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The wheat durable, multipathogen resistance gene *Lr34* confers partial blast resistance in rice

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Summary

The wheat gene *Lr34* confers durable and partial field resistance against the obligate biotrophic, pathogenic rust fungi and powdery mildew in adult wheat plants. The resistant *Lr34* allele evolved after wheat domestication through two gain-of-function mutations in an ATP-binding cassette transporter gene. An *Lr34*-like fungal disease resistance with a similar broad-spectrum specificity and durability has not been described in other cereals. Here, we transformed the resistant *Lr34* allele into the *japonica* rice cultivar Nipponbare. Transgenic rice plants expressing *Lr34* showed increased resistance against multiple isolates of the hemibiotrophic pathogen *Magnaporthe oryzae*, the causal agent of rice blast disease. Host cell invasion during the biotrophic growth phase of rice blast was delayed in *Lr34*-expressing rice plants, resulting in smaller necrotic lesions on leaves. Lines with *Lr34* also developed a typical, senescence-based leaf tip necrosis (LTN) phenotype. Development of LTN during early seedling growth had a negative impact on formation of axillary shoots and spikelets in some transgenic lines. One transgenic line developed LTN only at adult plant stage which was correlated with lower *Lr34* expression levels at seedling stage. This line showed normal tiller formation and more importantly, disease resistance in this particular line was not compromised. Interestingly, *Lr34* in rice is effective against a hemibiotrophic pathogen with a lifestyle and infection strategy that is different from obligate biotrophic rusts and mildew fungi. *Lr34* might therefore be used as a source in rice breeding to improve broad-spectrum disease resistance against the most devastating fungal disease of rice.

Introduction

Fungal plant diseases are a serious threat to cereal production. Breeding for effective and durable field resistance is the most sustainable strategy to reduce yield losses caused by pathogenic fungi. Fungal disease resistance genes in cereals can be broadly classified into three categories based on their specificity and durability. The first group contains genes that confer race-specific resistance against some, but not all races of a particular pathogen. These genes often encode intracellular immune receptor proteins belonging to the nucleotide-binding site–leucine-rich repeat family (NBS-LRR). NBS-LRR proteins directly or indirectly perceive pathogen-derived virulence effectors that are secreted into the cytoplasm of host cells in order to suppress basal immunity. This interaction triggers a strong resistance reaction called hypersensitive response that often results in death of the infected cell (Dodds and Rathjen, 2010). Mutations in pathogen effectors that escape recognition by plant NBS-LRR immune receptors have a strong selective advantage. For this reason, disease resistance based on single race-specific resistance genes is often not durable in the field and breakdown of NBS-LRR gene-based resistance is frequently observed in cereals (Park *et al.*, 2002; Pretorius *et al.*, 2000; Wilson and Talbot, 2009). The second group of cereal disease resistance genes confers race nonspecific resistance against all races of a particular pathogen. Examples include the wheat stripe rust resistance gene *Yr36* that encodes a protein with a kinase domain fused to a START lipid-binding domain (Fu *et al.*, 2009), the recessive rice blast resistance gene *pi21* that encodes a small proline-rich protein (Fukuoka *et al.*, 2009), or the recessive barley powdery mildew resistance gene *mlo* that encodes an integral membrane protein (Buschges *et al.*, 1997). The third group contains genes that

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confer race nonspecific resistance against multiple fungal pathogens simultaneously. Only three of these broad-spectrum, multipathogen resistance genes have been described in cereals to date. The three wheat genes *Lr34* (same as *Yr18/Sr57/Pm38*), *Lr46* (=*Yr29/Sr58/Pm39*) and *Lr67* (=*Yr46/Sr55/Pm46*) confer partial resistance at the adult plant stage against all races of the obligate biotrophic fungi leaf rust (*Puccinia triticina*), stripe rust (*Puccinia striiformis* f.sp. *tritici*), stem rust (*Puccinia graminis* f.sp. *tritici*) and powdery mildew (*Blumeria graminis* f.sp. *tritici*) (Ellis *et al.*, 2014). To our knowledge, this resistance-type has so far not been reported in cereals other than wheat.

