Expression of the wheat disease resistance gene Lr34 in transgenic barley leads to accumulation of abscisic acid at the leaf tip

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Abstract: Durable disease resistance genes such as the wheat gene Lr34 are valuable sources of resistance for agricultural breeding programs. Lr34 encodes an ATP-binding cassette transporter protein involved in the transport of the phytohormone abscisic acid. Lr34 from wheat is functionally transferable to barley, maize, rice and sorghum. A pleiotropic effect of Lr34 induces the development of a senescence-like phenotype, referred to as leaf tip necrosis. We used Lr34-expressing wheat and transgenic barley plants to elucidate the role of abscisic acid in the development of leaf tip necrosis. Leaf tips in Lr34-expressing wheat and barley showed an accumulation of abscisic acid. No increase of Lr34 expression was detected in the leaf tip. Instead, the development of ectopic, Lr34-induced leaf tip necrosis after removing the leaf tip suggests an increased flux of abscisic acid towards the tip, where it accumulates and mediates the development of leaf tip necrosis. This redistribution of abscisic acid was also observed in adult transgenic barley plants with a high Lr34 expression level growing in the field and coincided with leaf tip necrosis as well as complete field resistance against Puccinia hordei and Blumeria graminis f. sp. hordei. In a barley transgenic line with a lower Lr34 expression level, a quantitative resistance against Puccinia hordei was still observed, but without a significant redistribution of abscisic acid or apparent leaf tip necrosis. Thus, our results imply that fine-tuning the Lr34 expression level is essential to balance disease resistance versus leaf tip necrosis to deploy transgenic Lr34 in breeding programs.

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Expression of the wheat disease resistance gene *Lr34* in transgenic barley leads to accumulation of abscisic acid at the leaf tip

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1. Introduction

Crops are constantly challenged by pathogens. The use of resistance genes in crop breeding is the most sustainable approach to reduce yield losses caused by pathogens. However, many disease resistance genes lack durability and are quickly overcome by new virulent pathogen strains. Few known genes confer durable disease resistance and represent a valuable source for breeding. *Lr34* encodes an ATP-binding cassette transporter protein involved in the transport of the phytohormone abscisic acid. *Lr34* from wheat is functionally transferable to barley, maize, rice and sorghum. A pleiotropic effect of *Lr34* induces the development of a senescence-like phenotype, referred to as leaf tip necrosis. We used *Lr34*-expressing wheat and transgenic barley plants to elucidate the role of abscisic acid in the development of leaf tip necrosis. Leaf tips in *Lr34*-expressing wheat and barley showed an accumulation of abscisic acid. No increase of *Lr34* expression was detected in the leaf tip. Instead, the development of ectopic, *Lr34*-induced leaf tip necrosis after removing the leaf tip suggests an increased flux of abscisic acid towards the tip, where it accumulates and mediates the development of leaf tip necrosis. This redistribution of abscisic acid was also observed in adult transgenic barley plants with a high *Lr34* expression level growing in the field and coincided with leaf tip necrosis as well as complete field resistance against *Puccinia hordei* and *Blumeria graminis* f. sp. *hordei*. In a barley transgenic line with a lower *Lr34* expression level, a quantitative resistance against *Puccinia hordei* was still observed, but without a significant redistribution of abscisic acid or apparent leaf tip necrosis. Thus, our results imply that fine-tuning the *Lr34* expression level is essential to balance disease resistance versus leaf tip necrosis to deploy transgenic *Lr34* in breeding programs.

**Keywords:**
Abscisic acid (ABA)  
Barley  
Durable disease resistance  
Fungal pathogens  
Leaf tip necrosis  
*Lr34*  
Senescence

**ABSTRACT**

Durable disease resistance genes such as the wheat gene *Lr34* are valuable sources of resistance for agricultural breeding programs. *Lr34* encodes an ATP-binding cassette transporter protein involved in the transport of the phytohormone abscisic acid. *Lr34* from wheat is functionally transferable to barley, maize, rice and sorghum. A pleiotropic effect of *Lr34* induces the development of a senescence-like phenotype, referred to as leaf tip necrosis. We used *Lr34*-expressing wheat and transgenic barley plants to elucidate the role of abscisic acid in the development of leaf tip necrosis. Leaf tips in *Lr34*-expressing wheat and barley showed an accumulation of abscisic acid. No increase of *Lr34* expression was detected in the leaf tip. Instead, the development of ectopic, *Lr34*-induced leaf tip necrosis after removing the leaf tip suggests an increased flux of abscisic acid towards the tip, where it accumulates and mediates the development of leaf tip necrosis. This redistribution of abscisic acid was also observed in adult transgenic barley plants with a high *Lr34* expression level growing in the field and coincided with leaf tip necrosis as well as complete field resistance against *Puccinia hordei* and *Blumeria graminis* f. sp. *hordei*. In a barley transgenic line with a lower *Lr34* expression level, a quantitative resistance against *Puccinia hordei* was still observed, but without a significant redistribution of abscisic acid or apparent leaf tip necrosis. Thus, our results imply that fine-tuning the *Lr34* expression level is essential to balance disease resistance versus leaf tip necrosis to deploy transgenic *Lr34* in breeding programs.

