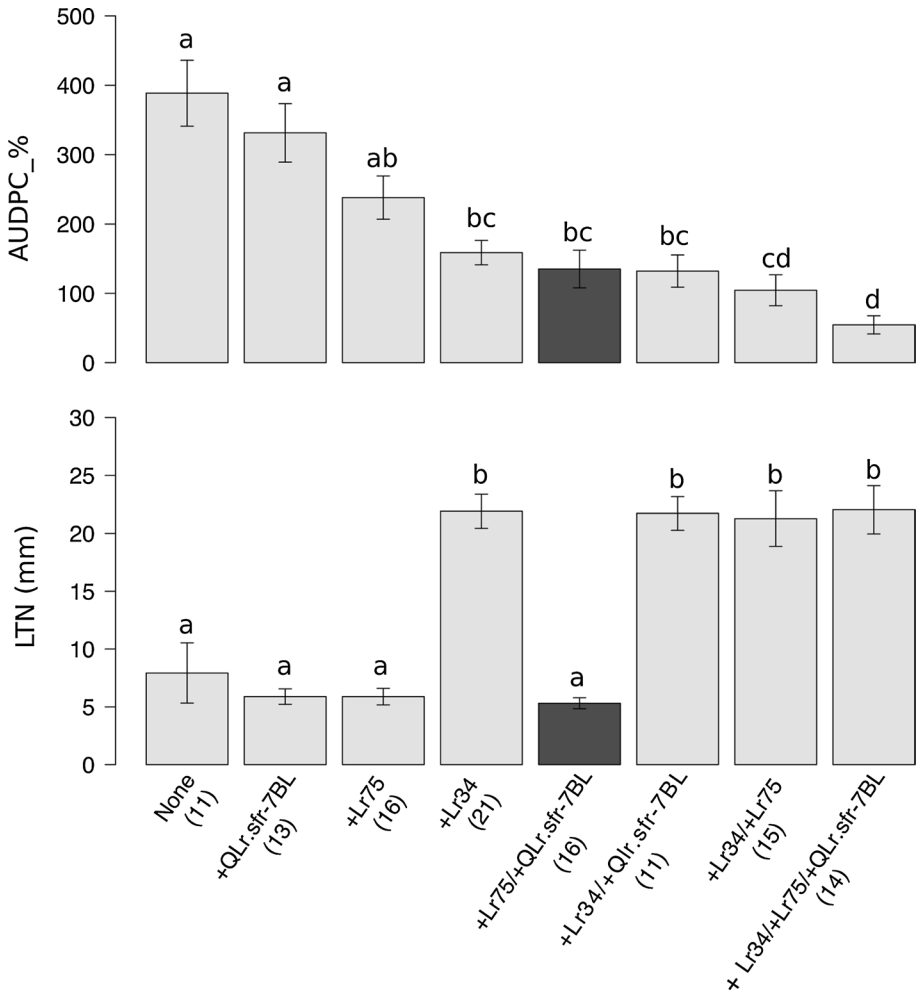

Fig. 1 Phenotypic effect of different leaf rust resistance QTL combinations. The phenotypic data of Schnurbusch et al. (2004) were re-evaluated for area under disease progress curve (AUDPC_ %) (*top graph*) and leaf tip necrosis (LTN) in millimeter (mm) (*bottom graph*) on groups of RIL lines. The group of RILs with both *Lr75* and *QLr.sfr-7BL* is highlighted in *black*. *Numbers* in *brackets* indicate the number of RIL lines present in each class. *Letters* indicate *lines* with equivalent resistance levels ($p > 0.05$, Tukey's HSD test) and *error bars* represent standard errors of the mean

(Arina*3/Forno) were screened with the flanking markers of the *Lr75* region (*barc128–gwm131*). Twelve of the 101 backcross lines showed the 'Forno' alleles for both the flanking markers in the *Lr75* region. By screening these twelve lines with *Lr34* diagnostic markers, two lines were positive for *Lr34* and were therefore excluded. From the remaining 10 lines, one line, 'Arina*LrFor'*, showed the 'Forno' alleles for the flanking markers of both *Lr75* and *QLr.sfr-7BL* region.

