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A combination of leaf rust resistance gene *Lr34* and lesion mimic gene *lm* significantly enhances adult plant resistance to *Puccinia triticina* in wheat

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Leaf rust caused by *Puccinia triticina* is an economically-important disease in wheat worldwide. A combination of different types of resistance genes may significantly enhance rust resistance under rust-favorable conditions. To investigate the interactions between the rust resistance gene *Lr34* and the lesion mimic gene *lm* on 1BL in Ning 7840, a segregating F_{8-10} population of 180 recombinant inbred lines was developed from Ning 7840/Chokwang and evaluated for both lesion mimic expression and leaf rust response at the adult plant stage in a greenhouse. A major quantitative trait locus (QTL), derived from Sumai 3, was co-localized with *Lr34* on chromosome 7D and explained 41.5% of phenotypic variations for rust severity and 22.1% for leaf tip necrosis (LTN). The presence of *Lr34* was confirmed by *Lr34*-specific markers *cssfr1* and *cssfr2* in Ning 7840 and Sumai 3. Unlike *Lr34*, *lm* conditioned a spontaneous lesion mimic phenotype and had a significant effect on reducing uredinial size, and a smaller effect on severity. Additive effects were observed between *lm* and *Lr34* for severity and LTN, and an epistatic effect was observed for infection type. Single marker analysis also identified several other QTL with minor effects on severity, infection type, or LTN.

***Triticum aestivum*, *Puccinia triticina*, lesion mimic, slow rusting gene *Lr34*, leaf rust resistance**

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Leaf rust, caused by *Puccinia triticina* Erikss., is an economically-important wheat disease worldwide. More than 60 leaf rust resistance genes and quantitative trait loci (QTL) have been described in wheat [1], most of which are race-specific. Race-specific resistance genes may stop the development of epidemics by limiting the initial inoculum or reproduction after infection [2]. Nevertheless, the resistance provided by these genes can be temporary because virulent races of the pathogen emerge or increase to overcome race-specific resistance. In contrast, race-nonspecific genes provide durable resistance to a broad spectrum of races and may function by slowing down the rate of infection development, thus slowing down the spread of disease in the field [2].

In wheat, only a few leaf rust resistance genes function in a race-nonspecific manner, such as *Lr34* [3,4] and *Lr46* [5,6], which are also called "slow rusting genes". Many cultivars, including Jupateco 73, Forno, Sunvale, and Chinese Spring have been reported to carry *Lr34* since it was first described in cultivar Frontana in 1966 [7]. *Lr34* confers a non-hypersensitive reaction (HR) type of resistance [6] and was recently cloned as an ABC transporter [8]. This gene remains durable, and virulence towards plants carrying *Lr34* has not been observed to date [8]. *Lr34* increases the latent period and reduces uredinial size and receptivity [4]. Although such resistance provides durable adult plant resistance, the effect on reducing rust damage is still dependent on environmental conditions and *Lr34* alone cannot

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provide adequate protection from rust infection during a severe epidemic. A combination of *Lr34* and other genes may be required to provide sufficient protection during severe rust epidemics.

Lesion mimic (LM) spontaneously occurs in plants and resembles HR-like phenotypes in the absence of pathogen infection [9]. Because plants with LM usually demonstrate enhanced resistance to a broad spectrum of pathogen races in a race-nonspecific manner, it is thought that LM is involved in plant defense signaling pathways [9–11]. In rice, LM mutants *ebr3* [10,12], *spl* [13] and *blm* [14] provide race-nonspecific resistance to rice blast. In *Arabidopsis*, LM mutants *lsd1* and *acd* showed enhanced resistance to the bacterial pathogen *Pseudomonas syringae* and oomycete *Peronospora parasitica* [15–17]. In wheat, both naturally-occurring [18] and chemical- or transgenic-induced LM have been reported [19–22]. These mutants express HR-like phenotypes in pathogen-free environments and exhibit enhanced resistance to biotrophic pathogens, including *P. triticina*.

Previous experiments indicated that wheat line Ning 7840 exhibits LM symptoms on the leaf blade after heading that resemble the typical wheat response to early leaf rust infection. A recessive gene, *lm*, on chromosome 1BL controlled LM expression, and lines with LM demonstrated a significantly lower infection response compared with a line without LM [18]. In the current study, investigations of resistance components in the recombinant inbred line (RIL) population from Ning 7840/Chokwang indicated that Ning 7840 also carries *Lr34*. The main objectives in this study were to elucidate the effects of HR-like *lm* and non-HR *Lr34* on adult plant response to leaf rust and to reveal additional chromosomal loci involved in rust resistance.

