Orthologous receptor kinases quantitatively affect the host status of barley to leaf rust fungi

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Initial paragraph

Global food security depends on cereal crops with durable disease resistance. Most cereals are colonized by rust fungi, which are pathogens of major significance for global agriculture[1]. Cereal rusts display a high degree of host specificity and one rust species or forma specialis generally colonizes only one cereal host[2]. Exploiting the non-host status and transferring non-host resistance genes between cereal crop species has been proposed as a strategy for durable rust resistance breeding. The molecular determinants that define the host status to rusts however are largely unknown. Here, we show that orthologous genes underlying the quantitative *Rphq2* locus from cultivated barley[3] and *Rph22* from wild bulbous barley[4] affect the host status to leaf rusts. Both genes encode lectin receptor-like kinases. We transformed *Rphq2* and *Rph22* into an experimental barley line that has been bred for susceptibility to non-adapted leaf rusts, which allowed us to quantify resistance responses against various leaf rust species. *Rphq2* conferred a much stronger resistance to the leaf rust of wild bulbous barley than to the leaf rust adapted to cultivated barley, while for *Rph22* the reverse was observed. We hypothesize that adapted leaf rust species mitigate perception by cognate host receptors by lowering ligand recognition. Our results provide an example for orthologous genes that link quantitative host and non-host resistance to cereal rusts and they form the basis to exploit non-host resistance in molecular breeding.

Main text

Innate immunity in animals and plants is triggered by the perception of pathogen-derived molecules by surface-localized and intracellular receptors[5-7]. The most durable type of disease resistance is non-host resistance, which refers to the immunity of an entire plant species to all races or strains of a potential pathogen species[8,9]. Similar to host resistance against adapted pathogens (a pathogen that is capable of parasitizing a given plant species), non-host resistance is often the result of induced defense responses and it has been hypothesized that both resistance mechanisms may be controlled by similar molecular factors[10-12]. Dissecting the genetic basis of non-host resistance and isolating genes that determine the host status of a cereal crop species to a particular pathogen species is challenging. The main reason for this is that non-

host resistance affects an entire plant species and that most interspecific hybrids that would allow researchers to genetically dissect non-host resistance are infertile. The cereal rusts have provided an ideal experimental system to study genetic components of host and non-host resistance. Several closely related rust species and formae speciales that colonize various cultivated cereals and their wild relatives have been described. Cereal rusts generally show a very narrow host range and only colonize one particular host species. Cultivated barley (*Hordeum vulgare*) is a host to the fungal pathogen *Puccinia hordei* that causes the barley leaf rust disease. *P. hordei* however is not able to infect bulbous barley (*H. bulbosum*)[13], a close wild relative of cultivated barley that diverged about six million years ago[14]. On the other hand, cultivated barley is generally a non-host to *P. hordei-bulbosi* and *P. triticina*, the causal agents of leaf rusts of bulbous barley and wheat (*Triticum aestivum*), respectively. It has been observed that *P. triticina* and *P. hordei-bulbosi* isolates are able to form a few colonies on only a limited number of cultivated barley lines, and only at seedling stage[11]. Such a very low level of susceptibility in a plant species to a pathogen of a different host is sometimes referred to as 'marginal host' or 'near non-host' status of that plant species. The genetic basis of non-host resistance against cereal rusts ranges from simple qualitative inheritance to complex quantitative genetic architectures[15-18]. *Rphq2* was initially identified in the old Dutch *H. vulgare* cultivar 'Vada' as a quantitative disease resistance locus against *P. hordei*[19,20]. It was later found that *Rphq2* also had an effect on non-adapted rusts when the gene was crossed into a barley line that allowed for some growth of non-adapted rust species[3,17]. This led to the hypothesis that the *Rphq2* locus confers partial resistance to both adapted and non-adapted rusts. Whether this resistance was conferred by the same or different genes at the *Rphq2* locus however remained unknown. *Rphq2* had previously been mapped to a 195 kb interval spanned by three Vada bacterial artificial chromosome (BAC) clones[3]. A second partial *P. hordei* resistance locus, *Rph22*, was independently mapped to exactly the same genetic interval on barley chromosome 2H[4]. *Rph22* was artificially introgressed into cultivated barley from bulbous barley in 1996 through interspecific hybridizations. Both loci result in a similar partial and non-hypersensitive resistance response in cultivated barley, but the effect of the introgressed *Rph22* is stronger[4] (Fig. 1a; Supplementary video). By generating additional recombinants, we further reduced the *Rphq2* genetic interval to 0.05 cM

delimited by flanking markers WBE115 and WBB118 (Fig. 1b and Supplementary Table 1). The corresponding 122 kb physical interval in 'Vada' contained five candidate genes (Fig. 1c), of which only one, a gene encoding a putative legume-like (L-type) lectin receptor-like kinase (Hv-LecRK; Fig. 1d) was expressed (Supplementary Fig. 1). To test for the involvement of this candidate receptor-like kinase in disease resistance, a 4,553 bp genomic fragment including the complete *Hv-LecRK* coding region and 2,243 bp of upstream sequence was transformed into barley line Golden SusPtrit[21]. Golden SusPtrit is an experimental barley line that has been bred for susceptibility to various non-adapted leaf rusts at seedling stage and that is amenable to transformation[16,21]. All six tested transgenic T1 families expressing *Hv-LecRK* showed partial resistance to *P. hordei*, measured as an increase in relative latency period (time after inoculation until half of the infection sites produce visible sporulation) by 10-20% compared to null segregants without the transgene (Fig. 2a). Hence, genetic mapping, the expression patterns of the five candidate genes, and transgenic complementation provide strong evidence that *Hv-LecRK* is *Rphq2*. To clone the *H. bulbosum* derived *Rph22*, we amplified an orthologous *Hb-LecRK* gene from the *Rph22* containing introgression line 182Q20[4] using *Rphq2*-derived PCR primers. A molecular marker confirmed that *Hb-LecRK* originated from the *H. bulbosum* donor and not from the recurrent *H. vulgare* parent Golden Promise (Supplementary Fig. 2a). Furthermore, the *Hb-LecRK* candidate gene was genetically mapped to the small interval that had been previously identified to contain *Rph22*[4] (Supplementary Fig. 2b). Similar to *Rphq2*, *Hb-LecRK* transgenic T1 families showed an increased relative latency period at seedling stage by 10-30% when challenged with *P. hordei*, indicating that *Hb-LecRK* is *Rph22* (Fig. 2b). While both genes prolonged latency period, only *Rph22* resulted in a significant reduction of the relative infection frequency (the number of sporulation sites formed). We detected no or only a marginal reduction of infection frequency in *Rphq2* expressing transgenic families (Fig. 2c and Supplementary Fig. 3). The phenotypic difference in infection frequency was supported by fungal biomass quantification using qPCR (Supplementary Fig. 4). These results confirm the previous observations that *Rph22* provides stronger *P. hordei* resistance than *Rphq2*. We refer to *Rphq2-P. hordei* as a homologous host gene-pathogen interaction because *Rphq2* originates from cultivated barley, which is a host to *P. hordei*. *Rph22-P. hordei* represents