**ARTICLE INFO**

**ABBREVIATIONS:** ABA, Abscisic acid; AUDPC, Area under the disease progress curve; *Bgh*, *Blumeria graminis* f. sp. *hordei*; LTN, Leaf tip necrosis.

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The gene \textit{Lr34} is associated with the development of leaf tip necrosis (LTN) in adult wheat plants (Singh, 1992). LTN also occurs in the absence of a pathogen and varies depending on the environment (Dyck, 1991). In the wheat flag leaves, a gradient of leaf rust disease symptoms could be observed that coincides with the development of LTN: there are fewer and smaller pustules towards the LTN-exhibiting tip of the leaf and the pustules are more abundant and larger towards the base of the leaf, respectively (Hubbert et al., 2007; Kolmer, 1996). Krattinger et al. (2009) showed that \textit{Lr34}-expression results in induction of senescence-associated genes and degradation of chlorophyll in wheat. In transgenic barley, three senescence-associated genes were induced in \textit{Lr34}-res-transpiring plants (Risk et al., 2013). Based on these observations, the development of LTN was described as a senescence-like process (Krattinger et al., 2009). In contrast to wheat, the expression of \textit{Lr34} in barley and rice led to an early LTN development already at seedling stage, starting from the oldest leaf and progressing towards the younger leaves. Furthermore, higher expression levels of \textit{Lr34} resulted in more pronounced LTN and reduction of plant vigor and yield parameters (Krattinger et al., 2016; Risk et al., 2013). The negative impact of LTN in barley was reduced by expressing \textit{Lr34} under the pathogen inducible promoter \textit{Hv-Ger4c}, which did not compromise the resistance phenotype in greenhouse and near-field conditions (Bonì et al., 2018; Himmelbach et al., 2010). In maize, \textit{Lr34} expression led to LTN development in adult plants and no effect on plant vigor was observed in controlled conditions (Sucher et al., 2017). Interestingly, \textit{Lr34}-expressing sorghum did not show the typical LTN phenotype, but a reduction in panicle weight (Schnippenkoetter et al., 2017).

\textit{Lr34} encodes an ATP-binding cassette transporter (Krattinger et al., 2009) that is localized in the plasma membrane (Deppe et al., 2018). The \textit{Lr34} protein encoded by the resistance conferring allele, \textit{Lr34res}, differs by two amino acids from the susceptible variant \textit{Lr34sus}, although only one of the amino acid changes is sufficient to confer resistance (Chauhan et al., 2015). Krattinger et al. (2019) showed that \textit{Lr34res} mediates transport of the phytohormone abscisic acid (ABA). ABA is involved in diverse processes such as regulation of plant development and response to biotic and abiotic stresses (Cutler et al., 2010; Dejonghe et al., 2018). The \textit{Lr34res}-expressing rice seedlings showed LTN, reduced stomatal conductance and hypersensitivity towards ABA (Krattinger et al., 2019). This pleiotropic phenotype fits well with increased ABA signaling. Krattinger et al. (2019) hypothesized that altered ABA fluxes might change the leaf physiology of \textit{Lr34res}-expressing rice plants, which results in the induction of a constitutive defense response and creates a more hostile environment for the pathogens. Surprisingly, no elevated concentration of ABA was observed in \textit{Lr34res}-expressing rice leaves compared to the sister line (Krattinger et al., 2019). Thus, it remains unclear how \textit{Lr34res} expression leads to the pleiotropic phenotypes including disease resistance.

Here, we studied the distribution of ABA in \textit{Lr34res}-expressing wheat and barley plants and found an accumulation of ABA at the leaf tip. This redistribution of ABA was also observed in adult barley plants growing in the field and co-occurred with leaf tip necrosis. Removal of the upper half of the leaves led to ectopic accumulation of ABA and development of LTN at the cut site suggesting that \textit{Lr34res} mediates an increased flux of an LTN-inducing compound that is likely to be ABA.