1 Materials and methods

1.1 Plant materials and leaf rust evaluation

A population of 180 F_{8-10} RIL was developed from the cross Ning 7840/Chokwang by single seed descent. Ning 7840 is a Chinese wheat breeding line that has adult plant leaf rust resistance, exhibits leaf tip necrosis (LTN), and expresses the LM trait after heading. The Korean wheat cultivar Chokwang does not demonstrate LM or LTN. The two parents and the resulting RIL were vernalized at 4°C in a growth chamber for 7 weeks and then transplanted into 10 cm × 10 cm plastic pots in a greenhouse at 17±2°C (night) and 22±5°C (day) with supplemental light for 12 h. Experiments were arranged in a randomized complete block design with two replicates (pots) of five plants per replicate. The experiment was performed twice in the greenhouses at Kansas State University, Manhattan, KS, USA, in spring 2008 and 2009. LM was recorded as either presence or absence of LM symptoms on the flag leaf.

Flowering plants were inoculated with *P. triticina* (isolate PRTUS55), which is virulent to both Ning7840 and Chokwang at the seedling stage, to evaluate adult plant leaf rust response in the RIL population. Ning 7840 showed high resistance to PRTUS55 and Chokwang showed moderate resistance at the adult stage. The inoculated plants were incubated in a mist chamber at 15°C with 100% relative humidity for 12 h and then moved to a greenhouse at 17±2°C (night) and 22±5°C (day) with 12 h of supplemental light during the daytime. Rust symptoms were scored 14 d after inoculation for severity (percentage of infected flag leaf area) as described by Peterson et al. [23]. Infection type (IT) was scored following the protocol by Roelfs et al. [24], and the two modifiers "+" and "–" from McIntosh et al. [25] was also introduced to describe IT. The scale for IT was: 0, necrotic flecks; 1, small uredinia surrounded by necrotic tissue; 2, small to medium uredinia surrounded by chlorotic or necrotic tissue; 3, medium uredinia with or without chlorosis; 4, large uredinia without chlorosis; X, random distribution of variable sized uredinia on a single leaf; Y, variable sized uredinia with larger pustules at the leaf tip; Z, variable sized uredinia with larger pustules at the leaf base; +, pustules larger than average; and –, pustules smaller than average. Since X, Y, Z, + and – are modifiers of IT and qualitative, IT ratings were converted to numerical scales for statistical and QTL analyses following the "maximum rule"; only the highest IT score was recorded for a single leaf. For example, if an IT score of a flag leaf was 3+4, then 4 was recorded. Similarly, the modifiers X, Y, and Z were also digitalized. For the RIL carrying *Lr34* that displayed Z patterns with a larger pustule size at the leaf base than at the leaf tip, only the IT at the base was scored; + or – was numerically transformed to +/– 0.25. For example, an IT 3+ was converted to 3.25 and 3– was converted to 2.75. Consequently, all of the qualitative modifiers were converted to corresponding numerical values.

1.2 Genotyping and data analysis

Parents were initially screened using a genome-wide set of simple sequence repeat (SSR) markers with 12 markers per chromosome. Additional markers in chromosomal regions where markers had been correlated with rust resistance were screened. A total of 176 markers polymorphic between parents were used to genotype all RIL. DNA extraction and PCR amplification procedures followed Yu et al. [26]. Amplified PCR fragments were separated in an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) and scored using GeneMarker 1.6 (Softgenetics Inc., State College, PA). Ambiguous data were removed following visuall inspection. *Lr34*-specific markers, *cssfr1* and *cssfr2*, which were designed based on a 3 bp polymorphism on exon 11 between *Lr34* alleles, were amplified in Ning 7840 (Anhui 11/Aurora//Sumai 3) and its parents following the procedure described by Lagudah et al. [27]. Agarose gels (1%) were used to resolve PCR products.

Composite interval mapping (CIM) was performed with Windows QTLCartographer version 2.5 [28] using data from individual experiments and the means of two experiments. A LOD value of 3.0 was set as the threshold to claim the presence of a QTL. If the QTL was below the threshold in CIM, then a single marker regression model was applied to reveal associations between marker loci and severity or IT. Analysis of variance (ANOVA) was performed to evaluate differences in rust response between *lm* and *Lr34* alone and in combination. Statistical analysis was conducted using Matlab software (MathWorks Inc, Natick, Massachusetts, 2007).