a heterologous interaction because *Rph22* was only recently introgressed into cultivated barley from *H. bulbosum*, which is a non-host to *P. hordei*[13]. We hypothesize that the *Rph22-P. hordei* interaction has not been subjected to extensive co-evolution. When we challenged the various transgenic families with *P. hordei-bulbosi,* a leaf rust that is non-adapted to cultivated barley but adapted to *H. bulbosum,* we observed the opposite effect of the two genes on infection frequency. The heterologous *Rphq2-P. hordei-bulbosi* interaction resulted in a clear reduction of infection frequency, while the homologous *Rph22-P. hordei-bulbosi* interaction only reduced the number of infection sites marginally (Fig. 2d). Both genes however gave an equally strong resistance response to the wheat leaf rust pathogen *P. triticina,* a non-adapted pathogen to both cultivated and wild bulbous barley (Fig. 2e, f). The phenotypic effect of *Rph22* against *P. hordei* was even more pronounced at adult plant stage where many transgenic families showed near immunity to *P. hordei* (Fig. 2g and Supplementary Fig. 5). We hypothesize that the different resistance responses of homologous and heterologous interactions are the result of a direct interaction between a pathogen-derived molecule and the respective receptor-like kinase. Inoculation experiments were performed in the uniform genetic background of *H. vulgare* line Golden SusPtrit and all downstream components contributing to the *Rphq2*-mediated and *Rph22*-mediated resistance are thus from *H. vulgare*. An indirect effect, for example through the silencing of a downstream signaling component by a pathogen-derived virulence effector, would most likely have resulted in a leaf rust species-dependent pattern of resistance responses, independent of the origin of the resistance gene. We observed however that the resistance outcome depends on both the origin of the resistance gene and the leaf rust species, which suggests that adapted rusts evolved the ability to partially evade recognition by the cognate receptor, possibly by lowering ligand binding (Supplementary Fig. 6). The predicted Hv-LecRK and Hb-LecRK proteins showed 85.2 % amino acid identity. Most of the polymorphisms were found in the putative extracellular domain, whereas the kinase domain showed a high degree of conservation (Supplementary Fig. 7). Similarly, the extracellular domains of Hv-LecRK and Hb-LecRK were highly diverged from a protein version present in the *H. vulgare* cultivar Golden Promise (GP_Hv-LecRK), while the intracellular domains were highly conserved (Supplementary Fig. 7). GP_Hv-LecRK is not associated with *P. hordei*

resistance[21]. The extracellular domain of Hv-LecRK from Vada showed 73 amino acid exchanges, 23 amino acid insertions and six deletions compared to the Golden Promise protein version, resulting in an amino acid identity of only 65%. The extracellular domain of Hb-LecRK was 53% identical to GP_Hv-LecRK, while the intracellular domain was 97% identical. A high degree of differentiation of extracellular domains has also been reported for other receptor-like kinases involved in disease resistance[22]. Allele sequences from 21 different barley cultivars identified nine different *LecRK* haplotypes, which indicates that quantitative non-host resistance genes are not generic and vary between different cultivars (Supplementary Fig. 8). Transient infiltration of *Hv-LecRK* (Vada and Golden Promise alleles) and *Hb-LecRK* into *Nicotiana benthamiana* resulted in similar levels of cell death and production of reactive oxygen species (ROS), whereas a kinase dead form did not result in cell death or ROS production. These results indicate that Hv-LecRK and Hb-LecRK are able to initiate kinase-dependent cell death at comparable intensity[23] (Fig. 3a, b).

Receptor-like kinases in plants represent a large family of surface-localized proteins that perceive external and internal stimuli. Localization experiments in *N. benthamiana* confirmed that both Hv-LecRK and Hb-LecRK localize to the plasma membrane (Fig. 3c). Lectin receptor kinases have been associated with the perception of various structurally unrelated ligands, including extracellular ATP[24], 3-hydroxy fatty acid metabolites[25], and peptides[26]. The subfamily of L-type lectin receptor kinases (LecRK) comprises 43 members in *Arabidopsis* and 69 members in rice[27]. Several LecRKs are involved in disease resistance[27]. For example, the closest *Arabidopsis* homologs of Hv-LecRK and Hb-LecRK, LecRK-IX.1 and LecRK-IX.2, have been shown to regulate resistance against various species of the oomycete pathogen *Phytophthora*[23]. Transcript levels of *Hv-LecRK* and *Hb-LecRK* were induced 0-24 hours after pathogen inoculation (Supplementary Fig. 9).

From a breeding perspective, our work highlights the benefits and potential risks of introgressing resistance genes from distant relatives, a strategy that is widely applied in cereal disease resistance breeding[28]. The phenotypic effect of *Rph22* against *P. hordei* was much stronger compared to the endogenous *Rphq2*.

However, our results indicate that *P. hordei* might evolve the ability to evade *Rph22*-mediated perception over time. On the other hand, a possible determinant of the non-host status of cultivated barley to *P. hordei-bulbosi* has been removed by replacing *Rphq*2 with *Rph22*, which might lower the barrier for a host jump of *P. hordei-bulbosi* to cultivated barley. The widespread planting of a wheat cultivar lacking a major determinant of host status to blast pathogens for example has most likely resulted in the emergence of wheat blast as a new and devastating disease of wheat[29]. In summary, we show that two orthologous L-type lectin receptor-like kinases quantitatively contribute towards determining the host status of barley to leaf rust pathogens, which constitutes a first important step to exploit non-host resistance in molecular rust resistance breeding.

References

1. Savary, S. *et al.* The global burden of pathogens and pests on major food crops. *Nature Ecology & Evolution* **3**, 430-439 (2019).
2. Dracatos, P.M., Haghdoust, R., Singh, D. & Park, R.F. Exploring and exploiting the boundaries of host specificity using the cereal rust and mildew models. *New Phytologist* **218**, 453-462 (2018).
3. Yeo, F.K. *et al.* Haplotype divergence and multiple candidate genes at *Rphq2*, a partial resistance QTL of barley to *Puccinia hordei*. *Theoretical and Applied Genetics* **129**, 289-304 (2016).
4. Johnston, P.A., Niks, R.E., Meiyalaghan, V., Blanchet, E. & Pickering, R. *Rph22*: mapping of a novel leaf rust resistance gene introgressed from the non-host *Hordeum bulbosum* L. into cultivated barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* **126**, 1613-1625 (2013).
5. Jones, J.D., Vance, R.E. & Dangl, J.L. Intracellular innate immune surveillance devices in plants and animals. *Science* **354**, aaf6395 (2016).
6. Dardick, C. & Ronald, P. Plant and animal pathogen recognition receptors signal through non-RD kinases. *PLoS Pathogens* **2**, e2 (2006).