2. Materials and methods

2.1. Plant material and growth conditions

For the measurement of apoplastic ABA, the wheat near isogenic lines Thatcher \textit{Lr34} containing the endogenous resistant allele \textit{Lr34res} and Thatcher containing the endogenous susceptible allele \textit{Lr34sus} were used (Dyck, 1977). Wheat plants were grown in the greenhouse (20\degree C, 16 h photoperiod, 60\% humidity). The \textit{Lr34res}-expressing barley lines BG 9 described in Risk et al. (2013) and GLP 8 described in Bonì et al. (2018) with their corresponding sister lines BG 9 sib and GLP 8 sib have been used in this study. \textit{Lr34res} expression in BG 9 is controlled by the native wheat promoter, while expression in GLP 8 is driven by the pathogen inducible promoter \textit{Hv-Ger4c}. For ABA measurement and leaf cutting experiments the barley plants were grown in pots with Einheitserde (Einheitserde Profi Substrat, Einheitserde Werkverband e.V., Germany) in growth chambers (Fitoclima 2.500, Aralab, Portugal; 17/13 \degree C, 16 h photoperiod, 65\% humidity).

2.2. Field experiment and disease scoring

The \textit{Lr34res}-expressing barley lines BG 9 and GLP 8 were grown together with their sister lines at the “Protected Site” of the Swiss center for agricultural research Agroscope in Zürich during the field season 2020. The “Protected Site” is designated to field trials with genetically modified organisms (Romeis et al., 2013). For each of the three different treatments (fungicide treatment, powdery mildew infection and rust infection) four microplots (1.32 × 1 m) of each plant genotype were grown in a randomized block design. For the powdery mildew infection, infections rows with the susceptible cultivar Golden Promise were sown. Plants that were artificially inoculated with the \textit{Blumeria graminis f. sp. hordei} (Bgh) isolate 5 (Bonì et al., 2019) were transferred into the infection rows 26 days after sowing. The scoring for powdery mildew started 60 days after sowing at the onset of the disease and was performed as previously described (Brummer et al., 2011). For the barley rust infection trial, the infection rows consisting of Golden Promise were sown on both sides of the microplots. barley plants that were artificially inoculated with the \textit{P. hordei} pathotype 1.2.1 (Risk et al., 2013) were placed in the infection rows 60 days after sowing. Additionally, we sprayed spores mixed with Fluorinert™ FC-43 (3M, Belgium) on the 84-day-old plants. Starting from 12 days after spraying, the percentage of leaf area covered with rust pustules of the flag leaf was scored every two to three days until the plants started to mature. Fungicide control treatments were planted with Amistar Xtra (Syngenta, Switzerland) 37 days after sowing and with Prosper (Bayer, Leverkusen, Germany) 104 days after sowing (after sampling). A 3 m wide border consisting of a mixture of the three barley cultivars KWS Atirka, Sydney and Explorer (Delley Samen und Pflanzen AG, Delley-Portalban, Switzerland) was sowed surrounding the transgenic barley field. The 1,000-grain weight was measured of six plots per line, using plots from different treatments.

2.3. Measurement of whole leaf ABA concentration

For the measurement of ABA distribution in barley plants grown in growth chambers, the youngest fully expanded leaf of one-month-old plants was cut into six different leaf parts as shown in Fig. 1b. To reach 100 ± 20 mg per sample, 3–6 leaves were pooled as one of the five biological replicates. To analyze the distribution of ABA in barley leaves in the field, samples were taken 75 days after sowing from fungicide-treated plots. The second youngest fully expanded leaf was sampled, cut in three equally long pieces (base, middle and tip) and the middle part was discarded. Then, the midrib and blade were separated and collected independently. To get sufficient and similar amounts of material, eight leaves were pooled for the tip midrib, six for the tip blade, four for the base midrib and blade as one of the five biological replicates. Only one half of the leaf was used for all the blade samples (see Fig. 1b). For the comparison of the ABA concentrations in \textit{Bgh} infected and fungicide-treated plants, the second youngest fully expanded leaves of 84-day-old plants were sampled. Three leaves were divided in three equally long segments and the distal halves of the middle segments were pooled together as one of the five biological replicates. To measure the ABA concentration in the LTN experiment, 2 cm-long segments were cut 1 cm below the cut site. For the non-cut leaves, the segments were sampled 1 cm below the middle of the leaf. The seven biological replicates each represent a pool of two segments. All the segments were transferred to weighted 2 ml tubes with glass beads and frozen in liquid nitrogen or dry ice for the sampling in the field. Subsequently, the...
samples were weighed and ground with the Geno/Grinder® (SPEX SamplePrep, Metuchen, NJ, USA).