2 Results

2.1 Phenotypic variations of parents and RIL

When inoculated with *P. triticina* (isolate PRTUS55), the mean percentage of infected leaf area was 15.6% for Ning 7840 (5.6% in 2008 and 25.6% in 2009) but 40.0% for Chokwang (16.4% in 2008 and 63.3% in 2009). A continuous distribution of severities within the RIL population, ranging from 2.0% to 82.5% (Figure 1). Transgressive segregation for increased resistance and susceptibility suggested that both Ning 7840 and Chokwang contributed different minor genes.

Ning 7840 produced an IT of 22+, Chokwang 3+4, and the RIL had IT values ranging from 22+ to 4 with a majority with 3 in both experiments. Most RIL with LM symptoms showed low IT (≤22+), and an IT of 3+4 was not observed in any RIL with LM. Most RIL without LM had high IT, except for six RIL (RIL 31, 60, 76, 80, 102 and 104) that had a consistent IT of 22+ in both experiments. Average rust severity for RIL with LM was 27.2% while it was significantly ($P < 0.0001$) higher for non-LM RIL (42.2%). RIL having both high IT (3+4) and high severity (>80.0%) always lacked LM.

2.2 Chromosomal loci associated with leaf rust resistance in the Ning 7840/Chokwang population

Ning 7840 showed LTN and a Z-pattern pustule distribution

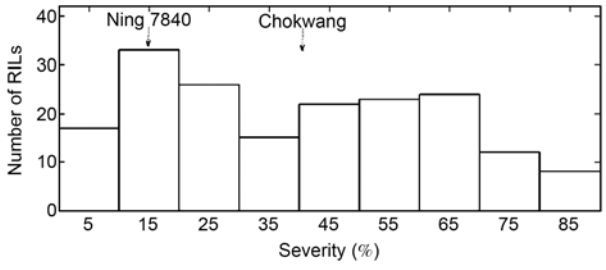

Figure 1 Frequency distribution of average leaf rust severity for the RIL population from Ning 7840/Chokwang in 2008 and 2009 experiments.

with more and larger pustules at the leaf base and gradually decreasing pustule size toward the leaf tip in the adult plant stage. Both LTN and Z-pattern pustule distribution are the characteristics of the slow-rusting resistance gene *Lr34*. QTL mapping identified a major QTL responsible for both low rust severity and LTN on the short arm of chromosome 7D in the individual experiments in 2008 and 2009 as well as when using the means of the two experiments. This QTL was flanked by SSR markers *Xgwm295* and *Xbarc352* and its peak coincided with the marker *csLV34*, which is a marker closely linked with *Lr34* (Figure 2). The identified QTL explained 41.5% of the phenotypic variation for rust severity and 22.1% of the phenotypic variation for LTN.

Sumai 3 and Aurora were the parents of Ning 7840 (Anhui 11/Aurora//Sumai 3) and both showed LTN of different sizes, but only Sumai 3 showed a consistent Z-pattern pustule distribution in both experiments. PCR amplifications of *Lr34*-specific markers, *cssfr1* and *cssfr2*, generated a DNA fragment (517 bp) for *cssfr1* only with Ning 7840 and Sumai 3, while a PCR fragment (523 bp) for *cssfr2* was produced only with Chokwang, Anhui 11, and Aurora. *cssfr1* is a positive control marker for *Lr34*, and produced a PCR fragment of 517 bp in wheat lines carrying *Lr34*, but generated no products in wheat lines lacking *Lr34*. *cssfr2* is a negative control marker for *Lr34* and produced a fragment of 523 bp in wheat lines lacking *Lr34* but generated no products in wheat lines carrying *Lr34* [27], indicating that *Lr34* in Ning 7840 was derived from Sumai 3.

In addition to *Lr34* and *lm*, other genes with minor effects were involved in resistance to PRTUS55. Single marker analysis identified four additional markers associated with rust severity (Table 1). These additional markers were located on four chromosomes. *Xsts3B-189* on 3BS and *Xgwm3* on 3DS were positively associated with severity, and Ning 7840 contributed the favorable alleles. Markers *Xgwm469* on 6DS and *Xgwm361.2* on 7BS displayed negative associations with severity, suggesting Chokwang contributed alleles for rust resistance at these two loci.