7. Mogensen, T.H. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical Microbiology Reviews* **22**, 240-273 (2009).

8. Lee, H.A. *et al.* Current understandings of plant nonhost resistance. *Molecular Plant-Microbe Interactions* **30**, 5-15 (2017).

9. Fonseca, J.P. & Mysore, K.S. Genes involved in nonhost disease resistance as a key to engineer durable resistance in crops. *Plant Science* **279**, 108-116 (2019).

10. Schulze-Lefert, P. & Panstruga, R. A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. *Trends in Plant Science* **16**, 117-125 (2011).

11. Niks, R.E. & Marcel, T.C. Nonhost and basal resistance: how to explain specificity? *New Phytologist* **182**, 817-828 (2009).

12. Cevik, V. *et al.* Transgressive segregation reveals mechanisms of *Arabidopsis* immunity to *Brassica*-infecting races of white rust (*Albugo candida*). *Proc Natl Acad Sci U S A* **116**, 2767-2773 (2019).

13. Anikster, Y. Host specificity versus plurivority in barley leaf rusts and their microcyclic relatives. *Mycological Research* **93**, 175-181 (1989).

14. Blattner, F.R. Multiple intercontinental dispersals shaped the distribution area of *Hordeum* (Poaceae). *New Phytologist* **169**, 603-614 (2006).

15. Bettgenhaeuser, J. *et al.* The genetic architecture of colonization resistance in *Brachypodium distachyon* to non-adapted stripe rust (*Puccinia striiformis*) isolates. *PLoS Genetics* **14**, e1007637 (2018).

16. Atienza, S.G., Jafary, H. & Niks, R.E. Accumulation of genes for susceptibility to rust fungi for which barley is nearly a nonhost results in two barley lines with extreme multiple susceptibility. *Planta* **220**, 71-79 (2004).

17. Jafary, H., Albertazzi, G., Marcel, T.C. & Niks, R.E. High diversity of genes for nonhost resistance of barley to heterologous rust fungi. *Genetics* **178**, 2327-2339 (2008).

18. Gilbert, B. *et al.* Components of *Brachypodium distachyon* resistance to nonadapted wheat stripe rust pathogens are simply inherited. *PLoS Genetics* **14**, e1007636 (2018).
19. Qi, X., Niks, R.E., Stam, P. & Lindhout, P. Identification of QTLs for partial resistance to leaf rust (*Puccinia hordei*) in barley. *Theoretical and Applied Genetics* **96**, 1205-1215 (1998).
20. Marcel, T.C., Aghnoum, R., Durand, J., Varshney, R.K. & Niks, R.E. Dissection of the barley 2L1.0 region carrying the '*Laevigatum*' quantitative resistance gene to leaf rust using near-isogenic lines (NIL) and subNIL. *Molecular Plant-Microbe Interactions* **20**, 1604-1615 (2007).
21. Yeo, F.K. *et al.* Golden SusPtrit: a genetically well transformable barley line for studies on the resistance to rust fungi. *Theoretical and Applied Genetics* **127**, 325-337 (2014).
22. Hurni, S. *et al.* The maize disease resistance gene *Htn1* against northern corn leaf blight encodes a wall-associated receptor-like kinase. *Proc Natl Acad Sci U S A* **112**, 8780-8785 (2015).
23. Wang, Y. *et al. Arabidopsis* lectin receptor kinases LecRK-IX.1 and LecRK-IX.2 are functional analogs in regulating *Phytophthora* resistance and plant cell death. *Molecular Plant-Microbe Interactions* **28**, 1032-1048 (2015).
24. Choi, J. *et al.* Identification of a plant receptor for extracellular ATP. *Science* **343**, 290-294 (2014).
25. Kutschera, A. *et al.* Bacterial medium-chain 3-hydroxy fatty acid metabolites trigger immunity in *Arabidopsis* plants. *Science* **364**, 178-181 (2019).
26. Bouwmeester, K. *et al.* The lectin receptor kinase LecRK-I.9 is a novel *Phytophthora* resistance component and a potential host target for a RXLR effector. *PLoS Pathogens* **7**, e1001327 (2011).
27. Wang, Y. & Bouwmeester, K. L-type lectin receptor kinases: New forces in plant immunity. *PLoS Pathogens* **13**, e1006433 (2017).
28. Wulff, B.B. & Moscou, M.J. Strategies for transferring resistance into wheat: from wide crosses to GM cassettes. *Frontiers in Plant Science* **5**, 692 (2014).
29. Inoue, Y. *et al.* Evolution of the wheat blast fungus through functional losses in a host specificity determinant. *Science* **357**, 80-83 (2017).

30. Mascher, M. *et al.* A chromosome conformation capture ordered sequence of the barley genome. *Nature* **544**, 427-433 (2017).

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Author contributions

Y.W., X.Q., R.E.N. and S.G.K. designed research. Y.W., S.S., H.V., P.D. and P.A.J. performed molecular experiments. Y.W., S.S., H.V., P.D., A.V. and R.E.N. performed rust inoculations and phenotypic scorings. G.H. and J.K. stably transformed barley line Golden SusPtrit. Y.W. and I.B. performed subcellular localization. Y.W., R.E.N. and S.G.K. wrote the paper. All authors have read and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper

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Figure legends

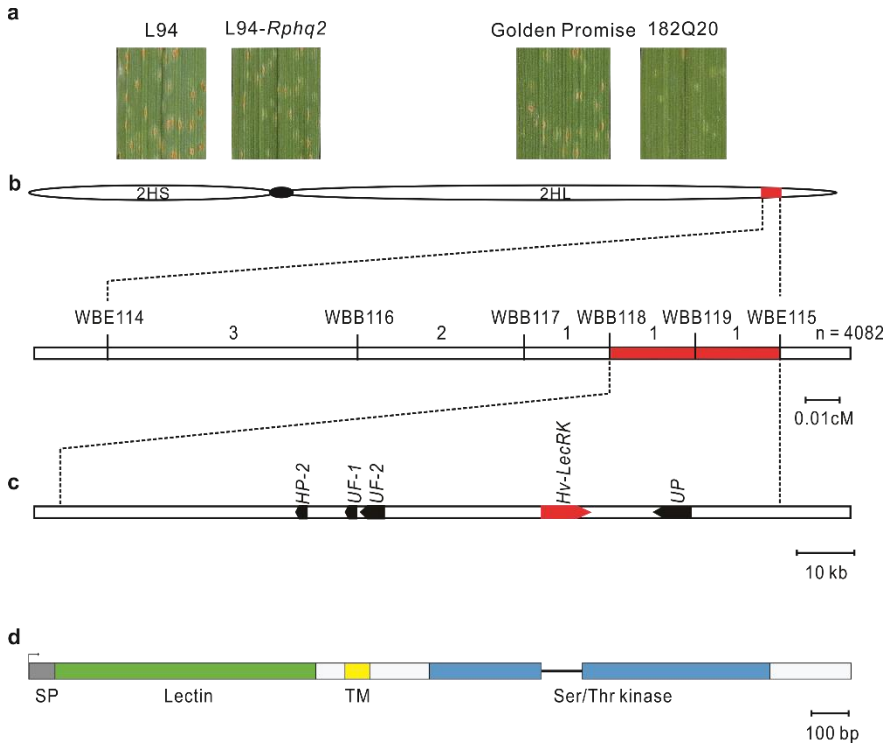

Fig. 1 *Rphq2/Rph22* phenotype and mapping. **a,** Phenotypic effect of *Rphq2* and *Rph22* against *Puccinia hordei*. L94-*Rphq2* is a backcross line that contains *Rphq2* from the resistant barley cultivar Vada in the genetic background of the highly susceptible barley line L94. 182Q20 is an introgression line that contains *Rph22* from the related wild bulbous barley *Hordeum bulbosum* in the genetic background of barley cultivar Golden Promise. **b**, Genetic map of the *Rphq2* interval. The number of recombinants is indicated between the DNA markers. n=4,082 gametes of the three mapping populations. **c**, The corresponding 122 kb physical interval contains five predicted genes. HP = hypothetical protein, UF = unknown function, UP = uncharacterized protein, Hv-LecRK = *Hordeum vulgare* lectin receptor-like kinase. Genes were *de novo* annotated using the TriAnnot pipeline with support from transcriptome sequencing of *P. hordei* inoculated Vada leaves[3]. The Morex reference assembly[30] shows a highly diverged haplotype in the *Rphq2* region and gene content and order were not conserved between Morex and Vada. Only *Hv-LecRK* was supported by the transcriptome reads of Vada[3]. **d**, Gene structure of the candidate gene *Hv-LecRK*. Exons are represented as rectangular bars, and intron as black line between the exons. The predicted protein domains are indicated by different colors. SP = signal peptide; Lectin = legume lectin domain; TM = transmembrane domain; Ser/Thr kinase = serine/threonine kinase domain.

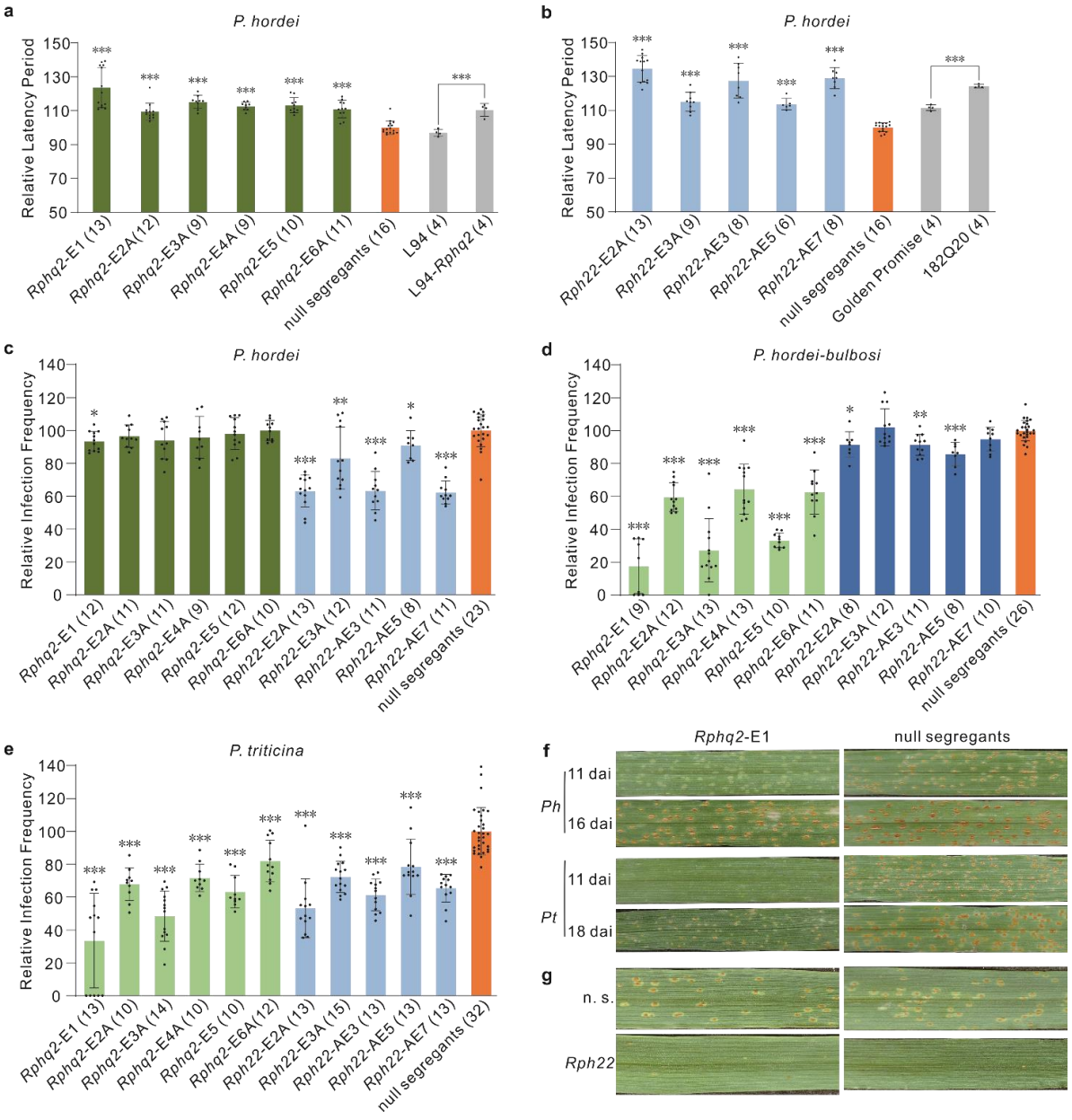

Fig. 2 Resistance mediated by *Hv-LecRK* and *Hb-LecRK* is stronger against non-adapted than against adapted leaf rust species. **a**, **b,** Measurement of relative latency period of *P. hordei* infections on *Rphq2* (**a**) and *Rph22* (**b**) expressing transgenic families. **c-e**, Measurement of relative infection frequency for the three rust pathogens *P. hordei* (**c**), *P. hordei-bulbosi* (**d**), and *P. triticina* (**e**) on transgenic plants expressing *Rphq2* (green) and *Rph22* (blue) compared to null segregants (orange). Dark blue and green bars represent homologous plant gene–rust interactions, whereas light blue and green bars represent heterologous interactions; **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 (two-tailed *t*-test transformants vs null segregants). Null segregants are set to 100%. Number in brackets refer to the number of biological replicates. Values and error bars represent means and standard deviations. **f**, Representative images showing the effects of *Rphq2* against *P. hordei* (*Ph*) and *P. triticina* (*Pt*) at seedling stage in the *Rphq2* expressing transgenic family *Rphq2*-E1 compared to null segregants. dai = days after inoculation. **g**, Representative images showing the effect of *Rph22* against *P. hordei* at adult plant stage. n.s. = null segregant. Inoculation experiments in **f** and **g** were repeated three times independently with similar results.