The lines, 'Arina*LrFor*' and 'Arina*Lr34*' were evaluated together with 'Arina' and 'Forno' for leaf rust resistance in Switzerland and Australia (Table 1). In addition, the lines 'Arina*Lr75*' and 'Arina*QLr.sfr-7BL*' were evaluated for leaf rust resistance only in Switzerland in 2016. 'Arina*Lr75*' and 'Arina*QLr.sfr-7BL*' both showed a weak partial resistance response with a final disease severity of 40–60 % and 50–70 %, respectively (Table 1; Fig. 2). The susceptible control 'Arina' had leaf rust infection levels of 60–100 % except for 2014 crop season where 'Arina' had leaf rust infection levels of 50–80 %. This was due to the emergence of stripe rust and low temperature at the time of rust development which resulted in the minimum infection level of 50 % in 'Arina'. Despite the relatively weak contributions towards resistance of *Lr75* and *QLr.sfr-7BL* alone, the gene combination in 'Arina*LrFor*' resulted in good levels of partial leaf rust resistance comparable or even stronger than *Lr34*. (Table 1; Fig. 2). 'Arina*LrFor*' displayed a slow-rusting response with a final leaf area coverage ranging from 14 to 40 % in comparison to 'Arina*Lr34*' which had final leaf area coverage of 5–56 %. 'Forno' displayed a near-immune response which is due to the combination of *Lr75*, *Lr34, QLr.sfr-7BL* and several minor QTLs.

The lines 'Arina*LrFor*' and 'Arina*Lr34*' along with 'Arina' and 'Forno' were also tested for leaf rust resistance in Australia. Similar to the results obtained for the Swiss environment, 'Arina*LrFor*' showed increased leaf rust resistance in the field in Australia (Table 1; Online Resource 3). Hence, 'Arina*LrFor*' showed good levels of partial leaf rust resistance in two environments.

Slow-rusting resistance genes are generally associated with a longer latency period, lower uredinial density and smaller uredinial size (Das et al. 1993). The line 'Arina*LrFor*' has a slow-rusting phenotype as shown in Fig. 2. Measurement of pustule density on the flag leaves of 'Arina', 'Forno', 'Arina*LrFor*' and 'Arina*Lr34*' showed that 'Forno' displayed a significantly lower number of pustules than the other three lines and, in agreement with the near-immune phenotype, no significant increase in the pustule

Table 1 Adult plant field leaf rust response of 'Arina*LrFor'*, 'Arina', 'Forno', 'Arina*Lr75'*, 'Arina*QLr.sfr-7BL'* and 'Arina*Lr34'* at Agroscope Reckenholz, Zurich, Switzerland and Cobbitty, Australia

Genotype	Infection type (IT) (0–4 scale)	Rust severity (%)				
	Reckenholz, Switzerland	Reckenholz, Switzerland				Cobbitty, Australia
	2012	2013	2014	2015	2016	2014
Arina	4	60–80	50–80	90–100	60–80	60–70
Forno	1	0	0	0	0	0
Arina*LrFor*	1–2	5–20	5–40	20–40	20–40	20–40
Arina*Lr34*	3–4	–[a]	15–60	30–60	5–10	15–30
Arina*Lr75*	–[b]	–[b]	–[b]	–[b]	40–60	–[b]
Arina*QLr.sfr-7BL*	–[c]	–[c]	–[c]	–[c]	50–70	–[c]

[a] Arina*Lr34* was not included in the 2013 field trial

[b] Arina*Lr75* was not included in the 2012, 2013, 2014 and field trials

[c] Arina*QLr.sfr-7BL* was not included in the 2012, 2013, 2014 and field trials

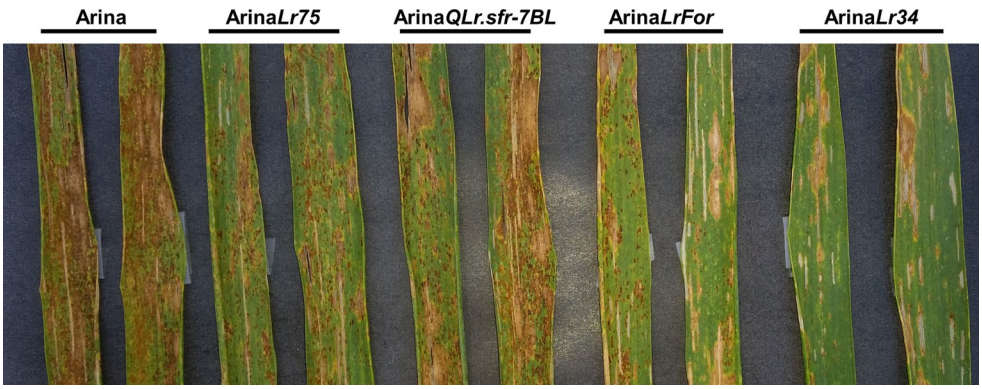

Fig. 2 Leaf rust infection on the flag leaves of 'Arina', 'Arina*LrFor'*, 'Arina*Lr75'*, 'Arina*QLr.sfr-7BL'* and 'Arina*Lr34'* Photographs were taken on field-infected plants in Switzerland in 2016

