The Lr34 adult plant rust resistance gene provides seedling resistance in durum wheat without senescence

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Abstract: The hexaploid wheat (Triticum aestivum) adult plant resistance gene, Lr34/Yr18/Sr57/Pm38/Ltn1, provides broad-spectrum resistance to wheat leaf rust (Lr34), stripe rust (Yr18), stem rust (Sr57) and powdery mildew (Pm38) pathogens, and has remained effective in wheat crops for many decades. The partial resistance provided by this gene is only apparent in adult plants and not effective in field-grown seedlings. Lr34 also causes leaf tip necrosis (Ltn1) in mature adult plant leaves when grown under field conditions. This D genome-encoded bread wheat gene was transferred to tetraploid durum wheat (T. turgidum) cultivar Stewart by transformation. Transgenic durum lines were produced with elevated gene expression levels when compared with the endogenous hexaploid gene. Unlike nontransgenic hexaploid and durum control lines, these transgenic plants showed robust seedling resistance to pathogens causing wheat leaf rust, stripe rust and powdery mildew disease. The effectiveness of seedling resistance against each pathogen correlated with the level of transgene expression. No evidence of accelerated leaf necrosis or up-regulation of senescence gene markers was apparent in these seedlings, suggesting senescence is not required for Lr34 resistance, although leaf tip necrosis occurred in mature plant flag leaves. Several abiotic stress-response genes were up-regulated in these seedlings in the absence of rust infection as previously observed in adult plant flag leaves of hexaploid wheat. Increasing day length significantly increased Lr34 seedling resistance. These data demonstrate that expression of a highly durable, broad-spectrum adult plant resistance gene can be modified to provide seedling resistance in durum wheat.

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The *Lr34* adult plant rust resistance gene provides seedling resistance in durum wheat without senescence

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**Summary**

The hexaploid wheat (*Triticum aestivum*) adult plant resistance gene, *Lr34/Yr18/Sr57/Pm38/Ltn1*, provides broad-spectrum resistance to wheat leaf rust (*Lr34*), stripe rust (*Yr18*), stem rust (*Sr57*) and powdery mildew (*Pm38*) pathogens, and has remained effective in wheat crops for many decades. The partial resistance provided by this gene is only apparent in adult plants and not effective in field-grown seedlings. *Lr34* also causes leaf tip necrosis (*Ltn1*) in mature adult plant leaves when grown under field conditions. This D genome-encoded bread wheat gene was transferred to tetraploid durum wheat (*T. turgidum*) cultivar Stewart by transformation. Transgenic durum lines were produced with elevated gene expression levels when compared with the endogenous hexaploid gene. Unlike nontransgenic hexaploid and durum control lines, these transgenic plants showed robust seedling resistance to pathogens causing wheat leaf rust, stripe rust and powdery mildew disease. The effectiveness of seedling resistance against each pathogen correlated with the level of transgene expression. No evidence of accelerated leaf necrosis or up-regulation of senescence gene markers was apparent in these seedlings, suggesting senescence is not required for *Lr34* resistance, although leaf tip necrosis occurred in mature plant flag leaves. Several abiotic stress-response genes were up-regulated in these seedlings in the absence of rust infection as previously observed in adult plant flag leaves of hexaploid wheat. Increasing day length significantly increased *Lr34* seedling resistance. These data demonstrate that expression of a highly durable, broad-spectrum adult plant resistance gene can be modified to provide seedling resistance in durum wheat.

**Introduction**

Wheat rust diseases caused by *Puccinia graminis* f. sp. *tritici* (stem rust), *P. striiformis* f. sp. *tritici* (stripe/yellow rust) and *P. triticina* and *P. graminis* (leaf rust) remain a major threat to world production of hexaploid bread wheat (*T. aestivum* L., 2n = 6x = AABBDD) and tetraploid durum (pasta) wheat (*T. turgidum* L. subsp. *durum*, 2n = 4x = 28, AABB) (Chen et al., 2014; Huerta-Espino et al., 2011; Kolmer, 2005; Singh et al., 2011a). Resistance to these wheat rust pathogens is achieved most economically using resistance genes derived from wheat landraces and wild relative species. However, most wheat rust resistance genes are ultimately overcome by pathogen evolution to virulence.