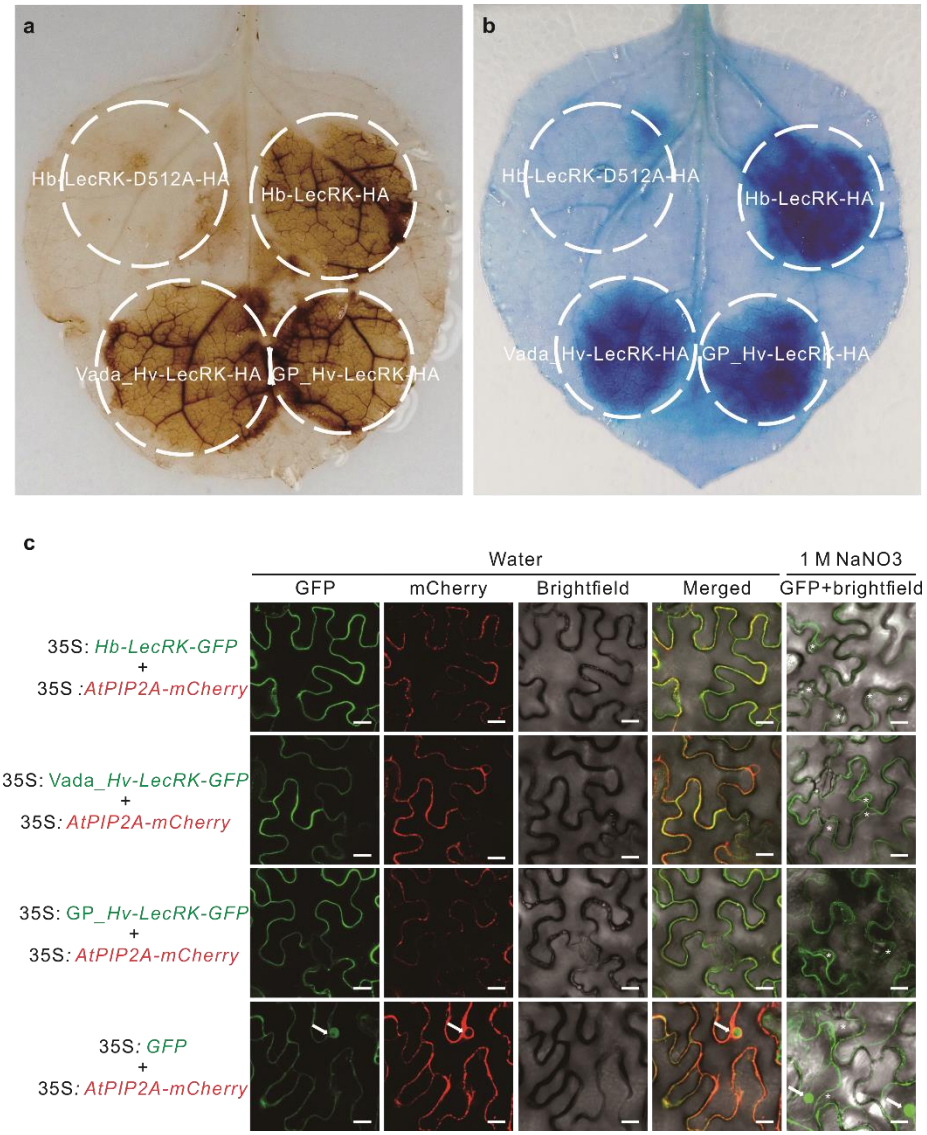

Fig. 3 Molecular characterization of Hv-LecRK and Hb-LecRK proteins. a,**b,** H_2O_2 production (**a**) and cell death (**b**) in leaves of *Nicotiana benthamiana* after infiltrations with three lectin receptor like kinase genes originating from Vada (*Rphq2*), *Hordeum bulbosum* (*Rph22*) and the susceptible barley cultivar Golden Promise (GP). Hb-LecRK-D512A is a kinase dead form of Hb-LecRK. **c**, Subcellular localization of GFP-tagged Hb-LecRK and Hv-LecRK. GFP alone and the mCherry tagged plasma membrane marker protein AtPIP2A were used as controls. Cells were plasmolyzed in 1 M NaNO3 for 5 min. Asterisks indicate areas between plasmolyzed cells, arrows indicate the nucleus, scale bars = 20 µm. Infiltration and localization experiments were repeated three times independently with similar results.

Methods

Pathogen inoculations

The rust strains used in this study are *Puccinia hordei* isolate 1.2.1, a monospore culture derived from isolate 1.2[31] collected at Wageningen in 1972. The *P. hordei-bulbosi* isolate was collected from Kalaybar, Iran in 2010. The *P. triticina* isolate B9414 (also referred to as *P. triticina* INRA) was kindly provided by Dr H. Goyeau, INRA, France[32]. The different leaf rust species were propagated on their respective hosts (*P. hordei-bulbosi* on *H. bulbosum* and *P. triticina* on *T. aestivum*) prior to inoculation experiments on *H. vulgare* (Supplementary Fig. 10). For seedling inoculations, barley grains were sown in two rows on both sides of a 37x39 or 55x28 cm tray and kept in a greenhouse compartment or growth cabinet at 20°C, 8 hours dark, 16 hours light. Inoculations were carried out 10 days after the sowing. For each seedling, the second leaf was removed, and the first leaf was pinned to the soil with the adaxial side facing upwards. A mix of 1 mg urediniospores of *P. hordei* isolate 1.2.1 or 1.5 mg urediniospores of heterologous rusts (*P. hordei-bulbosi* Iran or *P. triticina* isolate B9414) and 100 mg of lycopodium powder (Sigma) was blown over the plants in a settling tower[33], which facilitates the uniform distribution of urediniospores (Supplementary Fig. 11). Spore deposition is about 60 urediniospores cm^{-2} per mg applied inoculum. Ten minutes after inoculation in the settling tower, the trays were moved into a dew chamber to allow spore germination in the dark at 18°C. Infection levels were assessed using latency period 50 (LP50, measured as time period until 50% of the pustules appeared)[34] and infection frequency (IF, measured as number of pustules per cm^2, 15 days after inoculation). Relative latency period (RLP50) and relative infection frequency were calculated relative to the average LP50 and IF of null segregants. Pictures were taken 10 to 18 days after inoculation. For adult plant inoculations, barley plants were grown until flag leaf stage. Two leaves below the flag leaf were brushed with fresh urediniospores of *P. hordei* isolate 1.2.1 diluted 200 times by lycopodium powder. Inoculated plants were then put into a dew chamber at 18°C and no light overnight. Then, plants were put back into a growth room. Pictures were taken 10 days after inoculation. For the quantification of rust biomass, three leaf samples of each line were collected for DNA extraction 10 days after inoculation. Three