number was observed over time (84, 87 and 93 days after inoculating the spreader rows) (Table 2). On the other hand, 'Arina' showed a constant increase in the number of pustules from day 84 to 93 after inoculating the spreader rows. Both 'Arina*LrFor*' and 'Arina*Lr34*' showed an intermediate response with a slower increase of pustule density observed in 'Arina*LrFor*' compared to 'Arina*Lr34*' (Table 2).

'Arina*LrFor*' shows race-specific resistance at seedling stage

Partial adult plant resistance genes often do not confer seedling resistance. In order to determine the seedling responses of *Lr75* and *QLr.sfr-7BL* individually or in combination we infected 'Arina*LrFor*', 'Arina*Lr75*' and 'Arina*QLr.sfr-7BL*' along with 'Arina' and 'Forno' at the seedling stage in the greenhouse. For the two isolates 91,047 and 95,219 'Arina*LrFor*' was as susceptible as 'Arina' and showed a moderate infection type (IT = 3+) with medium sized uredia with or without chlorosis. Surprisingly, for some isolates (90,035, 96,002, 93,012, 95,001 and 96,209), Arina*LrFor* showed a stronger resistance reaction (;2) than either of its two parents 'Forno' and 'Arina' (Fig. 3). For these isolates 'Forno' showed a mesothetic infection type with various pustule sizes and hypersensitive flecks (X). A similar IT was previously reported for the leaf rust resistance gene *Lr14a* located on chromosome 7BL (Mcintosh et al. 1995). The differential line 'Thatcher *Lr14a*' showed a similar albeit slightly stronger mesothetic resistance reaction than 'Forno'. Hence, it is likely that *QLr.sfr-7BL* in 'Forno' is *Lr14a* and that this gene interacts with another resistance gene in 'Arina', which most likely is *Lr13* resulting in the strong resistance response of 'Arina*LrFor*'. 'Arina*Lr75*' showed an IT similar to the susceptible cv. 'Arina', indicating that *Lr75* is ineffective at the seedling stage. The seedling reaction of 'Arina*QLr.sfr-7BL*' was comparable to 'Arina*LrFor*', supporting the hypothesis that the seedling resistance in 'Arina*LrFor*' is most likely due to the interaction of *Lr13* gene present in 'Arina' background with *QLr.sfr-7BL* (Fig. 3).

Table 2 Pustule density on the flag leaves at three time points (84, 87 and 93 days after inoculating the spreader rows, dai) on 'Arina', 'Forno', 'Arina*LrFor*' and 'Arina*Lr34*' during the year 2014

Genotype	Number of pustules/cm^2		
	84dai	*87dai*	*93dai*
Arina	5.1 ± 5.1^{b}	$16.7 \pm 12.7^{c'}$	$60.4 \pm 33.3^{c''}$
Forno	0.2 ± 0.2^{a}	$0.6 \pm 0.9^{a'}$	$1.3 \pm 1.9^{a''}$
Arina*LrFor*	1.2 ± 0.7^{b}	$3.2 \pm 2.7^{b'}$	$14.0 \pm 9.5^{b''}$
Arina*Lr34*	4.0 ± 4.5^{b}	$11.4 \pm 10.8^{b'c'}$	$28.3 \pm 15.2^{b''}$

Letters indicate lines with similar infection levels for each time point ($p > 0.05$, Tukey's HSD test)