Six additional markers were associated with IT. They were assigned to five chromosomes: 3AS, 3BS, 3DS, 6AS and 7BS (Table 1). Markers *Xbarc323* on 3DS, *Xsts3B-189* on 3BS, *Xbarc3* on 6AS, and *Xgwm43* on 7BS were positively associated with IT, suggesting that Ning 7840 contributed alleles associated with resistance. Markers *Xbarc321* on 3AS and *Xgwm361.2* on 7BS were negatively associated with IT, suggesting that Chokwang carried alleles associated with resistance at these loci.

In addition to *Lr34*, three other loci were identified in Ning 7840 associated with LTN (Table 1). *Xwmc710* on 4BS was negatively associated with the length of LTN ($P < 0.01$), suggesting that the Ning7840 allele at this locus extended LTN. The other two markers, *Xgwm539* on 2DL and *Xgwm 361.2* on 7BS, were positively correlated to the length of LTN, suggesting Ning7840 alleles at these two loci reduced the length of LTN.

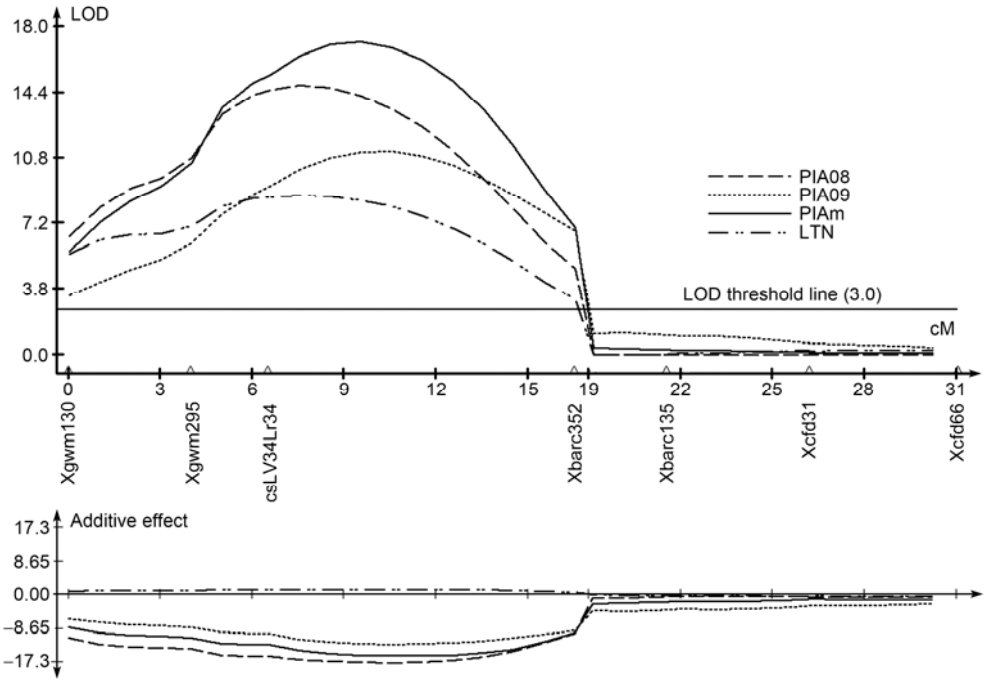

Figure 2 LOD curves for the *Lr34* locus showing the QTL effect and location on chromosome 7D using percentages of infected flag leaf area (PIA) from 2008 (PIA08), 2009 (PIA09), the means over the two experiments (PIAm), and LTN data from 2009.

Table 1 Additional marker loci associated with severity, infection type (IT) and leaf tip necrosis (LTN) in the RIL population from Ning 7840/ Chokwang in 2008 and 2009 greenhouse experiments, as indicated by linear regression analysis using Matlab software at $\alpha = 0.01$

Trait	Marker	Effect	VE[a)]	*P* value	Chromosome
Severity	*Xsts3B-189*	4.408	0.029	<0.001	3BS
	Xgwm469	–4.13	0.029	<0.01	6DS
	Xgwm361.2	–4.08	0.028	<0.01	7BS
	Xgwm3	3.983	0.027	<0.01	3DS
IT	*Xbarc323*	0.102	0.05	<0.0001	3DS
	Xbarc321	–0.155	0.047	<0.0001	3AS
	Xsts3B-189	0.139	0.039	<0.001	3BS
	Xgwm361.2	–0.224	0.038	<0.0001	7BS
	Xbarc3	0.086	0.036	<0.0001	6AS
	Xgwm43	0.19	0.03	<0.01	7BS
LTN	*Xwmc710*	–0.729	0.056	<0.0001	4BS
	Xgwm539	0.705	0.05	<0.001	2DL
	Xgwm361.2	0.603	0.039	<0.01	7BS

a) Proportion of phenotypic variances explained by the marker.