non-inoculated leaf samples were collected form newly emerged leaves of *Rphq2*-E2A, *Rphq2*-E5, *Rph22*-E2A and *Rph22*-AE3. DNA was extracted using a CTAB protocol. DNA concentrations were measured on a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific) and DNA quality assessed by agarose gel electrophoresis. Then, DNA was diluted to 20 ng/µl and later used as template for quantitative PCR (qPCR). qPCR was performed by using the rust specific primer pair ITS1rustF10d/ITS1rustR3c[35] and the barley specific primer pair Hv-GAPDH-genomic-F/Hv-GAPDH-genomic-R (Supplementary Table 2) on an ABI PRISM 7000 SDS (Applied Biosystems). The $2^{-\Delta\Delta CT}$ method was used to normalize and calibrate rust gene amplification values relative to the barley endogenous *Hv-GAPDH* control as described previously[36].

Map-based cloning of *Rphq2*

In the original mapping population, recombination between the *Rphq2* flanking markers WBE114 and WBE115 was suppressed due to major haplotype differences between the two parents Vada and SusPtrit[3]. In order to further reduce the number of candidate genes, we searched for a barley genotype with the Vada 'PAA' haplotype at the *Rphq2* locus[3], but with a susceptibility level as high as in L94 at the seedling stage. One accession in the screen by Yeo et al. (2016), UNAe3.1, had the same haplotype as Vada in combination with a *P. hordei* LP almost as short as on L94. Other relevant materials were two doubled haploid lines, SG76 and SG123, from the mapping population 'SusPtrit x Golden Promise'[21]. At the WBE114 to WBE115 interval, Golden Promise has the Vada haplotype PAA[3] but no allele for partial resistance to *P. hordei*[21]. The only QTL for resistance to *P. hordei* in Golden Promise was mapped to chromosome 6H. SG76 and SG123 carry the Golden Promise haplotype at the WBE114 to WBE115 interval, and the SusPtrit susceptibility allele at the 6H locus[21]. These two lines indeed were about as susceptible to *P. hordei* as L94. In 2,041 F_2 seedlings derived from the resistant parent 'L94-*Rphq2'* and susceptible UNAe3.1, SG76, and SG123, we found eight recombinants between WBE114 and WBE115 (Supplementary Table 1), amounting to a genetic distance of 0.2 cM. Additional markers, V.Perox-2, V.HP-1, V.SINA, V.Perox-3, V.Leg-Lec-

D, V.HP-2, V.UF-1 and V.UP were developed based on polymorphisms found within the eight predicted genes in the WBE114 – WBE115 target interval[3]. Marker V.Perox-2, V.HP-1, V.Leg-Lec-D, and V.UP were also named as WBB116, WBB117, WBB118, and WBB119, respectively (Fig. 1b and Supplementary Tables 1 and 2). The eight recombinants were genotyped with these eight markers (Supplementary Table 1). Thirty F3 progeny of each recombinant F2 plant were phenotyped for LP of *P. hordei* isolate 1.2.1, and genotyped for markers WBE114 or WBE115. Progenies for which the seedlings that were homozygous for the Vada allele gave significantly higher RLP values than seedlings homozygous for the allele of the susceptible parent indicated that the resistance gene was located between WBB118 and WBE115 (Fig. 1b and Supplementary Table 1), reducing the number of candidate genes to five.

Cloning and mapping of *Hb-LecRK*

We cloned the *Hv-LecRK* orthologue from the *Rph22*-containing introgression line 182Q20[4] by PCR. Conserved PCR primers, LecRK-Nested PCR-F1, LecRK-Nested PCR-F2 and LecRK-Nested PCR-R were designed based on the *Hv-LecRK* sequences from Vada and SusPtrit[3], as well as Morex, Barke and Bowman[30] (Supplementary Table 2). Through a nested PCR, we amplified the coding sequence and part of the promoter sequence of *Hb-LecRK*. We were able to obtain additional promoter sequence through inverse PCR[37]. One hundred ng of 182Q20 genomic DNA were digested with restriction enzymes AluI, AvaIII, BanI, BseDI, HincII, PstI, PvuI, ScaI, SduI, SpeI and XmiI (Thermo Fisher Scientific), respectively, followed by DNA purification and circularization. Primer pairs 182Q20-LecRK-ATG-F/Hb-LecRK-IPCR1-R1 and Hb-LecRK-IPCR1-F2/Hb-LecRK-IPCR1-R2 (Supplementary Table 2) were used for first round inverse PCR, and 619 bp upstream sequence was obtained. New primer pairs Hb-LecRK-IPCR2-F1/Hb-LecRK-IPCR2-R1 and Hb-LecRK-IPCR2-F2/Hb-LecRK-IPCR2-R2 (Supplementary Table 2) were designed based on the 619 bp sequence. A second round of inverse PCR was performed, which revealed another 2,017 bp of upstream sequence. In the end, a 5,088 bp sequence, including the coding region and 2,764 bp of upstream region was obtained from 182Q20.

To confirm that *Hb-LecRK* is located in the same genetic interval as *Rph22*, we designed an InDel marker, LecRK-indel (Supplementary Table 2), based on the sequences of *Hb-LecRK* and *Hv-LecRK*, which resulted in 234 bp and 195 bp amplicons from 182Q20 and Golden Promise, respectively (Supplementary Fig. 2a). DNA was freshly extracted from the leaves of 173 F_4 lines developed for the original *Rph22* mapping population designated the '182Q20_F4_Popn'[4]. PCRs were performed by using the Thermo Scientific ThermoPrime Taq DNA Polymerase system. PCR products were separated on 1.8% agarose gels in 1xTBE running buffer for 20 min at 240V and visualized under UV light after staining with ethidium bromide. The LecRK-indel genotypes for all 173 lines were identical to those obtained for the markers H35_26334 and H35_45139, which were previously shown to co-segregate with the phenotypic response of *Rph22* to leaf rust infection[4]. The results from a set of non-redundant lines and markers are shown in Supplementary Fig. 2b.