$'$, $''$ indicates significant differences between the lines per time point

Genetic mapping of *Lr75*

Based on our results, *Lr75* can be considered as a partial leaf rust resistance QTL. We therefore decided to further narrow down the *Lr75* interval. To define an *Lr75* target interval we tested 63 publically available, 1BS specific SSR markers (http://wheat.pw.usda.gov/GG2/index.html). Of the 63 SSRs, nine were 1B-specific and polymorphic (*barc128, cwem6c, cfa2158, gpw4069, wmc230, gwm11, gwm18, wmc277* and *wmc156)* between 'Arina' and 'Forno' and were added to the genetic map of the 'Arina' × 'Forno' RIL population (Schnurbusch et al. 2004). This resulted in the establishment of an 8 cM target region spanning the *Lr75* gene with *wmc230* and *gwm18* as the distal and proximal flanking markers, respectively. For precise genetic mapping and phenotypic analysis of *Lr75*, a near isogenic line (NIL) population consisting of 2067 F_2 individuals from a cross of 'Arina*LrFor*' and 'Arina' (Arina*4/Forno) was used. Out of these, 234 lines showed a recombination between the two flanking markers *wmc230* and *gwm18*. These recombinants were further screened with two *QLr.sfr-7BL*-associated SSR markers (*gwm344* and *gwm146*). Only recombinants without the *QLr.sfr-7BL* QTL were selected for further mapping in order to avoid interference from the 7BL QTL during phenotyping. This resulted in 65 BC_3F_2 recombinants that were phenotyped qualitatively as BC_3F_3–BC_3F_5 families in comparison to the parental lines.

Using the available sequence information of 'Chinese Spring', the 1BS physical map and synteny information of *Brachypodium*, rice and sorghum, 98 SSR primers were designed (Online Resource 2), out of which 8 were polymorphic between the parents. Of these 8 markers, six (*swm271, swm275, swm276, swm278, swm281* and *swm294*) were mapped in the target interval in the BC_3F_2 fine mapping population (Fig. 4a). The other two markers, *swm216* and *swm247* were mapped at a distance of 0.16 and 0.31 cM proximal to *gwm18*, respectively. The addition of the 8 new SSR markers placed *Lr75* between the distal marker *gwm604* and proximal marker *swm271* at a distance of 1.6 and 2.7 cM, respectively (Fig. 4a).

Deletion bin mapping

In order to physically map *Lr75*, we used the cytogenetic stocks of chromosome 1B of wheat cv. 'Chinese Spring'. Marker *wmc230* amplified on none of the 1BS deletion lines but amplified in all lines with a deletion on the long arm. The marker *swm271* on the other hand did not amplify on deletion lines 1BS1-0.35 and 1BS10-0.50 (Fig. 4b–d).