The majority of cereal disease resistance genes that have been identified and cloned so far belong to the race-specific NBS-LRR gene family. In contrast to race-specific resistance genes, resistance based on race nonspecific genes is usually more durable in the field (Lagudah, 2011). Of the three wheat multipathogen resistance genes, only *Lr34* has been cloned to date. It encodes a full-size ATP-binding cassette (ABC) transporter belonging to the ABCG (formerly PDR) subfamily (Krattinger *et al.*, 2009; Risk *et al.*, 2012). Full-size ABCG transporter proteins share a conserved domain structure consisting of two nucleotide-binding domains and two transmembrane domains. These proteins use the energy derived from ATP hydrolysis to shuttle various substrates across cellular membranes (Rea, 2007). *Lr34* has been used in wheat breeding since the early 20th century, and no increased pathogen adaptation has been observed (Kolmer *et al.*, 2008). The gene delays pathogen development by prolonging the latency period and decreasing infection frequency (Rubiales and Niks, 1995). This partial disease resistance phenotype is also referred to as slow-rusting or slow-mildewing. So far, only one resistance-conferring *Lr34* allele, named *Lr34res,* has been identified in the wheat gene pool. This particular allele emerged after wheat domestication about 8000 years ago and is the result of two gain-of-function mutations that are critical for disease resistance (Dakouri *et al.*, 2010; Krattinger *et al.*, 2011, 2013). These two mutations resulted in a deletion of a phenylalanine residue and a conversion of a highly conserved tyrosine to histidine in the resistant LR34 ABC transporter version. Both amino acid changes are located in the first transmembrane domain of the transporter. *Lr34res* is only found in cultivated bread wheat (*Triticum aestivum* L.), but not in wild wheat progenitors which is in agreement with the emergence of this allele after domestication. Most wild wheat progenitors and susceptible wheat cultivars carry an expressed *Lr34* allele called *Lr34sus* with a complete open reading frame encoding a putatively functional ABC transporter. The function of *Lr34sus* however is not yet known. Furthermore, rice and sorghum carry an expressed *Lr34* ortholog, but only the susceptible *Lr34* haplotype for the two critical amino acid polymorphisms was found in these orthologous *Lr34* genes in rice and sorghum (Krattinger *et al.*, 2013). *Lr34res* is associated with a senescence-like process called leaf tip necrosis (LTN) (Singh, 1992). The development of LTN in adult wheat plants is dependent on the genetic background and environmental conditions. Risk *et al.* (2013) showed that *Lr34res* is functionally transferable to the close wheat relative barley (*Hordeum vulgare* L.) where *Lr34res* but not *Lr34sus* conferred partial resistance against the barley-specific diseases barley leaf rust (*Puccinia hordei*) and barley powdery mildew (*B. graminis* f.sp. *hordei*). In contrast to wheat, where *Lr34res* is ineffective at seedling stage under normal temperatures, *Lr34res* conferred seedling resistance in barley. However, *Lr34res*-barley also developed a severe LTN during the seedling stage resulting in poor plant growth and reduced seed production.

Rice (*Oryza sativa* L.) is one of the most widely grown crops and provides a daily staple for more than half of the world's population. In this study, we transformed *Lr34res* into the *japonica* rice cultivar Nipponbare. *Lr34res*-carrying rice plants showed increased resistance against the hemibiotrophic fungal pathogen *Magnaporthe oryzae,* the causal agent of rice blast disease. These results show that a broad-spectrum disease resistance gene can be functional across different cereal species against diverse biotrophic and hemibiotrophic pathogens.

Results

Transgenic expression of *Lr34res* confers partial rice blast resistance

A construct containing the genomic sequence of the wheat resistant *Lr34res* allele under its native promoter (Risk *et al.*, 2013) was transformed into the *japonica* rice cultivar Nipponbare. Homozygous lines of five independent transgenic events were selected based on the presence and expression of *Lr34res*. Southern blot analysis indicated that three events (numbers 8, 11 and 19) carried single T-DNA insertions, one event (16) had two co-segregating insertions and one event (5) showed multiple co-segregating copies (Figure S1). In wheat and barley, *Lr34res* confers resistance against obligate biotrophic pathogens belonging to the cereal rusts (*Puccinia* spp.) and cereal powdery mildews (*B. graminis*; Krattinger *et al.*, 2009; Risk *et al.*, 2013). Rice however is immune to all powdery mildew and rust fungi (Ayliffe *et al.*, 2011; Cheng *et al.*, 2015). The five transgenic events were subjected to infection experiments with the hemibiotrophic fungal pathogen *M. oryzae* because (i) *M. oryzae* proliferates biotrophically during the initial 72 h of the infection (Wilson and Talbot, 2009) and (ii) rice blast is the most damaging fungal disease for rice production worldwide (Dean *et al.*, 2012). We microscopically monitored and rated early invasive, biotrophic fungus growth on leaf sheath cells of 1-month-old plants. Invasive hypha development was delayed in the four transgenic lines 8, 11, 16 and 19 compared to sib lines without *Lr34res* (Figures 1a and S2). Most infection sites showed low levels of invasive growth (levels 1 and 2) in the transgenic lines after 28 h and only 15%–29% of the penetration sites developed invasive hyphae of levels 3 and 4 (high levels of invasive growth). In contrast, 75% of the penetration sites showed high levels of invasive growth (levels 3 and 4) in the sib lines after 28 h. After 40 h, more than 40% of the infection sites spread to multiple cells (level 4) in the sib lines, compared to <10% after 28 h (Figure S3). The delay in pathogen development by *Lr34res* was still apparent 40 h postinfection and only 12% of the penetration sites spread to more than one cell in the transgenic line 19. This slowdown in disease development in *Lr34res*-containing rice is very similar to the partial resistance characteristic for *Lr34res* in wheat and barley. The multicopy line 5 showed silencing of *Lr34res*-based disease resistance. While T2 plants showed a reduction in disease progression, there was no difference between transgenic and sister plants at the T5 generation (Figure S4). To test whether the delay in early invasive growth was sufficient to also reduce macroscopic rice blast symptoms, we spray-inoculated rice plants and evaluated necrotic lesions 7 days after infection. The four transgenic lines 8, 11, 16 and 19 developed less and smaller lesions compared to sib lines when infected with *M. oryzae* isolate FR13 (Figures 1b and S5).