Plasmid construction

A 4,553 bp genomic sequence of *Hv-LecRK* including 2,243 bp of upstream sequence and a 4,295 bp genomic sequence of *Hb-LecRK* including 2,003 bp of upstream sequence were amplified by using primer pairs Hv-LecRK-Full length-F/Hv-LecRK-Full length-R and Hb-LecRK-Full length-F/Hb-LecRK-Full length-R (Supplementary Table 2), and cloned into the entry vector pNOS-ABM (DNA-Cloning-Service, Hamburg, Germany). The constructs were then introduced into the binary vector p6id35STE9 (DNA-Cloning-Service, Hamburg, Germany) using the SfiI cloning sites. These constructs were transformed into *Agrobacterium tumefaciens* AGL1 and used for stable transformation of barley line Golden SusPtrit[21].

The entire open reading frames (ORFs) without stop codons of *Hv-LecRK* and *Hb-LecRK* were amplified from the cDNA of Vada, Golden Promise (GP) and 182Q20 by using primers Vada/GP-LecRK-ATG-F, 182Q20-LecRK-ATG-F, Vada-LecRK_TAG and 182Q20/GP-LecRK_TAG (Supplementary Table 2), and then cloned into the pCR™8/GW/TOPO vector (Thermo Fisher Scientific). Finally, the CDS constructs

were introduced into vectors pGWB5[38] to generate *35S:Vada_Hv-LecRK-GFP*, *35S:GP_Hv-LecRK-GFP*, and *35S:Hb-LecRK-GFP*, and into pGreen0229-HA[39] to generate *35S:Vada_Hv-LecRK-HA*, *35S:GP_Hv-LecRK-HA* and *35S:Hb-LecRK-HA* by using Gateway LR Clonase II (Thermo Fisher Scientific). The point mutation in the pCR™8/GW/TOPO construct of Hb-LecRK-D512A was generated through PCR by using the primer pair Kinase-DFGtoAFG-F and Kinase-DFGtoAFG-R (Supplementary Table 2), and then introduced into pGreen0229-HA to generate *35S:Hb-Lec-D512A-HA*. The constructs in the pGWB5 backbone were transformed into *A. tumefaciens* GV3101. The constructs with pGreen0229-HA backbone were transformed into *A. tumefaciens* GV3101 carrying the pSOUP plasmid.

Barley transformation

Stable barley transformations were produced as described previously[40]. In total, six independent *Hv-LecRK* transgenic T0 plants and ten independent *Hb-LecRK* transgenic T0 plants were generated. Positive plants were confirmed by PCR using primer pair 35S-F2-Catrin/GH-HYG-R5 (Supplementary Table 2).

RNA Isolation, RT-PCR and RT-qPCR

For the expression study of the five candidate genes, *P. hordei* isolate 1.2.1 inoculation was performed as described above, using 10 mg urediniospores on L94, L94-*Rphq2*, Golden Promise and 182Q20 seedlings. Samples were taken at 0, 6, 12, 24, 48, 72, 96, 120, 144 hours after inoculation (hai). Total RNA was extracted using TRIZOL reagent (Invitrogen), and RNA samples were treated with DNase I (Thermo Fisher Scientific) to remove genomic DNA. First strand cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad). For RT-PCR, primer pairs of five candidate genes HP-2-F/HP-2-R, UF-1-F/UF-1-R, UF-2-F/UF-2-R, Hv-LecRK-F/Hv-LecRK-R, UP-F/UP-R and the endogenous control *glyceraldehyde-3-phosphate dehydrogenase* (*Hv-GAPDH*), Hv-GAPDH-F/Hv-GAPDH-R were used (Supplementary Table 2). RT-qPCR was performed by using primer pairs LecRK-QF/LecRK-QR and Hv-

GAPDH-QF/Hv-GAPDH-QR (Supplementary Table 2) on a CFX96 Touch™ Deep Well Real-Time PCR Detection System (BioRad). The $2^{-\Delta\Delta CT}$ method was used to normalize and calibrate transcript values relative to the endogenous *Hv-GAPDH* control.

For the expression analyses on transgenic *Rphq2* and *Rph22* plants, 32 T1 grains of each of the six *Hv-LecRK* primary transgenic (T0) plants (*Rphq2*-E1, *Rphq2*-E2A, *Rphq2*-E3A, *Rphq2*-E4A, *Rphq2*-E5, and *Rphq2*-E6A) and of the six *Hb-LecRK* T0 plants (*Rph22*-E2A, *Rph22*-E3A, *Rph22*-AE1, *Rph22*-AE3, *Rph22*-AE5 and *Rph22*-AE7) were sown in two groups, 16 grains for each group, and six grains of L94, L94-*Rphq2* or Golden Promise, 182Q20 were sown along with each group. Leaf samples were taken from each of the transformants and some of the controls to extract DNA. PCR was applied by using the primer pair Vada-Hv-LecRK-Spec-F/Vada-Hv-LecRK-Spec-R (Supplementary Table 2) for *Hv-LecRK* transformants and primer pair Hb-LecRK-Spec-F/Hb-LecRK-Spec-R (Supplementary Table 2) for *Hb-LecRK* transformants to identify transgene-positive plants. Group 1 seedlings were inoculated with 10 mg *P. hordei* isolate 1.2.1 urediniospores mixed with 100 mg lycopodium powder (Sigma). Group 2 seedlings were inoculated with only 100 mg of lycopodium powder (Sigma) as mock control. For each line, leave samples from transgene positive plants or controls were collected together and then divided into three parts as three biological replicates at 24 hai. Total RNA was extracted using RNeasy Plant Mini Kit (QIAGEN) and treated with RQ1 RNase-Free DNase (Promega). First strand cDNA was synthesized using the SuperScript® First-Strand Synthesis System (Thermo Fisher Scientific). RT-qPCR was performed by using primer pairs Vada-Hv-LecRK-QF/Vada-Hv-LecRK-QR (to amplify Hv-*LecRK*), Hb-LecRK-QF/Hb-LecRK-QR (to amplify *Hb-LecRK*) and Hv-GAPDH-QF/Hv-GAPDH-QR on an ABI PRISM 7000 SDS (Applied Biosystems) using SYBR® GREEN. The $2^{-\Delta\Delta CT}$ method was used to normalize and calibrate transcript values relative to the endogenous *Hv-GAPDH* control. The RT-qPCR results are shown in Supplementary Fig. 12.