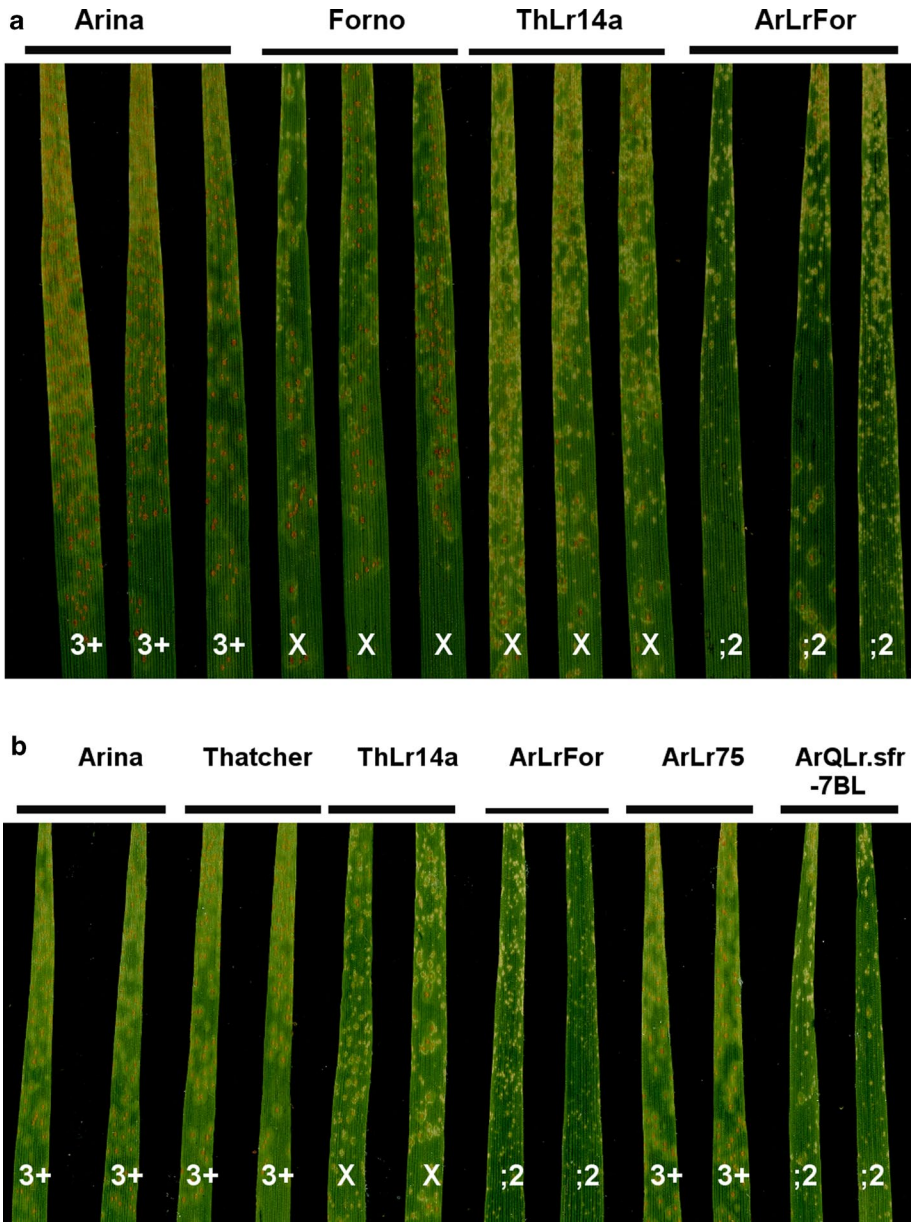

Fig. 3 Seedling infection assay on **a** 'Arina', 'Forno', 'Thatcher*Lr14a'* (Th*Lr14a*) and 'Arina*LrFor'* (Ar*LrFor*) and on **b** 'Arina', 'Thatcher', 'Thatcher-*Lr14a*', 'Arina*LrFor*' (Ar*LrFor*), 'Arina*Lr75*' (Ar*Lr75*) and 'Arina*QLr.sfr-7BL'* (Ar*QLr.sfr-7BL*) using isolate 96,209. Infection type response was scored based on a 0–4 scale (Roelfs et al. 1992). Two images represent results from two independent infection experiments with the same isolate 96,209

Hence, the use of the cytogenetic stocks physically placed the markers *wmc230* and *swm271* and the gene towards the distal end of chromosome 1BS (Fig. 4). Singh et al. (2013b) mapped *Lr71* close to the centromere in the deletion bins 1BS10-0.50 and 1BL6-0.32 respectively (Fig. 4).

Discussion

In this study we introgressed two leaf rust resistance QTLs, *Lr75* and *QLr.sfr-7BL,* from 'Forno' into the leaf rust susceptible Swiss winter wheat cv. 'Arina'. Marker-assisted introgression of these two QTLs resulted in high levels of partial adult plant leaf rust resistance in the field. Further, genetic mapping of *Lr75* with SSR markers placed this gene towards the distal end of chromosome 1BS. The only reported leaf rust resistance gene present on chromosome 1BS in the close proximity of *Lr75* is *Lr71* (Singh et al. 2013b). *Lr75* can be distinguished from *Lr71* by the marker *gwm18* which was reported to be the distal flanking marker of *Lr71* (Singh et al. 2013b), whereas *gwm18* mapped proximal to the *Lr75* gene. In addition, deletion bin mapping also physically separates *Lr75* from *Lr71*. The deletion bin mapping of the SSR markers *wmc230* and *swm271* mapped *Lr75* towards the distal end whereas deletion bin mapping mapped *Lr71* towards the centromere on chromosome 1BS as reported by Singh et al. (2013b). Hence, both genetic and physical mapping of *Lr75* on chromosome 1BS with SSR markers showed that it is a novel gene as no other leaf rust resistance gene has been described in the target region of *Lr75*.