The extraction and measurement of the ABA concentration in barley plants was conducted according to Glauer et al. (2014). In brief, 990 μl of the solvent (ethylacetate:formic acid, 99.5:0.5) and 10 μl of an internal standard solution containing $[^{13}H_2](+)$-cis,trans-ABA (OL ChemIm, Olomouc, Czech Republic) at a concentration of 100 ng/ml in water were added to the ground plant material and the glass beads. Samples were vortexed thoroughly and then mixed with the Geno/Grinder® for 3 min at 1,500 rpm. Subsequently, the samples were centrifuged at 14,000 g for 3 min and the supernatant was transferred to a new 2 ml tube. Another 500 μl of solvent was added to the pellet. The mixing and centrifugation steps were repeated, and the supernatant was added to the first one. Then, the solvent was evaporated using a Speed Vac Plus SC110A (SAVANT Instruments, India) to dryness. The pellet was resuspended in 100 μl of 70% methanol, vortexed thoroughly, sonicated for 1 min and vortexed again. Subsequently, the sample was transferred to a 0.2 ml tube and centrifuged for 2 min at 14,000 rpm. Finally, the supernatant was transferred to a conical glass insert in an appropriate vial for analysis by ultra-high performance liquid chromatography-tandem mass spectrometry. Five μl of extract was injected in an Ultimate 3000 RSLC (Dionex-ThermoFisher Scientific, Waltham, MA, USA) coupled to a 4000 QTRAP (AB Sciex, Foster City, CA, USA) equipped with a Turbo V ion source. The chromatographic separation was performed on a 50 x 2.1 mm Acquity UPLC BEH C18 column (Waters, Milford, MA, USA) and ABA and ABA-d$_6$ were monitored using the multiple reaction monitoring transitions m/z 263/153 and 269/159, respectively. Linear calibration curves weighted by 1/x and built from calibration points at 0.1, 0.5, 2, 20, and 100 ng/ml, all containing ABA-d$_6$ at 10 ng/ml, were applied to quantify ABA. For the ABA distribution in field grown plants, plant-wise means of blade and vein samples were calculated.

2.4. Measurement of apoplastic ABA in wheat plants

Apoplastic fluid was extracted using the infiltration-centrifugation technique (Nouchi et al., 2012). Briefly, the wheat flag leaves of ~9-week-old plants around anthesis were harvested. Thatcher Lr34 leaves, which did not show any LTN at the time of sampling, were sampled and a 5 cm leaf segment from the tip was used for apoplastic fluid extraction. Flag leaves of three plants were pooled for one of the 7–8 biological replicates. Infiltrations were done in 30 ml syringes filled with 100 mM KCl. Centrifugations were done at 1,000 g for 10 min and the volume of the collected apoplastic fluid was determined. Of this liquid, ABA concentrations were measured using the Phytodetek® ABA Test kit (Agdia-Biofords, Grigny, France) according to the manufacturer’s instruction.

2.5. Transgene expression analysis of field grown barley plants

For the analysis of the Lr34res transcript distribution, the second youngest fully expanded leaf in the fungicide-treated plots was sampled and cut in the six different parts as done for the analysis of the ABA distribution in the field. Each of the five biological replicates consisted of a pool of six leaves for the tip midrib, four for the tip blade, three for the base midrib and blade. Only one half of the leaf was used for all the blade samples (see Fig. 1b). To compare the expression between powdery mildew infected and fungicide-treated plots, the sampling was performed the same way as for the measurement of the ABA concentration. The second youngest fully expanded leaves of 84-day-old plants were sampled and the distal halves of the middle third of three leaves were pooled together as one of the five biological replicates. All the samples were transferred to weighed 2 ml tubes with glass beads and frozen in dry ice. Subsequently, the samples were weighed and ground with the Geno/Grinder®. The mRNA was extracted using the Dynabeads® mRNA
DIRECT™ Kit 61011 and 61012 (Thermo Fisher scientific). In brief, Lysis Binding/Buffer (125 µl/10 mg plant tissue) was added to the ground and frozen leaf tissue. The samples were vortexed and spun down in a tabletop centrifuge at 4 °C and full speed for 4 min. Subsequently, 290 µl supernatant were mixed with 25 µl of the washed beads in RNase free tubes and incubated for 5 min. The beads were collected with a magnet and the supernatant was discarded. Then, the beads were washed twice with Wash A (300 µl) and twice with Wash B (300 and 200 µl). For the elution, the beads were mixed with 30 µl cold 10 mM Tris and transferred to fresh tubes. For the reverse transcription, 3 µl RNA were reverse transcribed in a total volume of 10 µl with the iScript™ Advanced cDNA Synthesis Kit (Bio-Rad). RT-qPCR was performed on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) with the Kapa SYBR® FAST qPCR Master Mix (Kapa Biosystems). In a reaction volume of 6 µl, 2.4 µl of 1:20 diluted cDNA template and 250 nM primers were used. Primer sequences and efficiencies for expression analysis are listed in Suppl. Table 1.