Wheat rust resistance genes have been broadly categorized into two groups, all-stage or seedling resistance genes and adult plant resistance (APR) genes. All-stage resistance genes, function at all stages of plant development and although often race specific, can provide high levels of resistance. Nine cloned all-stage wheat rust resistance genes each encode a nucleotide-binding site leucine-rich repeat protein (NLR), a large class of disease resistance proteins characterized in numerous plant species (Cloutier et al., 2007; Feuillet et al., 2003; Huang et al., 2003; Liu et al., 2014; Mago et al., 2015; Pertyanova et al., 2013; Saintenac et al., 2013; Steuernagel et al., 2016). These proteins recognize pathogen molecules (effectors) introduced into plant cells or alternatively effector-mediated modifications of host proteins, leading to defence activation (reviewed by Dodds and Rathjen, 2010). Pathogen effector genes can rapidly evolve to avoid plant recognition, making NLR resistance genes ineffective.

Unlike all-stage resistance, APR occurs in mature wheat plants only and tends to provide partial resistance, although specific APR gene combinations show additive resistance effects (Singh et al., 2011b). Some APR genes provide resistance to all isolates of a pathogen species (broad spectrum) and in some cases resistance to multiple pathogen species. For example, the *Lr34/Yr18/Sr57/Pm38/Ltn1* gene (hereafter called *Lr34*) provides resistance to *P. triticina* (Lr), *P. striiformis* f. sp. *tritici* (Yr), *P. graminis* f. sp. *tritici* (Sr) and *Blumeria graminis* (Pm). This gene also confers a leaf tip necrosis phenotype (*Ltn*) on flag leaves when plants are field-grown (Dyck, 1991; Hultbert et al., 2007; Lagudah et al., 2006; Risk et al., 2012; Shah et al., 2011). Similarly, the *Lr67/Yr46/Sr56/Pm39/Ltn3* gene (hereafter referred to as *Lr67*) shows broad-spectrum, partial resistance to rust and mildew pathogens (Herrera-Foessel et al., 2014). In contrast, the *Yr36* APR gene provides *P. striiformis* f. sp. *tritici* resistance only (Uaay et al., 2005).

The molecular basis of APR is poorly understood when compared with NLR-mediated resistance. The three APR genes described above have been cloned and encode an ABC-type transporter (*Lr34*), a hexose transporter (*Lr67*) and a protein
kinase fused to a START domain (Yr36) (Fu et al., 2009; Krattinger et al., 2009; Moore et al., 2015). The Lr67 protein may act as a dominant negative regulator of hexose transport (Moore et al., 2015), while the Yr36 protein increases chloroplast H₂O₂ accumulation by phosphorylation of a thylakoid-associated ascorbate peroxidase (Guo et al., 2015). The mode of action of the Lr34 transporter and molecules it transports are unknown. The deletion of a single phenylalanine codon in the D genome-encoded Lr34-susceptible allele converts it to a functional Lr34 resistance gene (Chauhan et al., 2015). This mutation is believed to have occurred after the domestication of wheat (Krattinger et al., 2013).

Lr34 has been used extensively in hexaploid wheat cultivation for many decades and remained durable to all pathogen races over this long period of time (Dyck et al., 1966; Kolmer et al., 2008). It provides partial resistance that is insufficient to prevent yield losses from rust diseases unless supplemented with additional resistance genes. Lr34 partial resistance occurs in mature plants (60 days postgermination) and is often associated with Ltn (Dyck, 1991; Singh, 1992). Ltn, which appears to be accelerated leaf senescence, is particularly apparent in flag leaves (Krattinger et al., 2009; Risk et al., 2012) and has been used as a phenotypic marker for Lr34 identification (Shah et al., 2011). Ltn, however, is influenced by both the environment and genetic background (Shah et al., 2010, 2011; Singh, 1992). Lr34 resistance occurs in mature flag leaves after the onset of Ltn (Krattinger et al., 2009) with leaf tips showing the greatest resistance (Hulbert et al., 2007). Flag leaf tips show up-regulation of abiotic stress-responsive genes in the absence of pathogen infection and higher levels of pathogenesis-related (PR) protein expression upon P. triticina infection (Hulbert et al., 2007). This apparent abiotic stress response in uninfected, mature Lr34 tissue is suggested to prime the plant defence response for elevated expression upon rust pathogen challenge (Hulbert et al., 2007).

Expression of Lr34 in transgenic barley seedlings results in a very deleterious phenotype due to the induction of rapid, developmental leaf senescence (Risk et al., 2013). Unlike Lr34 wheat plants, these barley plants show constitutive induction of defence pathways in the absence of pathogen infection (Chauhan et al., 2015) and are resistant to pathogens at the seedling and adult plant stage (Risk et al., 2013). Barley does not contain orthologous Lr34 sequences (Krattinger et al., 2011), and co-expression of the Lr34-susceptible allele appears to help attenuate negative effects in this species (Chauhan et al., 2015).