2.3 Relationship between *lm* and *Lr34* in terms of rust response

The LM phenotype had a marked effect on rust infection type and severity ($P < 0.0001$) and a marginally significant effect on LTN ($P = 0.012$) (Table 2). The presence of *Lr34* significantly reduced both severity and LTN ($P < 0.0001$), but not IT. The interaction between *lm* and *Lr34* was marginally significant for IT ($P < 0.05$), but not for severity and LTN. Since *lm* and *Lr34* have much larger effects, to analyze the interactions between *lm* and *Lr34*, the RIL were classified into four groups: *lm+Lr34+*, *lm+Lr34–*, *lm-Lr34+*, and *lm-Lr34–*. Variations in rust severity were significant among the four groups (Table 3). The two groups with *Lr34* had significantly lower severities than the groups without *Lr34*. The group with both resistance alleles showed the lowest severity (14.5%), and the group without any of the resistance alleles showed the highest severity (54.4%). The group having *Lr34* alone had significantly lower rust severity than the group having *lm* alone, suggesting that *Lr34* had a larger effect on the reduction of rust severity than *lm*, although both *lm* and *Lr34* played a role in reducing disease severity.

A significant difference existed between the groups with *lm* compared with those without the allele ($P < 0.0001$) (Table 3). RIL in the group with *lm* had a smaller uredinial size than those in the group without *lm*, indicating that *lm*, rather than *Lr34*, played a significant role in reducing IT in Ning 7840.

The differences in the mean length of flag leaf tip necrosis were significant among the four groups ($P < 0.001$) (Table 3). The group with both genes displayed the longest LTN, whereas the group without either exhibited the shortest LTN. On average, the presence of *lm* alone increased LTN by 1.2 cm, and the presence of *Lr34* increased it by 2.1 cm. *Lr34* and *lm* appeared to have an additive effect on LTN and intergenic interaction was not significant (Table 2, $P = 0.512$).

Table 2 ANOVA analysis of the effects of *lm* and *Lr34* on severity, infection type (IT), and leaf tip necrosis (LTN) of RIL from Ning 7840/Chokwang in 2008 and 2009 greenhouse experiments

	Severity					IT			LTN	
	DF[a)]	*SS*	*F*	*P*	*MS*	*F*	*P*	*MS*	*F*	*P*
lm	1	9974.542	28.402	2.989×10^{-7}	12.539	102.893	2.498×10^{-19}	48.636	6.433	0.012
Lr34	1	28707.217	81.742	2.778×10^{-16}	0.115	0.947	0.332	284.028	37.568	5.638×10^{-9}
lm×*Lr34*	1	434.812	1.238	0.267	0.560	4.596	0.033	3.264	0.432	0.512
Error	176	351.194			0.122			7.560		

a) *DF*, degree of freedom; *SS*, sums of squares; *MS*, mean square; *F*, *F* statistics; *P*, probability.

Table 3 Pair-wise comparisons of gene effects on rust severity, infection type (IT), leaf tip necrosis (LTN), and Z-pattern rust distribution on flag leaves among four *lm* and *Lr34* gene combinations in a RIL population from Ning 7840/Chokwang in 2008 and 2009 greenhouse experiments

Group[a)]	No. of RIL	Severity[b)]	IT[b)]	LTN[b)]	Z pattern[c)]
lm+*Lr34*+	44	14.47±3.57 a	2.7±0.07 a	7.7±0.46 c	+
lm+*Lr34*–	35	43.19±4.01 c	2.6±0.07 a	5.3±0.52 a	–
lm-*Lr34*+	55	31.96±3.20 b	3.1±0.06 b	6.2±0.41 b	+
lm-*Lr34*–	46	54.43±3.49 d	3.3±0.06 b	4.1±0.45 a	–

a) Gene combination in RIL. *lm*+, RIL with LM phenotype; *lm*–, RIL without LM phenotype; *Lr34*+, RIL carrying *Lr34*; and *Lr34*–, RIL without *Lr34*. b) Mean ± standard error; a, b, c, and d indicate significant differences between groups at $LSD_{0.05}$. c) More and larger pustules occurred at the leaf base and gradually decreased in size toward the leaf tip in adult plants; wheat lines carrying *Lr34* displayed a typical Z-pattern. +, presence of Z pattern; and –, absence of Z-pattern.