Furthermore, lines 16 and 19 were tested with three additional rice blast isolates originating from Korea and China. Similar to the results obtained with FR13, transgenic lines showed reduced lesion formation compared to the sib lines for all three isolates (Figure 1c). These results show that *Lr34res* in rice confers partial resistance against multiple rice blast isolates.

Expression level-dependent development of LTN in Lr34-carrying rice plants

In wheat and barley, *Lr34res* is associated with a senescence-like process called LTN (Krattinger *et al.*, 2009). Transgenic rice plants also developed the typical LTN (Figures 2a and S6). In contrast to barley where LTN was very strong and ultimately affected entire leaves (Risk *et al.*, 2013), LTN in rice was restricted to the tip of the leaf. The rice phenotype resembles the LTN found in adult wheat plants rather than the uncontrolled necrosis of barley. Lines 11, 16 and 19 developed LTN from early seedling stage on with the strongest LTN observed in line 19. LTN development in this particular line started at the two-leaf stage when the plants were approximately 10 days old. LTN first developed on the older lower leaves and subsequently appeared on younger upper leaves as the plants matured. The early development of LTN had a severe negative impact on the formation of axillary shoots, overall plant vigour and spikelet production (Figure 2b, Table S1). Line 8 on the other hand did not develop LTN at seedling stage but only in adult plants after about 1 month. In contrast to the lines with early LTN development, axillary shoot formation in line 8 was not or only marginally compromised. Plants of transgenic line 8 had 12 tillers on average whereas plants of the respective sib line had 14.8 tillers per plant on average (*t*-test $P = 0.115$, $n = 6$, Table S1). The number of spikelets per panicle did not differ between line 8 (43 spikelets/panicle) and the sib line (46 spikelets/panicle, *t*-test $P = 0.25$, $n = 6$, Table S1) but was significantly reduced in lines 16 and 19. Both lines 19 and 8 carry single T-DNA insertions. The difference in strength of LTN can therefore not be attributed to variation in copy number (Figure S1). RT-qPCR analysis revealed that line 8 showed *Lr34res* expression levels that were 4–10 times lower than in the other three lines at seedling stage and that *Lr34res* expression increased in adult plants of line 8 about five times (Figure 3). Line 11 also showed an increase of *Lr34res* expression from seedling to adult plant stage. However, *Lr34res* expression levels in this line were already ~4 times higher compared to line 8 at seedling stage. A similar increase of *Lr34res* expression levels from seedlings to adult plants was not observed