In contrast to barley, rice encodes an Lr34 orthologue (Krattinger et al., 2011). Expression of the wheat Lr34 gene in rice was associated with early leaf tip necrosis and deleterious pleiotropic effects in most cases, although not as extreme as observed in barley (Krattinger et al., 2016). In some lines, leaf tip necrosis occurred in seedlings at the two-leaf stage and plants subsequently showed a severe negative impact on auxillary shoot formation, plant vigour and spikelet production (Krattinger et al., 2016). However, a single line with low seedling expression was recovered that was only marginally compromised. Remarkably, this gene also provided resistance to Magnaporthe oryzae, the hemibiotrophic causal agent of rice blast disease. Deletion of the critical phenylalanine codon present in the rice Lr34 orthologue did not result in disease resistance (Krattinger et al., 2016).

Given the remarkable durability of Lr34, we have introduced this hexaploid wheat (ABD) D genome-encoded gene into durum wheat (AB) by transgenesis as a potentially useful source of disease resistance. Amongst these durum transgenics, several lines showed obvious seedling resistance to leaf rust, stripe rust and powdery mildew diseases, a phenotype not associated with the endogenous Lr34 gene. A strong correlation between seedling resistance and transgene expressions level was observed. Unlike barley and rice, no deleterious accelerated senescence or developmental phenotypes were observed in these seedlings. Lr34 is therefore potentially of significant benefit for durum wheat germplasm improvement and, with elevated expression, can provide seedling resistance that is not conferred by the endogenous hexaploid wheat gene.

Results

Lr34-mediated leaf rust resistance occurs in hexaploid wheat seedlings when grown at a constant 10 °C throughout P. triticina challenge (Risk et al., 2012). However, these seedlings are not resistant when grown at higher temperatures (Risk et al., 2012; Rubiales and Niks, 1995; Singh and Gupta, 1992). Lr34 cold-induced seedling resistance was exploited to screen transgenic durum cultivar Stewart plants containing a Lr34 transgene (Figure S1) for P. triticina resistance. T1 seedlings from 12 lines were infected with P. triticina and after inoculation grown at a constant 10 °C with a 16-h light/8-h dark photoperiod. Potentially resistant progeny were observed in 10 T1 families. On susceptible plants, pustules were present after 20 days postinoculation (dpi), which increased in size by 36 dpi. Resistant seedlings were not immune to P. triticina, but had an obvious reduction in pustule size (Figure S2). Homozygous Lr34 lines were identified using PCR and confirmed by DNA blot analysis of DNAs from T2 seedlings using a restriction enzyme/probe combination that also determined transgene copy number (Figure S3). Four independent homozygous T1 lines were produced that contained a single transgene and potentially showed weak (36-4), moderate (17-1, 41-2) and high (39-2) levels of seedling leaf rust resistance at 10 °C.

Homozygous T2 progeny were re-screened for seedling leaf rust resistance at 10 °C. For each line, P. triticina growth was quantified 30 dpi (Risk et al., 2012) by pooling equivalent leaves from 10 to 15 seedlings and the relative amount of fungal chitin present per gram of fresh tissue determined using a chitin assay (Ayliffe et al., 2013). Chitin levels were expressed as fluorescence units of bound WGA-FITC, a fluorophore-conjugated lectin that specifically binds chitin. A clear reduction in fungal biomass was apparent in homozygous Lr34 lines compared with Stewart control plants (Figure 1a, black columns). Hexaploid wheat cultivar Thatcher carrying the endogenous D genome Lr34 gene (Th+Lr34) had significantly less rust disease compared with Thatcher control plants, as expected (Figure 1a).

These genotypes were then tested for P. triticina resistance when plants were grown at 22 °C with a 16-h light/8-h dark photoperiod. Under these conditions, Stewart control plants were moderately susceptible (3C on the Stakman scale, where 0 is immune and 4 is highly susceptible (Stakman et al., 1962)) to the P. triticina isolate used, with obvious macroscopic rust growth and sporulation occurring (Figure 2a). Remarkably, an obvious increase in seedling resistance occurred in T2 seedlings of these durum lines compared with nontransgenic control plants 14 dpi in three replicated experiments (Figures 1a, 2a, 5a, 6a). As expected, Th+Lr34 seedlings had high levels of infection when grown at 22 °C, albeit less than the Tc control (Figures 1a, 2a). The relative levels of rust growth on transgenic seedlings at 22 °C

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expression levels and disease resistance in these durum lines (Figure 1b). All four lines had significantly greater (4.5- to 10-fold) seedling Lr34 expression compared with the endogenous Th+34 gene (Figure 1b), presumably explaining the durum seedling resistance observed for this APR gene under greenhouse conditions. Increased Lr34 expression in these lines is likely due to transgene integration into more transcriptionally favourable regions of the genome.