3 Discussion

Ning 7840 carries the non-HR resistance gene *Lr34* and an HR-like resistance gene *lm*, and both conferred partial resistance to leaf rust in adult plants. *Lr34* is widely recognized as reducing severity or receptivity as measured by the percentage of infected leaf area and accounts for 20%–50% of the phenotypic variation in various reports [4,29–33]. In this study, the effect of the rust resistance QTL at the *Lr34* locus was consistent with previous reports [4,34]. In contrast, *lm* increased rust resistance by reducing IT, a component different from that of *Lr34*. Thus, these two genes showed a complementary effect on overall rust resistance.

The Z-pattern pustule distribution on infected wheat leaves is a typical response of *Lr34* and is characterized by more and larger pustules at the leaf base than at the leaf tip [4,34] (Figure S1(a)). Therefore, *Lr34* should reduce IT, at least at the leaf tip. The insignificant association between *Lr34* and IT in this study was due to the "maximum rule" used in the IT scoring system where IT is scored only at the leaf base, not at the tip. On average, RIL with both genes had a slightly higher IT than RIL with *lm* alone and a slightly lower IT at the leaf base than RIL carrying *Lr34* alone (Table 3). Therefore, the expression of *lm* appeared to be negatively affected by expression of *Lr34*. This was also supported by the observation that LM symptoms appearing in the earlier stages of infection were reduced when LM was expressed in the presence of *Lr34*. This result could be due to the differences in the chronological expression patterns of the HR-like *lm* and the non-HR *Lr34*. The effect of *Lr34* was more pronounced at the grain filling stage than at other stages, and resistance at the flag leaf was most effective [4,8]. LM spots initially appeared around the heading stage [18]. Thus, the appearance of LM occurred earlier than the peak expression of *Lr34* (i.e., at the grain filling stage) and the later expression of non-HR *Lr34* might negatively affect *lm* expression, resulting in slightly higher IT for RIL with both genes than for RIL with *lm* alone (Table 2).

Either *Lr34* or *lm* alone may not be sufficient to control leaf rust under diverse climatic conditions. Singh et al. [35] reported that cultivars with *Lr34* alone showed approximately 40% of severity, but could reach unacceptably severe levels in epidemics. Results from this study suggest that *Lr34* and *lm* have different but complementary functions. The effect of *Lr34* on severity reduction was superior to reductions in uredinial size, whereas *lm* functioned in the opposite manner. Seventeen of 21 lines consistently showing low severity (< 10.0%) across the two experiments exhibited LM phenotype, LTN and Z-pattern pustule distribution, suggesting that a combination of race-nonspecific HR-like *lm* and non-HR *Lr34* may significantly improve adult plant resistance to a desirable level. It is commonly known that RIL with low IT do not always have low severity and vice versa. Only lines simultaneously having low IT and low severity were resistant in nature, and they usually carried both *Lr34* and *lm* (Figure S1(c)). Our results indicate that a combination of the two distinct types of resistance genes could enhance the level of adult plant resistance. However, whether the expression of *lm* has a negative impact on yield remains to be determined.

In addition to *Lr34* and *lm*, the transgressive segregation of the RIL for severity (Figure 1) suggests that additional genes are involved in conferring resistance to PRTUS55. Single marker association analysis detected four and six additional markers that were significantly associated with severity and IT, respectively (Table 1). *Xsts3B-189* on the short arm of chromosome 3B of Ning 7840, previously associated with the fusarium head blight resistance gene *Fhb1*

[36], was positively associated with both severity and IT, suggesting a gene on 3BS might also contribute to rust resistance. The marker *Xgwm361.2* on 7BS chromosome was negatively associated with both severity and IT (Table 1). Thus, the associated locus in Ning 7840 might suppress the expression of leaf rust resistance to increase both severity and IT.

Results from this study indicated that Ning 7840 provided a unique source of resistance for improving adult plant leaf rust resistance in wheat. Resistance in Ning 7840 was mainly mediated by two distinct types of resistance genes (HR-like and non-HR) that conditioned complementary components of adult plant resistance. A combination of *Lr34* and *lm* resulted in reduced severity and smaller uredinial size.

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Supporting Information

Figure S1 Typical phenotypes of both *lm* and *Lr34* alone and in combination during PRTUS55 infection. (a) Typical phenotype of *Lr34* with Z pattern; (b) susceptible line with high IT and high severity; (c) the RIL carrying *Lr34* and *lm* plus one or more genes with a highly reduced to almost immune phenotype; (d) typical phenotype of *lm*.

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