2.6. Statistical analysis

Data were presented as single data points and boxplots showing the median, lower and upper quantile. The statistical analysis was performed in RStudio version 1.2.5001 using Tukey’s honest significance test and Nemenyi’s test for multiple comparison of normally or not normally distributed samples, respectively. For pair-wise comparison Student’s t-test and Wilcoxon test were used for normally or not normally distributed data, respectively. A significance level of 5% was used for hypothesis testing. Dixon’s Q test (P < 0.01) was used to determine outliers, which were excluded from analysis.

3. Results

3.1. Accumulation of abscisic acid at the leaf tip in Lr34res-expressing wheat and barley plants

Since the whole-leaf concentrations of ABA did not differ between Lr34res-expressing rice plants and wild-type plants (Krattinger et al., 2019), we hypothesized that Lr34res alters the ABA distribution within the leaf. Therefore, apoplastic fluid was extracted of leaf tips and bases using the infiltration-centrifugation technique (Nouchi et al., 2012) and ABA concentrations were measured. We found increased ABA concentrations in the apoplastic fluid extracted from leaf tip tips of the wheat line Thatcher Lr34 (expressing Lr34res) compared to Thatcher (expressing Lr34sus). ABA concentrations in leaf bases of Thatcher and Thatcher Lr34 showed no differences in ABA concentrations (Fig. 1a).

The ABA concentrations were also measured in the transgenic barley line BG 9 and the corresponding sister line BG 9 sib grown in climate chambers. Line BG 9 has a high Lr34res expression level under control of the native wheat promoter (Chauhan et al., 2015; Risk et al., 2013). The complete tissue of different leaf parts was assessed. This allowed for a higher sample throughput and comparison of smaller leaf parts. To capture the stage of LTN initiation, the youngest fully expanded leaves that did not show leaf tip necrosis at the time of sampling were collected for the ABA measurements. They were cut into three parts along the proximal-distal axis and between blade and midrib of the leaf (Fig. 2b). As described in a previous study (Boni et al., 2018), the expression of Lr34res under the native wheat promoter was stronger than under the pathogen-inducible promoter Hv-Ger4c. This indicates that low expression levels are not sufficient to induce a detectable accumulation of ABA at the leaf tip. Furthermore, these results suggest that redistribution of ABA is not induced by an accumulation of Lr34res transcripts at the leaf tip. In previous studies, an increased expression of pathogenesis-related (PR) genes was observed in white leaves of Lr34res-expressing barley plants (Chauhan et al., 2015; Risk et al., 2013). The expression of five of these PR genes (PR1, 3, 5, 9 and 10) was analyzed in the same samples as for the Lr34res expression analysis to check for differential expression in different parts of the leaf. A correlation of ABA accumulation and PR gene expression at the leaf tip was observed for PR1, 3 and 10 in line BG 9 (Suppl. Fig. A.1). A similar trend was observed for PR3 and PR9. Evenly distributed PR gene expression was detected in line GLP 8, a line where ABA redistribution was not detectable with our experimental setup. This result indicates that ABA accumulation at the leaf tip is involved in Lr34res-mediated induction of PR genes, which might contribute to Lr34res-mediated disease resistance.

To test if the Lr34res-expressing barley plants behave similarly in the field as in near-field conditions (Boni et al., 2018), the disease resistance and possible pleiotropic effects of the transgenic barley plants were evaluated. We scored the field grown lines BG 9, GLP 8, their corresponding sister lines and the untransformed genotype Golden Promise for disease resistance against Bgh and P. hordei. Rust disease symptoms were scored every two to three days by estimating the percent of leaf area covered by pustules and from this data the area under the disease progress curve (AUDPC) was calculated. BG 9 was completely resistant to P. hordei in the field as no pustules were observed on the BG 9 plants (Fig. 2c). Line GLP 8 was partially resistant against P. hordei (Fig. 2c). Powdery mildew scoring was performed eight times starting from the onset of disease and the AUDPC was calculated. Line BG 9 showed complete Bgh resistance, whereas line GLP 8 with lower Lr34res expression was not significantly more resistant than the corresponding sister line (Fig. 2d). No difference in ABA concentration and Lr34res expression level was observed between Bgh infected and fungicide-treated plots (Suppl. Fig. A.2).