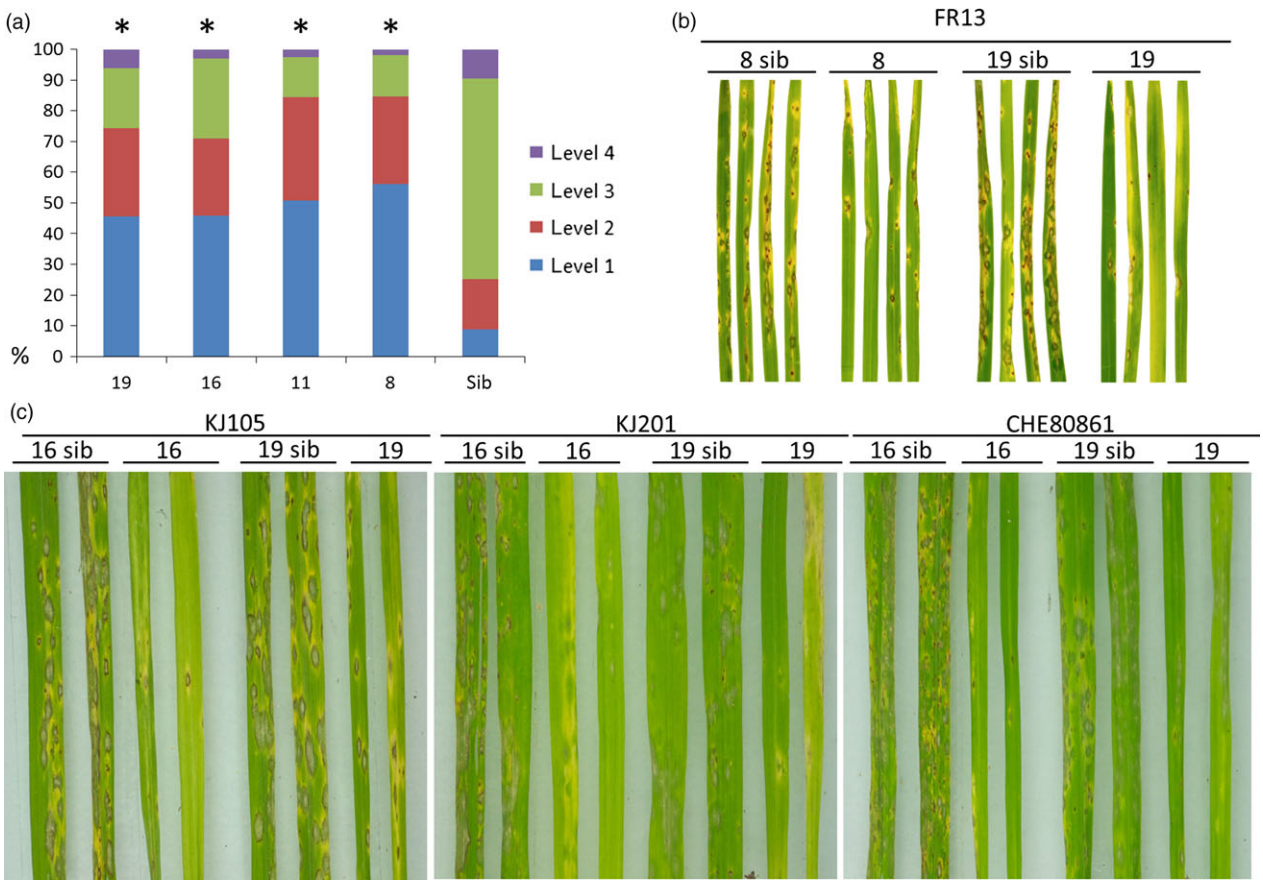

Figure 1 *Lr34res* confers partial resistance against *Magnaporthe oryzae* in transgenic rice. (a) Classification of biotrophic, invasive *M. oryzae* growth on rice leaf sheath cells 28 h after infection according to Saitoh *et al.* (2012). Levels 1–4 represent different lengths of invasive hyphae with level 1 being the shortest (see Experimental procedures for detail). The *y*-axis shows the percentages of appressorial penetration sites that belong to the different infection levels. At least three biological replicates were averaged for each line, and ~50 appressorial penetration sites were evaluated for each replicate. *Significant differences compared to sib lines (sib = pool of sibs from the four transgenic lines; Mann–Whitney *U*-test, $P < 0.01$). (b, c) Macroscopic development of different *M. oryzae* isolates on different transgenic lines and sib lines 7 days postinfection. Infection experiments in (b) were done by spray-inoculation at Aachen University and (c) by spray-inoculation at the Zhejiang Academy of Agricultural Sciences, China. Isolate FR13 was collected in France, KJ105 and KJ201 in Korea and CHE80861 in China.

in lines 16 and 19. All four lines showed comparable *Lr34res* expression levels at adult plant stage. Hence, the late development of LTN in line 8 can most likely be explained by the lower *Lr34res* expression levels at early growth stage. Importantly, the level of LTN at seedling stage did not correlate with level of disease resistance. Although line 8 showed the weakest LTN development, it showed resistance levels comparable to the other lines or even stronger (Figure 1a). The multicopy line 5 showed *Lr34res* expression levels that were 3–4 times higher than in the high-expressing line 19 in the T2 (resistant) generation. In the silenced T5 generation, *Lr34res* expression levels were 3–4 times lower than in the T2 generation, but they were still comparable to the *Lr34res* expression levels of line 19 (Figure S4b). These results indicate that the silencing in line 5 is due to both transcriptional and post-transcriptional mechanisms.

Site-directed mutagenesis of the orthologous rice *Lr34* gene

Rice cultivar Nipponbare contains an expressed orthologous *Lr34* gene—*OsABCG50*—with an intact open reading frame (Krattinger *et al.*, 2011). The predicted OsABCG50 protein shares 86% amino acid identity with LR34res. Because an *Lr34*-like, race nonspecific, multipathogen disease resistance with LTN has not been reported in rice, we hypothesize that OsABCG50 has a function different from disease resistance. This hypothesis is supported by the fact that OsABCG50 carries the susceptible haplotype for the two critical amino acid residues that distinguish LR34res from LR34sus in wheat (Krattinger *et al.*, 2013; Figure S7a). At the first position located in exon 11, *OsABCG50* has the nucleotide triplet 'TTA' encoding for a leucine residue (L550). LR34sus carries a phenylalanine at the orthologous position (F546). These two residues are predicted to be located within a transmembrane helix, and only LR34res has a deletion of a hydrophobic amino acid at this position (Krattinger *et al.*, 2013). At the second critical position, both LR34sus and OsABCG50 carry a tyrosine residue encoded by 'TAT' that is converted to a histidine in LR34res ('CAT'). According to Genevestigator (Zimmermann *et al.*, 2008), *OsABCG50* shows medium expression levels in all parts of the rice plant that are affected by rice blast, namely leaves, sheaths and panicles. We tested whether site-directed modification of the two critical residues in OsABCG50 might alter its function and result in broad-spectrum *M. oryzae* resistance. For this, we constructed the resistant OsABCG50 haplotype by deleting the leucine residue (△L550) and by converting the critical tyrosine to histidine (Y638H). A plasmid containing the altered allele—named *OsABCG50res*—was stably transformed into the *japonica* rice cultivar Nipponbare. *Lr34res* in wheat functions in the presence of *Lr34sus* and we therefore expected that the presence of the endogenous *OsABCG50*