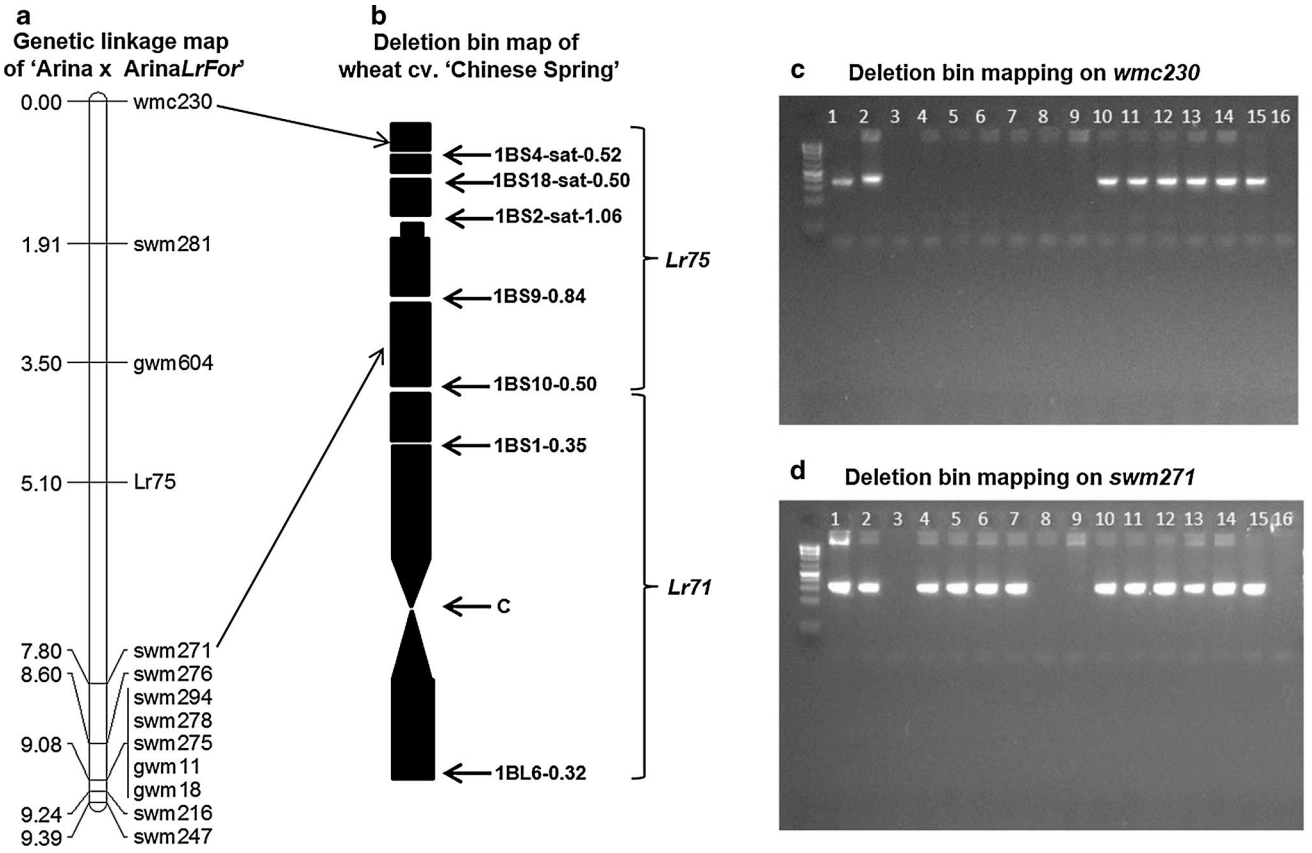

Fig. 4 Genetic linkage and physical deletion bin mapping of leaf rust resistance gene *Lr75*. **a** Genetic linkage map of the short arm of chromosome 1B of the 'Arina' × 'Arina*LrFor*' mapping population. Marker positions are shown in cM on the *left side* of the linkage map. **b** Deletion bin map of the short arm of chromosome 1B of wheat cv. 'Chinese Spring'. Physical bin localization of the markers *wmc230* and *swm271* is shown by arrowheads. **c** Deletion bin mapping of *wmc230*. **d** Deletion bin mapping of *swm271* on Chinese Spring, Arina*LrFor*, water, 1BS4-sat-0.52, 1BS18-sat-0.50, 1BS2-sat-1.06, 1BS9-0.84, 1BS10-0.50, 1BS1-0.35, 1BL11-0.23, 1BL6-0.32, 1BL1-0.47, 1BL2-0.69, 1BL3-0.85, DT1BS, DT1BL (*lanes 1–16*)

Breeding for slow-rusting resistance in European wheat germplasm

All the adult plant slow-rusting genes (*Lr34/Yr18/Sr57/Pm38, Lr46/Yr29/Pm39* and *Lr68*) that were obtained from the CIMMYT wheat germplasm are associated with LTN which is considered undesirable in European wheat breeding programme (Singh et al. 1998; Krattinger et al. 2009; Hiebert et al. 2010; Herrera-Foessel et al. 2012). Due to this reason, the wheat cultivars possessing these genes have not been widely grown and accepted in European wheat breeding. It has therefore become essential to identify additional sources of durable rust resistance in European wheat germplasm without LTN. In our study we described a novel slow-rusting gene, *Lr75* present on wheat chromosome 1BS. *Lr75* has shown to provide an additive effect when combined with another slow-rusting QTL, *QLr.sfr-7BL*. Both these QTLs are present in Swiss winter wheat cv. 'Forno' and are not associated with LTN. Another example of a non-LTN broad-spectrum APR gene is *Lr22a* which was introgressed from an *Aegilops tauschii* accession into cultivated wheat (Hiebert et al. 2007).