The strong powdery mildew and rust resistance of BG 9 was accompanied by strong LTN. In contrast, the GLP 8 plants did not show apparent LTN. In addition to strong LTN, the BG 9 plants showed earlier maturity. The plants dried faster and lodged after 111 days, which was two weeks before harvesting. The 1,000-grain weight of BG 9 but not the 1,000-grain weight of GLP 8 was significantly lower compared to the respective sister lines (Fig. 2e). Overall, the results of the field experiment indicate a positive correlation between the Lr34res expression characterized in the greenhouse and under near-field conditions (Boni et al., 2018; Chauhan et al., 2015; Risk et al., 2013). Since Lr34res is an adult plant resistance gene in wheat, we aimed to verify the accumulation of ABA at the leaf tip in adult plants in the field. Furthermore, we wanted to elucidate differential expression of Lr34res as a possible cause of the ABA redistribution. Hence, we compared the ABA concentration and Lr34res transcript levels at the same time point in field grown barley. Similar to the ABA distribution experiment performed in controlled conditions (Fig. 1b and c), leaves which did not yet show LTN were sampled to capture the initiation of premature senescence. In addition to the high expressing line BG 9, line GLP 8 and its sister line GLP 8 sib (Boni et al., 2018) were included in this experiment. Line GLP 8 expresses Lr34res under the pathogen-inducible promoter Hv-Ger4c (Himmelbach et al., 2010). Boni et al. (2018) showed that Lr34res-expression in GLP 8 is induced upon Bgh infection under greenhouse conditions, resulting in an overall lower Lr34res expression level than BG 9. As shown in Fig. 1, a redistribution of ABA to the leaf tip was detected in field grown BG 9, but this was not observed in field grown GLP 8 (Fig. 2a). In both lines, no difference in Lr34res transcript level was found along the proximal-distal axis and between blade and midrib of the leaf (Fig. 2b). As described in a previous study (Boni et al., 2018), the expression of Lr34res under the native wheat promoter was stronger than under the pathogen-inducible promoter Hv-Ger4c. This indicates that low expression levels are not sufficient to induce a detectable accumulation of ABA at the leaf tip. Furthermore, these results suggest that redistribution of ABA is not induced by an accumulation of Lr34res transcripts at the leaf tip. In previous studies, an increased expression of pathogenesis-related (PR) genes was observed in white leaves of Lr34res-expressing barley plants (Chauhan et al., 2015; Risk et al., 2013). The expression of five of these PR genes (PR1, 3, 5, 9 and 10) was analyzed in the same samples as for the Lr34res expression analysis to check for differential expression in different parts of the leaf. A correlation of ABA accumulation and PR gene expression at the leaf tip was observed for PR1, 3 and 10 in line BG 9 (Suppl. Fig. A.1). A similar trend was observed for PR3 and PR9. Evenly distributed PR gene expression was detected in line GLP 8, a line where ABA redistribution was not detectable with our experimental setup. This result indicates that ABA accumulation at the leaf tip is involved in Lr34res-mediated induction of PR genes, which might contribute to Lr34res-mediated disease resistance.

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level, the ABA accumulation at the leaf tip, the development of LTN and the disease resistance against Bgh and P. hordei. However, partial disease resistance against P. hordei was also observed in GLP 8 with a low Lr34res-expression level but without apparent LTN nor significant 1,000-grain weight reduction.

3.3. Development of leaf tip necrosis after leaf tip removal coincides with Lr34res-mediated ABA accumulation

To further investigate ABA accumulation at the leaf tip and LTN development, the distal half of Lr34res-expressing transgenic barley leaves was removed by cutting. For these experiments the youngest leaves were chosen, which were almost fully expanded and did not yet show LTN. After removal of the distal part of the leaves, Lr34res-expressing barley plants developed LTN at the cut site unlike the non-transgenic sister line (Fig. 3a). This suggests that the development of LTN is induced by a transportable signal that accumulates at the distal end of the leaf. In order to investigate whether ABA accumulates at the cut site, we measured the ABA concentration four days after cutting the leaves when the first signs of LTN became visible. Samples were taken from healthy tissue 1 cm below the cut site or the middle of the leaf in case of the non-cut mock samples. The cutting of the leaf resulted in an

![Fig. 2. Field experiment with the Lr34res-expressing barley lines BG 9, GLP 8, the corresponding sister lines (BG 9 sib and GLP 8 sib) and the background cultivar Golden Promise (GP). (a) ABA concentrations at the leaf base and tip of the second youngest fully expanded leaf of 75-day-old plants. * indicates P < 0.05, Student’s t-test. (b) Lr34res expression relative to the housekeeping gene ADP ribosylation factor in different parts of the second youngest fully expanded leaf of 75-day-old plants. Differing letters above the boxplots indicate P < 0.05 calculated by Tukey’s honest significance test. (c) Area under disease progress curve (AUDPC) of the P. hordei infection score. * indicates P < 0.05 between Lr34res-expressing line and their sister lines calculated with the Wilcoxon test. (d) AUDPC of the Blumeria graminis f. sp. hordei (Bgh) infection score. * indicates P < 0.05 between Lr34res-expressing line and their sister lines calculated with the Wilcoxon test. (e) 1,000-grain weight of six plots per line. Differing letters indicate P < 0.05 calculated by Tukey’s honest significance test. The boxplots representing the leaf tip samples are shaded in darker green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