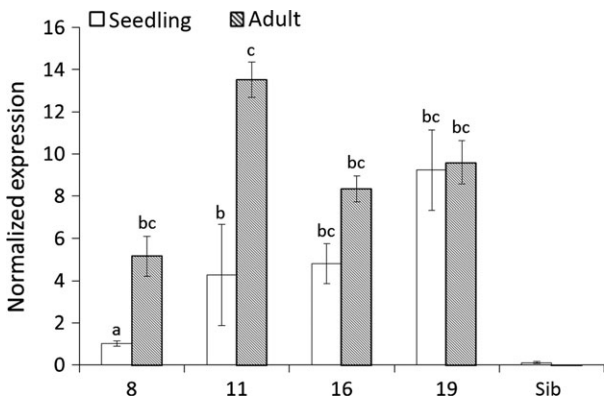

Figure 3 Normalized *Lr34res* expression in transgenic lines. Letters indicate lines with equivalent expression levels (Tukey's honest significance test, $P > 0.05$). Data are normalized to the reference gene *UBC* (Jain *et al.*, 2006) from three biological replicates, and error bars represent standard errors.

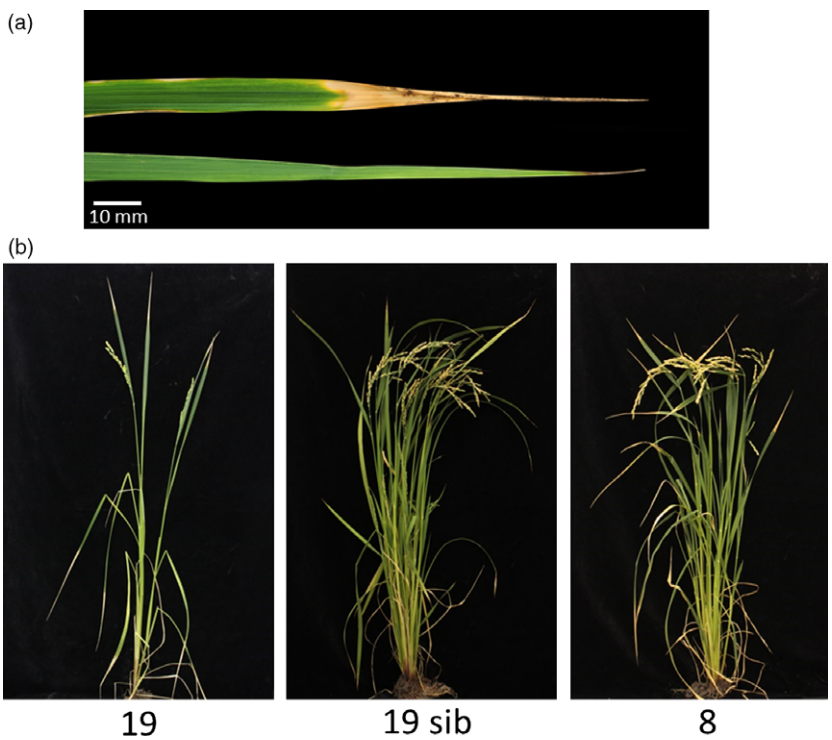

Figure 2 *Lr34res* results in a typical, senescence-like leaf tip necrosis (LTN) in transgenic rice. (a) Representative example of LTN on flag leaves of transgenic line 19 (upper leaf) compared to its sib line (lower leaf). Scale bar = 10 mm. (b) Early LTN development results in suppressed axillary shoot formation in line 19 (left). Line 8 only developed LTN at adult plant stage, and axillary shoot formation in this line was not affected (right).

copy would not interfere with a potential involvement of *OsABCG50res* in *M. oryzae* resistance. We identified two transgenic events (4.2 and 9.1; Figure S7b) that expressed *OsABCG50res*. Segregating T2 families of these two events were infected with rice blast isolate FR13, and early disease progression was monitored microscopically. There was no difference in early *M. oryzae* invasion between lines with *OsABCG50res* and segregating sib lines (Figure S7c). Furthermore, plants expressing *OsABCG50res* also did not develop LTN. These experiments indicate that targeted modification on *OsABCG50* based on the corresponding amino acid changes in *Lr34res* does not result in rice blast resistance.

Discussion

In this study, we showed that the wheat durable, multipathogen resistance gene *Lr34* confers resistance against multiple *M. oryzae* isolates in rice. Rice, together with wheat and maize, is one of the three most widely grown crops in the world, providing a daily food source for more than half of the global population. Rice blast is the most devastating and most economically damaging rice disease (Wilson and Talbot, 2009). It is a rapidly evolving pathogen, and occurrence of new *M. oryzae* races frequently resulted in breakdown of rice blast resistance in the past (Dean *et al.*, 2012; Huang *et al.*, 2014; Wilson and Talbot, 2009). More than 90 rice blast resistance genes have been identified in cultivated and wild rice germplasm until today, and 22 of them have been cloned. Most of the cloned blast resistance genes confer race-specific resistance and code for NBS-LRR immune receptors (Fukuoka *et al.*, 2014; Wang *et al.*, 2014). There are only very few examples of rice blast resistance genes that are active against a broad range of *M. oryzae* isolates. For example, rice plants containing the NBS-LRR gene *Pi9* are resistant against more than 100 rice blast isolates (Qu *et al.*, 2006). Similarly, the atypical NBS-LRR gene *Pb1* confers race nonspecific and durable blast resistance at adult plant stage. It is not yet understood why the pathogen cannot adapt to the *Pb1* resistance gene (Hayashi *et al.*, 2010; Inoue *et al.*, 2013). A third example is *Pi21* that encodes for a protein with a putative heavy metal-binding domain and a protein–protein interaction motif. This gene represents a susceptibility factor and loss-of-function of *Pi21* results in durable, race nonspecific and recessive rice blast resistance (Fukuoka *et al.*, 2009).