Very little information is available about leaf rust APRs in European wheat breeding material. Only a few studies looked at the existence of APR genes in ~100 European wheat lines (Winzeler et al. 2000; Park et al. 2001; Pathan and Park 2006). All studies reported the frequent occurrence of the *Lr13* APR gene in European wheat cultivars. Winzeler et al. (2000), detected varying levels of resistance shown by the cultivars carrying *Lr13* across Europe which indicates that virulence for *Lr13* exist. 'Arina' for example is known to possess *Lr13* but is susceptible to leaf rust throughout Europe (Pathan and Park 2006).

To our knowledge, only four studies reported on the identification of leaf rust APRs on chromosome 1BS in European wheat breeding material (Messmer et al. 2000; Schnurbusch et al. 2004; Singh et al. 2009; Buerstmayr et al. 2014). Other than 'Forno', cultivars 'Beaver' and 'Capo' possess 1BS QTLs. 'Beaver' has the 1BL/1RS translocation and the QTL identified in 'Beaver' can

therefore not be *Lr75*. The QTL in 'Capo', *QLr.ifa-1B* mapped close to the centromere but since no detailed study has been available on this QTL, it is not clear whether the genomic locations of *Lr75* and *QLr.ifa-1B* are identical. So far, 'Forno' seems to be the only source of *Lr75* and interestingly this gene has not been described in any other European wheat cultivar. However, apart from European wheat lines, two CIMMYT wheat lines, 'Pastor' and 'Parula' also possess a leaf rust resistance QTL on chromosome 1BS (William et al. 1997; Rosewarne et al. 2012). According to the study conducted by Singh and Rajaram (1992), the high level of resistance in 'Parula' is due to the combination of three slow-rusting APR genes, *Lr34, Lr46* and *Lr68* plus some minor genes. William et al. (1997) by using a RIL population developed from a cross of resistant cv. 'Parula' and moderately susceptible cv. 'Siete Cerros' identified a minor QTL on chromosome 1BS in 'Parula' which explained a phenotypic variance of 7–10 %. Another QTL on chromosome 1BS was detected in cv. 'Pastor' by Rosewarne et al. (2012). They reported this QTL to be present in the same genomic region as *Lr75*. Both these QTLs have not been characterized in detail and their precise genetic location is not available. Therefore, it is impossible to conclude if these QTLs are *Lr75* or not.

Slow-rusting APR genes are influenced by environment

The knowledge of an environmental influence on resistance genes allows wheat breeders to deploy resistance gene combinations most effectively in different regions. Our study showed that the combination of *Lr75* and *QLr.sfr-7BL* provided partial resistance in Switzerland and Australia. Similarly, *Lr34* also showed partial resistance at the adult plant stage in Switzerland and Australia. However, the level of resistance shown by these genes varied at the two locations. In Australia, the resistance provided by *Lr34* was stronger than that provided by the gene combination of *Lr75* and *QLr.sfr-7BL*, whereas in Switzerland, except for one crop season (2016), *Lr34* alone showed a weaker resistance response. Similar findings have also been reported in the literature where the environment plays a role in modifying the resistance response of slow-rusting genes. Herrera-Foessel et al. (2012) compared the leaf rust resistance response of *Lr68*, *Lr34*, *Lr46* and *Lr67* during three crop seasons (2008–2009, 2009–2010, 2010–2011) in the field at Ciudad Obregon, Mexico. They reported that except for one crop season (2010–2011), the effect of *Lr68* was smaller as compared to *Lr34*, *Lr46* and *Lr67*. In 2010–2011 however, *Lr68* showed a stronger resistance response than *Lr46*. Similar results were observed by Lillemo et al. (2011) while studying the additive effect of three APR genes, *Lr34/Yr18/Sr57/Pm38, Lr46/Yr29/Pm39* and *Lr68* in 'Avocet-YrA × Parula' F_6 RIL mapping populations across nine different environments. In agreement with Herrera-Foessel et al. (2012), they also observed a smaller resistance response of *Lr68* than *Lr34* and *Lr46* in Mexico. On the other hand, a stronger resistance response of *Lr68* as compared to *Lr34* was seen in Argentina and Uruguay (Lillemo et al. 2011). Interestingly, the combination of *Lr68* and *Lr34* showed stronger resistance than either gene alone in all the tested environments which suggests an additive effect of these two genes. In contrast, Silva et al. (2015) while studying the effect and interaction of *Lr68*, *Lr34* and *Sr2* genes in two wheat populations derived from 'Parula' at sites in Uruguay did not observe an additive effect of the combination of *Lr68* and *Lr34*. Instead, the effect of the combination of *Lr68* and *Lr34* was comparable to the effect of *Lr68* alone. These studies clearly showed that resistance gene combinations do not necessarily behave in the same manner in all environments. Stem rust resistance gene, *Sr2* is known to be tightly linked to seedling resistance gene *Lr27* (Mago et al. 2011) and *Lr27* was also reported to be responsible for reducing leaf rust severity (Bariana et al. 2007). Silva et al. (2015) studied the effect of the *Sr2* gene in reducing leaf rust in Uruguay. They observed that the stem rust resistance gene *Sr2* does not have any effect on leaf rust resistance when present alone but a significant increase in resistance level was seen when *Sr2* was present in combination with *Lr68*. From their study it was clear that *Sr2* alone is not strong enough to provide resistance and rather it enhances the effect of *Lr68*.