Phenotypic characterization of transgenic *Hv-LecRK* and *Hb-LecRK* plants

Sixteen T1 grains of each of the six *Hv-LecRK* T0 plants (*Rphq2*-E1, *Rphq2*-E2A, *Rphq2*-E3A, *Rphq2*-E4A, *Rphq2*-E5, and *Rphq2*-E6A) and of the six *Hb-LecRK* T0 plants (*Rph22*-E2A, *Rph22*-E3A, *Rph22*-AE1, *Rph22*-AE3, *Rph22*-AE5 and *Rph22*-AE7) were sown in two rows on both sides of a 55x28 cm tray. Three to six grains of regenerated non-transformed Golden SusPtrit, L94, L94-*Rphq2*, Golden Promise or 182Q20 were sown along with the transformants as references. Then, seedlings were kept in a growth room at 20ºC, 8 hours dark, 16 hours light. *P. hordei* isolate 1.2.1, *P. hordei-bulbosi* Iran and *P. triticina* isolate B9414 inoculations were carried out as described above. After inoculation, seedlings were moved into a growth room at 16 hours light and 15ºC, 8 hours dark and 10ºC. Transgene positive seedlings were identified by PCR as described above. Disease evaluation was performed as described above.

Twelve T1 grains of each of the six *Hv-LecRK* T0 plants and the six *Hb-lecRK* T0 plants were sown in 12 cm diameter pots, three plants per pot. Six grains of regenerated non-transformed Golden SusPtrit, L94, L94-*Rphq2*, Golden Promise, or 182Q20 were sown along with the transformants as references. Then plants were kept in a growth room at 20ºC, 8 hours dark, 16 hours light. Transgene positive seedlings were identified by PCR as described above. Adult inoculation was applied as described above. Pictures were taken 10 days after inoculation.

<u>*Agrobacterium tumefaciens*-mediated transient expression in *Nicotiana benthamiana* leaves</u>

A. tumefaciens strains carrying binary vectors described above and suppressor of post-transcriptional gene silencing - p19 were grown overnight at 28ºC in Lysogeny broth with appropriate antibiotics. *A. tumefaciens* cells were collected by centrifugation at 4,000 rpm for 10 min, and then re-suspended in MMA induction buffer (10 mM MES, 10 mM $MgCl_2$, 100 µM acetosyringone, pH 6.5). After adjusting the OD600 to 0.8, the cultures were incubated at 28ºC for 3 hours. *A. tumefaciens* suspensions carrying binary vectors were mixed with *A. tumefaciens* p19-silencing-suppressor strain at a ratio of 1: 1, and then syringe infiltrated into five-week-old *N. benthamiana* leaves.

462

463 DAB and Trypan blue staining

464 *N. benthamiana* leaves were harvested three days after *A. tumefaciens* infiltration. For DAB staining, *N.*
465 *benthamiana* leaves were immersed into DAB solution (1 mg/ml DAB-HCL, 10 mM Na_2HPO_4, 0.05% v/v
466 Tween 20) and vacuumed until leaves were infiltrated by solution. After five hours shaking at 100 rpm,
467 leaves were washed with dH_2O to remove the staining solution on the surface and destained by incubation
468 in a bleaching solution (ethanol: acetic acid: glycerol = 3: 1: 1) at 95^{o}C until no green color was left. For
469 Trypan blue staining, *N. benthamiana* leaves were boiled for 5 min in a 1:2 mixture of stock solution (10
470 ml lactic acid, 10 ml glycerol, 10 g phenol, and 0.02 g Trypan blue, dissolved in 10 ml dH_2O) and 96%
471 ethanol for staining, then leaves were destained in 2.5 g/ml chloral hydrate until good color contrasting was
472 observed.

473

474 Fluorescence imaging

475 *N. benthamiana* leaves were cut into small pieces (approx. 0.5 cm) that were mounted on a slide containing
476 water or 1 M $NaNO_3$ with the abaxial site facing the coverslip.

477 Fluorescence images were acquired using an upright Zeiss LSM 710 confocal laser scanning microscope
478 (Carl Zeiss) equipped with a LD LCI Plan-Apochromat 40×/1.20 W Korr water-immersion objective. GFP
479 fluorescence was excited with a 488 nm laser, detected with 509-550 nm wave length and the emission was
480 recorded at 530 nm. For mCherry, fluorescence was excited with a 561 nm laser, detected at 608 to 648 nm
481 and the emission was recorded at 628 nm. Images were processed using Zeiss ZEN 2.3 software and
482 CorelDRAW 2018.

483

484 Phylogenetic analyses

Rphq2 alleles from different barley lines were amplified from genomic DNA using primers LecRK-Nested PCR-F1, LecRK-Nested PCR-F2 and LecRK-Nested PCR-R. Sequences were aligned with Clustal X (version 2.1)[41] using default parameters. A neighbor-joining tree was constructed using the phylip package (version 3.695) with 100 bootstrap replicates[42].

Statistical methods

Statistical tests (t-tests and chi-square tests) were performed in excel. P-values and number of samples are indicated in figure and table legends.

Data availability statement

All data is available in the main text or the supplementary materials. GenBank accession numbers: MK512576 - MK512578, MK530949 -MK530950, MK569504, MN128516 – MN128521.

References

31. Parlevliet, J.E. Evaluation of the concept of horizontal resistance in the barley/Puccinia hordei host-pathogen relationship. *Phytopathology* **66**, 494-497 (1976).
32. Martinez, F., Niks, R.E., Moral, A., Urbano, J.M. & Rubiales, D. Search for partial resistance to leaf rust in a collection of ancient Spanish wheats. *Hereditas* **135**, 193-197 (2001).
33. Niks, R.E., Parlevliet, J.E., Lindhout, P. & Bai, Y. *Breeding crops with resistance to diseases and pests*, (Wageningen Academic Publishers, Wageningen, 2011).
34. Parlevliet, J.E. Partial resistance of barley to leafrust, *Puccinia hordei*. I. Effect of cultivar and development stage on latent period. *Euphytica* **24**, 21-27 (1975).

35. Barnes, C.W. & Szabo, L.J. Detection and identification of four common rust pathogens of cereals and grasses using real-time polymerase chain reaction. *Phytopathology* **97**, 717-727 (2007).

36. Zheng, Z. *et al.* Loss of function in *Mlo* orthologs reduces susceptibility of pepper and tomato to powdery mildew disease caused by *Leveillula taurica*. *PLoS One* **8**, e70723 (2013).

37. Ochman, H., Gerber, A.S. & Hartl, D.L. Genetic applications of an inverse polymerase chain reaction. *Genetics* **120**, 621-623 (1988).

38. Nakagawa, T. *et al.* Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J. Biosci. Bioeng.* **104**, 34-41 (2007).

39. Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S. & Mullineaux, P.M. pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Mol. Biol.* **42**, 819-832 (2000).

40. Hensel, G., Kastner, C., Oleszczuk, S., Riechen, J. & Kumlehn, J. *Agrobacterium*-mediated gene transfer to cereal crop plants: current protocols for barley, wheat, Triticale, and maize. *Int. J. Plant Genomics* **2009**, 835608 (2009).

41. Larkin, M.A. *et al.* Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947-2948 (2007).

42. Felsenstein, J. PHYLIP - Phylogeny Inference Package (Version 3.2). *Cladistics* **5**, 164-166 (1989).