The origin of *Lr34* in modern wheat breeding goes back to crosses made in Italy by the wheat breeder Nazareno Strampelli in the early 20th century (Kolmer *et al.*, 2008). *Lr34* has been extensively used in breeding since the Green Revolution, and it continues to provide race nonspecific and partial field resistance against the three wheat rust diseases and powdery mildew. *Lr34* therefore fulfils all the criteria of a durable disease resistance gene in wheat because it remained effective during its prolonged and widespread use in environments favourable to the disease (Johnson, 1984). We therefore consider it likely that the partial resistance described in this study might also be durable in rice. In wheat, *Lr34* is often combined with other partial adult plant resistance genes and this strategy resulted in wheat cultivars that show near-immune levels of broad-spectrum disease resistance (Ellis *et al.*, 2014). Pyramiding of partial resistance genes has also been successfully used to enhance blast resistance in rice (Fukuoka *et al.*, 2015). Rice blast infections can occur during all stages of rice development on leaves, stems and panicles (Wilson and Talbot, 2009). Our results showed that *Lr34* confers effective rice blast resistance on sheaths and leaves. *Lr34* might therefore be a valuable source to improve broad-spectrum rice blast resistance in rice cultivars in the future. The combination of *Lr34* with other broad-spectrum blast resistance genes like *pi21* or *Pb1* would be the most sustainable strategy to achieve long-lasting and effective blast resistance.

Despite the limited number of independent transgenic events generated during this study, we recovered a line that showed late LTN and high levels of disease resistance but no obvious negative impact on tiller formation and spikelet development. This is in contrast to barley, where all transgenic lines showed a dramatic reduction in growth and seed production (Risk *et al.*, 2013). Our data indicated that high *Lr34res* expression levels at seedling stage correlated with early LTN development and reduced plant vigour. Low seedling expression of *Lr34res* in line 8 on the other hand resulted in late LTN development and normal plant growth. The detrimental effects of early *Lr34res* expression might also explain why *Lr34res* in wheat evolved as an adult plant resistance gene that is not effective at seedling stage.

Wheat and rice shared their last common ancestor 40–54 million years ago (The International Brachypodium Initiative, 2010). The rice *Lr34* ortholog *OsABCG50* is most likely not involved in rice blast resistance and has the susceptible *Lr34* haplotype. It is therefore remarkable that all components required for the function of *Lr34res* are still present in rice. This suggests that the substrate transported by *Lr34* must be conserved in wheat, barley and rice. An obvious experiment was the artificial reproduction of the evolutionary events that gave rise to *Lr34res* in wheat by site-directed mutagenesis of *OsABCG50*. A successful outcome might have opened the possibility to increase durable blast resistance in rice nontransgenically through targeted genome editing of *OsABCG50* by TALEN or CRISPR/Cas. Our results however suggested that besides the two critical residues, additional sequence motifs of *Lr34res* might be required for disease resistance in rice. The replacement of larger *OsABCG50* segments spanning the two critical nucleotide triplets with the corresponding wheat sequence might still result in rice blast resistance as the transformation of the entire wheat *Lr34res* gene was functional. A future approach might therefore consist in the generation and testing of multiple rice *OsABCG50*—wheat *Lr34res* chimeric constructs to identify the smallest functional segmental replacement.

So far, *Lr34* was associated with resistance to obligate biotrophic pathogens of wheat and barley. In this study, we could show that *Lr34* also functions against a hemibiotrophic pathogen that has a different lifestyle from rusts and mildews. Obligate biotrophic pathogens remain in close association with their host cells throughout the entire infection cycle. *M. oryzae* represents a special case of hemibiotrophic lifestyle that lacks a distinct switch from biotrophy to necrotrophy. Instead, the growing front of the fungus remains biotrophic as it spreads from cell to cell but invaded cells lose viability by the time the fungus moves to the next cell (Kankanala *et al.*, 2007). The inhibition of this biotrophic fungus growing front by *Lr34res* is obviously sufficient to reduce macroscopic disease symptoms on rice leaves.

In this study, we show a proof of principle that *Lr34res* might serve as an effective source to increase broad-spectrum and durable rice blast resistance. Future field tests and infection experiments with additional relevant rice diseases will be necessary to assess the full benefit of *Lr34res* for rice breeding.