QLr.sfr-7BL in 'Forno' is most likely the leaf rust resistance gene *Lr14a*

In a survey of wheat leaf rust in Western Europe, Park et al. (2001) reported the presence of *Lr14a* in 'Forno'. This gene was mapped to the distal end of chromosome 7BL in a wheat consensus map (Gale et al. 1995). A major leaf rust resistance QTL, (*QLr.ubo-7B.2*) was also identified by Maccaferri et al. (2008) on chromosome 7BL within an 8.2 cM region in durum wheat cv. 'Creso'. Their study also postulated that this QTL is effective at both seedling and adult plant stages. The two microsatellite markers *gwm146* and *gwm344* were reported to be tightly linked to this QTL on chromosome 7BL. The same SSR markers were also reported to be closely linked to another gene, *LrLla* which was more precisely mapped on chromosome 7BL in a population of 98 F_3 lines derived from Chilean durum cv. Llareta INIA by Herrera-Foessel et al. (2008). They postulated this gene to be *Lr14a* based on the resistance response and chromosomal location. In addition, (Singh et al. 2013a) also identified a major QTL for leaf rust resistance on chromosome 7BL close to marker *gwm146* in French durum wheat cv. 'Sachem'. They reported that this QTL is effective at both seedling and adult plant stages

when tested in different environments in Mexico. The APR gene *Lr68* was also mapped on chromosome 7BL in the same genomic region as *QLr.sfr-7BL* by Herrera-Foessel et al. (2012). Like all the QTLs mentioned above, *QLr.sfr-7BL* identified from 'Forno' also shared the same genomic region on chromosome 7BL close to the markers *gwm344* and *gwm146*. In addition, seedling infection data have also shown that the QTL, *QLr.sfr-7BL* in 'Forno' is most likely *Lr14a* because of the mesothetic resistance response shown by both 'Forno' and *Lr14a* differential line, 'Thatcher-*Lr14a*'. Therefore, it is likely that this QTL is actually the *Lr14a* gene. *Lr14a* was transferred from emmer wheat to the bread wheat cv. 'Hope' and H-44. Park et al. (2001) reported the frequent occurrence of *Lr14a* in Europe and that 33.5 % of the area in France has been occupied by *Lr14a* alone or in combination with *Lr13*. Virulence against *Lr14a* was reported in Europe by Goyeau et al. (2010) and Park et al. (2001).

In this research we have successfully shown that marker-assisted introgression of two partial, non-LTN leaf rust resistance genes results in slow-rusting resistance in the susceptible Swiss winter wheat cv. 'Arina'. Introgression of this slow-rusting gene combination in different cultivars will be useful in improving leaf rust resistance in near future and impedes a greater value in breeding. For the optimal use of *Lr75*, this gene has to be tested with the local pathotypes of the region and if effective, can be combined with other effective rust resistance genes to minimize the spread of emerging virulent pathotypes.

Author contribution statement JS, LL, TW, SGK and BK planned and conceptualized the experiments. JS, LL SGK and UB performed field leaf rust infection experiments. JS, LL and SGK generated the mapping population. JS and LL performed the molecular work. JS and TW developed the strategy for marker development. JS, SGK and BK wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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