![Fig. 3. Removal of the distal half of the youngest leaf which was almost fully expanded and did not yet show LTN. One-month-old plants of the Lr34res-expressing barley plants developed LTN at the cut site unlike the non-transgenic sister line (Fig. 3a). This suggests that the development of LTN is induced by a transportable signal that accumulates at the distal end of the leaf. In order to investigate whether ABA accumulates at the cut site, we measured the ABA concentration four days after cutting the leaves when the first signs of LTN became visible. Samples were taken from healthy tissue 1 cm below the cut site or the middle of the leaf in case of the non-cut mock samples. The cutting of the leaf resulted in an...
increase of ABA below the cut site in BG 9 but not in the corresponding sister line (Fig. 3b). This result indicates that Lr34res increases the flux of ABA directly or an ABA-inducing compound towards the tip where it accumulates and induces the development of LTN.

4. Discussion

Lr34res has been extensively used in wheat breeding for more than hundred years because of its durable and broad-spectrum resistance against multiple fungal diseases (Kolmer et al., 2008). However, both the disease resistance mechanism as well as the basis of the pleiotropic effect LTN are still largely unknown. Krattinger et al. (2019) showed that Lr34res encodes an ABC transporter that mediates the import of the phytohormone ABA, but no change of total ABA content was measured in leaves of Lr34res-expressing rice plants. Hence, it was hypothesized that Lr34res-expression affects the ABA distribution. Here, we showed that ABA accumulated at the leaf tip in wheat as well as transgenic barley plants expressing Lr34res. The development of LTN after removal of the distal half of the leaf suggests that Lr34res mediated the transport of an LTN-inducing signal towards the leaf tip. In developmental senescence, increasing ABA levels induce the transcription factor NAC-LIKE ACTIVATED BY AP3/PI (NAP) that leads to nutrient remobilization and chlorophyll degradation (Liang et al., 2014; Schippers, 2015). In addition, it was shown that ABA induces the receptor protein kinase RPK1, which acts as a positive regulator of age-dependent senescence (Lee et al., 2011). These studies on the role of ABA in senescence together with our experiments suggest ABA as the LTN-inducing signal in Lr34res-expressing plants.

ABA is transported from root to shoot and shoot to root in the xylem and phloem, respectively (Hartung et al., 2002; Kuromori et al., 2018). In Arabidopsis, the ABA transporters AtABCG25, AtDTX50 and AtNPF4.6 are expressed in the vascular tissue and involved in loading and unloading of the xylem and phloem (Kanno et al., 2012; Kuromori et al., 2016; Zhang et al., 2014). In monocots, it is yet unclear how many ABA transporters are involved in the distribution of ABA. So far the two ABA transporters, OsPM1 from rice (Yao et al., 2018) and BdABCG25 from Brachypodium distachyon (Kuromori et al., 2020), have been identified and suggest a similar regulation of ABA long-distance transport in monocots as in Arabidopsis. However, none of the described ABA transporters is orthologous to Lr34res (Krattinger et al., 2011). The ABA importer OsPM1 is expressed in vascular tissue, guard cells and mature embryos and is involved in the regulation of stomatal closure as well as drought tolerance (Yao et al., 2018). BdABCG25 is a homologue of AtABCG25 and overexpression of BdABCG25 in Arabidopsis resulted in reduced ABA sensitivity of seedlings and reduced stomatal conductance similarly as AtABCG25 (Kuromori et al., 2020). Based on these studies and our data we suggest the following model: Lr34res mediates an increased ABA flux via the xylem to the leaf tip where it induces premature senescence. However, it is yet unclear if the premature senescence directly leads to partial fungal disease resistance or if the disease resistance is caused by a different mechanism. In barley, silencing of gene RBOH2 leads to premature leaf senescence and increased resistance against powdery mildew (Torres et al., 2017). In addition, Torres et al. (2017) reported that the leaf senescence and disease resistance are an age-dependent phenotype as it is the case for Lr34res. This indicates that the premature leaf senescence in Lr34res-expressing plants might have an influence on the disease resistance. In this study, we detected a correlation of PR gene induction and ABA accumulation in Lr34res-expressing barley plants that did not yet show any LTN. Also, the barley line GLP 8 with low Lr34res expression level but quantitative disease resistance shows neither LTN, PR gene induction nor ABA accumulation. Taken together, the results of this and previous studies indicate that Lr34res-expression mediates a certain level of disease resistance without detectable ABA redistribution and LTN development. Furthermore, ABA accumulation at the leaf tip might enhance Lr34res-mediated resistance by induction of PR gene expression. The exact mechanism how the Lr34res-mediated ABA redistribution regulates the quantitative disease resistance needs to be determined in the future.