Experimental procedures

Plasmid construction

The cloning of the genomic sequence of the resistant *Lr34res* allele with its native promoter and terminator into the binary vectors p6U and pWBVec8 was described in Risk *et al.* (2013) and resulted in plasmids *p6U:gLr34res* and *pWBVec8:gLr34res*, respectively. The genomic *OsABCG50* sequence including 2879 bp native promoter and 2025 bp terminator sequence was released from Nipponbare BAC clone OsJNBa0035B12 by *Eag*I digestion. The 16 521-bp *Eag*I fragment was subcloned into vector pWGEM-NZf(+) (Promega, Duebendorf, Switzerland). The entire cassette was then transferred into vector pWBVec8 (Wang *et al.*, 1998) through *Not*I. For the site-directed mutagenesis, a 2.6-kb fragment of *OsABCG50* was released from *pWGEM-NZf (+):OsABCG50* with a *Sac*II-*Eco*RI double digest and cloned into pGEM®-T Easy vector (Promega) digested with the same enzymes. To introduce the two mutations, two rounds of site-directed mutagenesis were performed using the Quick Change XL Site-Directed Mutagenesis kit (Agilent Technologies) with primers 5′ GGA GCA TTG TTT TTT TCC ATC ATG ATC ATG CTA AAT GGC ACA-3′ (△L550) and 5′ CAT CAA TCA GTC ACT TCG CTG CAT CGA TTT ATT GCT TCA TAC TT 3′ (Y638H). The altered *Sac*II-*Eco*RI fragment was then re-introduced into the digested *pWBVec8: OsABCG50* fragment to result *in pWBVec8:OsABCG50res*.

Agrobacterium-mediated transformation and selection of transgenic events

The binary plasmids *p6U:gLr34res* (events 5, 8, 11 and 16) and *pWBVec8:OsABCG50res* were transformed into the *Agrobacterium tumefaciens* strain EHA105 by electroporation according to Sambrook and Russel (2001). Fifty primary calli of Nipponbare were transformed as described in Sallaud *et al.* (2003). Plasmid *pWBVec8:gLr34res* (event 19) was cloned into Agrobacterium strain AGL1. Transformation with this plasmid was performed according to Upadhyaya *et al.* (2000). Primary transgenic T0 plants transformed with *Lr34res* were evaluated for T-DNA integration using the *Lr34res*-specific PCR marker *cssfr1* (Lagudah *et al.*, 2009). A primer pair that specifically distinguished *OsABCG50res* from the endogenous *OsABCG50* gene was used to check for insertion and expression of the *OsABCG50res* T-DNA (OsABCG50res_f: 5′-GAG CAT TGT TTT TTT CCA TCA TG-3′ and OsABCG50res_r: 5′-GCA GCG AAG TGA CTG ATT GAT G-3′). PCR positive T0 plants were evaluated for expression of the transgenes using *Lr34res*-specific cDNA primers (Risk *et al.*, 2013) or OsABCG50res_f–OsABCG50res_r. To determine copy number, 7.5 μg of genomic DNA of T0 and T1 plants was digested with *Xba*I (pWBVec8) and *Eco*RI or *Apo*I (p6U). Southern blots were performed as described in Risk *et al.* (2013) with a probe for the selectable marker gene *HPT*. Homozygous T2 lines for *Lr34res*-containing events were PCR-selected using *cssfr1* on ~20 individual T2 plants.

Fungal strain cultivation

Magnaporthe oryzae isolates were grown on oatmeal agar (30 g/L oatmeal, 5 g/L sucrose and 16 g/L agar) at room temperature, with 12-h light during 5 days. To enhance sporulation, the fungus was transferred to white light/blue light (Philips TL-D 15W BLB, Zofingen, Switzerland) for four additional days. Rice blast conidia were then harvested from plates by rinsing with sterile distilled water and raking with a spatula. Spores were filtered through two layers of gauze and suspended to a final density of 1–2.5 × 10^5 conidia/mL.

Leaf sheath infection assay

Rice plants were grown in a growth cabinet at 28/24 °C day/night, 75% humidity and 12-h photoperiod. Early development of *M. oryzae* was rated microscopically according to Saitoh *et al.* (2012). In brief, the inoculation of leaf sheaths was performed with a syringe on 3 cm leaf sheath segments of 5-week-old plants using a conidial suspension of 1 × 10^5 conidia/mL of isolate FR13. Infected fragments were then incubated for 28 or 40 h in a humid petri dish at room temperature in the dark. To investigate the level of infection, invasive hyphae were stained with lactophenol–trypan blue (30 mL ethanol, 10 mL glycerol, 10 mL lactic acid, 10 mg trypan blue and 10 mL distilled water). Whole leaf sheath segments were boiled for 1 min in the staining solution and decolorized in chloral hydrate (2.5 g of chloral hydrate in 1 mL ddH_2O) for at least 30 min ~50 appressorial sites were evaluated per leaf sheath segment using a Zeiss Axio Imager Z1 microscope (Feldbach, Switzerland). The different levels of invasive growth were classified according to Saitoh *et al.* (2012): level 1, invasive hypha length is shorter than 10 μm with no branch; level 2, invasive hypha length is 10–20 μm with 0–2 branches; level 3, invasive hypha length is longer than 20 μm and/or with more than two branches within one cell; level 4, invasive hypha is spread into more than one cell. At least three biological replicates were averaged for each sample, and ~50 appressorial penetration sites were evaluated for each replicate.

Rice blast spray-inoculation

Three-week-old seedling plants were spray-inoculated with conidial solutions of *M. oryzae* isolates FR13 (France), KJ105 (Korea), KJ201 (Korea) and CHE80861 (China). A conidial suspension of 2.5 × 10^5 spores/mL in gelatin (0.1% w/v)/Tween-20 (0.05% w/v) was sprayed on seedling plants. After spraying, plants were incubated in a dark moist chamber (26 °C, nearly 100% relative humidity) for 24 h and afterwards kept under a transparent tent in a climatic chamber (15 h light at 24 °C, 75% humidity and 9 h darkness at 23 °C, 80% humidity). Disease symptoms were evaluated 7 days after infection. At least three independent infection experiments were performed for each transgenic line. The avirulence spectrum of isolates KJ105, KJ201 and CHE80861 was analysed with PCR markers for seven known rice blast avirulence genes. The reaction of the European isolate FR13 on different rice lines has been evaluated by Roumen *et al.* (1997; Table S2). Based on these analyses, the four isolates are expected to show virulence on *Pish*, *Pik*^{s}, *Pif*, *PiCO39*, *Pia*, *Pii* and *Pita*.

Quantitative expression analysis

Expression analysis was carried out using RT-qPCR. Total RNA was extracted from 3-week-old seedling leaves and flag leaves using the SV Total RNA Isolation System (Promega). RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Zug, Switzerland), and RNA integrity was assessed on a 0.8% SB agarose gel. cDNA was synthesized from 1 μg of RNA using the i-Script™ cDNA Synthesis kit (BioRad, Cressier, Switzerland). Expression of *Lr34res* was measure using the *Lr34res* qPCR probe designed by Risk *et al.* (2012). RT-qPCR was carried out in a 10 μL reaction including 5 μL KAPA SYBR® FAST qPCR Master Mix (KAPA Biosystems, London, UK), 4 μL of 1 : 20 diluted cDNA template and 500 nM final concentration of forward and reverse primers. Samples were run on a CFX96 Touch™ Real-Time PCR Detection

System (BioRad). The ubiquitin-conjugating enzyme E2 (*UBC*; LOC_Os02g42314; UBC_f 5′-GAA TGT GCA TTT CAA GAC AGG-3′, UBC_r 5′-GCA ATT ATG GCT CTA CAA ACG G-3′) was used as reference gene (Jain *et al.*, 2006). Primers for *Lr34res* and *UBC* were designed on exon–intron junctions to avoid amplification of genomic DNA. Thermocycling conditions for both probes were 95 °C for 1 min, followed by a two-step PCR of 40 cycles of 95 °C for 3 s and 63 °C for 20 s. Primer efficiencies were determined through serial dilutions (*Lr34* = 94.5%; *UBC* = 103.5%), and no reverse transcriptase controls were included in each PCR run. RT-qPCR was performed on at least three biological replicates with technical triplicates. Data were analysed using the CFX MANAGER 3.1 software (BioRad). Statistical analysis was done using the REST software (Pfaffl *et al.*, 2002) and the JMP® statistical package (SAS Institute, Boeblingen, Germany).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Southern blots showing T-DNA copy number in the five independent transgenic *Lr34res* rice lines.

Figure S2 (a) Microscopic images of the four levels of early *Magnaporthe oryzae* invasive growth. Level 1, invasive hypha length is shorter than 10 μm with no branch; level 2, invasive hypha length is 10–20 μm with 0–2 branches; level 3, invasive hypha length is longer than 20 μm and/or with more than two branches within one cell; level 4, invasive hypha is spread into more than one cell. (b) Representative images of leaf sheath surface of lines 19 and 19 sib 28 h after *M. oryzae* infection. Most appressorial infection sites in line 19 are levels 1 and 2, whereas 19 sib shows many infection sites of levels 3 and 4. Sp = spore; Ap = appressorium; IH = invasive hypha; scale bar = 15 μm for (a) and (b).

Figure S3 Comparison of early invasive *Magnaporthe oryzae* growth in transgenic line 19 and corresponding sib line (sib) 28 h (left) and 40 h (right) postinfection (hpi).

Figure S4 Silencing of disease resistance phenotype in the multicopy line 5.

Figure S5 Representative examples of leaves of transgenic lines 11 and 16 and corresponding sib lines (sib) infected (spray-inoculation) with *Magnaporthe oryzae* isolate FR13, 7 days postinfection.

Figure S6 Leaf tip necrosis on flag leaves of the four *Lr34res* lines 8, 11, 16 and 19. Scale bar = 10 mm.

Figure S7 Site-directed modification of the rice *Lr34*-ortholog *OsABCG50* does not result in increased *Magnaporthe oryzae* resistance.

Table S1 Number of tillers and number of spikelets per panicle for three transgenic lines compared to their respective sib lines.

Table S2 Distribution of different known avirulence genes in three isolates.