The understanding of Lr34res-mediated resistance is complicated by the substrate versatility of ABCG-transporters. In yeast, they are known to transport multiple structurally unrelated substrates (Harris et al., 2021). Also, ABCG transporters in plants are involved in different physiological processes suggesting that members of this family transport several substrates (Gröfe and Schmitt, 2021). Therefore, Lr34res might also act as a transporter of several substrates contributing to the constitutive defense response in wheat, barley and rice (Bucher et al., 2017; Chauhan et al., 2015; Hulbert et al., 2007; Krattinger et al., 2019). Recent studies on Lr34 revealed several physiological changes suggesting other potential substrates of LR34. Deppe et al. (2018) showed an accumulation of phosphatidylserine at the exoplasmic leaflet of the plasma membrane and increased phosphatidic acid (PA) concentration at the cytoplasmic leaflet as a secondary effect in Lr34res and Lr34sus-expressing yeast cells and barley protoplasts. PA accumulation at the cytoplasmic leaflet is reported to regulate defense and ABA signaling in Arabidopsis (Mishra et al., 2006; Zhao, 2015). Another study showed an accumulation of the phenylpropanoid diglyceride 1-O-p-coumaroyl-3-O-feruloylglycerol (CFG) in Lr34res-expressing wheat cultivars (Rajagopalan et al., 2020). Since CFG is thought to be a storage compound of antimicrobial hydroxycinnamic acids (HCA) and fungal infection results in CFE depletion, these compounds might be a factor for Lr34res-mediated resistance. These results suggest that LR34 mediates either the redistribution of CFG or derivatives of HCAs, inhibits CFG transport or regulates the biosynthesis of CFG (Rajagopalan et al., 2020). However, to date transport assays were only performed for ABA, leaving the question open whether the mentioned physiological observations are indirectly caused by ABA transport or by the transport of an additional substrate.

In wheat, it was shown that the expression of Lr34res leads to a grain yield increase of 5% or 4.3% in fields protected by fungicides compared to Lr34sus-expressing lines (Johnston et al., 2017; Singh and Huerta-Espino, 1997). However, Lr34res lines showed a 2.9–65.8% higher yield than Lr34sus lines upon natural infection of leaf rust or stripe rust (Johnston et al., 2017; Singh and Huerta-Espino, 1997). The yield reduction in fungicide treated Lr34res plants is likely caused by LTN that results in a reduction of the photosynthetically active leaf area and premature plant senescence. In this study, we analyzed Lr34res-expressing transgenic barley plants under field conditions at adult plant stage and observed resistance against Bgh and P. hordei consistent with the results obtained in the greenhouse and under near-field conditions (Bonì et al., 2018; Chauhan et al., 2015; Risk et al., 2013). Field grown barley line BG 9 with a high Lr34res expression level and strong LTN coincided with a reduced 1,000-grain weight. Similar results were obtained from studies conducted in the greenhouse, however the 1,000-grain weight was not reduced in near-field conditions (Bonì et al., 2018). Interestingly, barley line GLP 8 with a lower Lr34res expression level did not show strong LTN and no significantly reduced 1,000-grain weight was observed, but still was partially resistant against P. hordei. However, barley line GLP 8 did not show significant resistance to Bgh compared to its sister line. Similarly to the barley line GLP 8, transgenic Lr34res-expressing sorghum lines did not develop LTN and were partially resistant to anthracnose (Colletotrichum sublineolum) and rust (Puccinia purpurea) (Schnippenkoetter et al., 2017). Furthermore, Lr34res-expressing maize lines were partially resistant to P. sorghi and E. turgidum with late development of LTN and without negative effects on plant growth (Sucher et al., 2017). Thus, fine-tuning the Lr34res expression allows to generate plants that show disease resistance with no or minor LTN and fitness penalty.

In this study, we found an ABA accumulation at the leaf tips of Lr34res-expressing transgenic barley that correlates with senescence, expression of defense genes and complete immunity to biotrophic fungal pathogens in the field. These results increase our understanding of Lr34res-mediated physiological effects and suggest a fine-tuning of Lr34-
expression level to enhance fungal disease resistance in the field while minimizing the negative effects of LTN. Thus, we suggest the use 34res as a transgenic source of resistance in breeding programs with different types of promoters and to test a large number of events to identify the optimal expression level for conferring resistance to a specific pathogen.

Author contribution statement

SB, BK, SGK and TK designed the research; SB, TK, GG and SGK carried out the experiments; SB, TK and GG analyzed the data; SB, TK, SGK and BK wrote the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2021.07.001.

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