As Lr34 also provides APR to P. striiformis f. sp. tritici (wheat stripe rust disease) (Singh, 1992b) and Blumeria graminis (wheat powdery mildew disease) (Spielmeyer et al., 2005), Lr34 durum lines were screened for seedling resistance to these pathogens (22 °C, 16-h light). An obvious reduction in seedling stripe rust growth was observed on transgenic lines both macroscopically and by relative fungal biomass quantification in three replicated experiments (Figures 1c, 2b, S4). The levels of disease resistance again largely correlated with transgene expression levels of each replicates (Figure 1b). Similarly, significantly less B. graminis growth occurred on two Lr34 durum lines tested with this pathogen using 14 biological replicates per genotype (Figure 3a).

To further investigate the Lr34 resistance of hexaploid wheat seedlings grown at 10 °C, transcript levels were quantified. Seedlings were grown at 22 °C (16-h light) and then half transferred to 10 °C growth conditions with the same light regime. After 3 days of acclimation, half of the seedlings in each cabinet were infected with P. striiformis f. sp. tritici and tissues harvested from infected and uninfected seedlings 3 dpi. A fourfold induction of Lr34 expression occurred in Tc+34 seedlings grown at 10 °C after P. triticina infection (Figure 3b) compared with uninfected seedlings. No equivalent increase in Lr34 expression occurred in Tc+34-infected seedlings grown at 22 °C. These data are consistent with similar studies that showed Lr34 induction in P. triticina-infected wheat seedlings grown at 10 °C, but not in infected seedlings grown under higher temperatures (Risk et al., 2012). The ability of Lr34 to provide seedling resistance in hexaploid wheat at 10 °C therefore appears due to elevated gene expression upon pathogen infection, although additional effects from these growth conditions (e.g. reduced pathogen growth rate and potential cold acclimation response) may contribute to resistance.

Microscopic analyses were undertaken on transgenic durum lines 17-1, 36-4, 39-2 and 41-2 and control Stewart seedlings...
Seedling resistance in *Lr34* durum wheat

after infection with either *P. triticina* or *P. striiformis* f. sp. *tritici*. On all lines, a mixture of infection sites occurred, ranging from small sites to large sporulating uredinia. Control Stewart seedlings showed extensive growth of both pathogens with infection sites usually producing sporulating uredinia (Figure 2c, g), while transgenic durum lines showed less hyphal growth, fewer uredinia and generally smaller infection sites albeit with haustoria (Figure 2d–f, h). Autofluorescent cells were uncommon in all lines infected with either pathogen, suggesting that cell death was not a predominant feature of the resistance response. These data are consistent with previous analysis of adult hexaploid *Lr34* wheat plants after *P. triticina* infection where reduced rust growth occurred without obvious cell death (Risk et al., 2012).

As rust growth does still occur on *Lr34* transgenic seedlings, the effect of additional plant growth following *P. striiformis* infection was examined 26 dpi. By this time, infected seedling leaves of all plants were becoming chlorotic and senescent, suggesting that little more growth of this biotrophic pathogen would occur due to leaf ageing (Figure S5). Relative fungal biomass quantification again showed significantly less stripe rust pathogen growth (Figure 3c) on *Lr34* durum seedlings that correlated with transgene expression levels (Figure 1b). These data suggest that rust pathogen growth on these seedlings does not ever reach that observed on nontransgenic control lines.

Expression of *Lr34* in barley causes strong seedling leaf necrosis that appears to be an accelerated senescence response (Risk et al., 2013). To assess potential deleterious effects of *Lr34* expression in durum wheat, T3 plants were grown to maturity in the glasshouse. No difference in plant tiller number, tiller height and seed weight yield occurred between control and transgenic durum lines (Figure S6). During plant development, no dramatic differences in leaf senescence rates were apparent (Figure 2k–m).

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![Figure 2](image-url)
transgenic controls (Figures 2n, 3d). Leaf tip necrosis of mature
increased leaf tip necrosis of flag leaves compared with non-
However, under field conditions, transgenic plants did show some
was undertaken. Two senescence genes were targeted, $S40$
(Krupinska et al., 2002) and CP-MIII (serine carboxypeptidase)
(Parrott et al., 2010), with $S40$ previously shown to be highly
induced in Th+Lr34 flag leaves (Krattinger et al., 2009). No
significant difference in $S40$ expression occurred between
Stewart and Lr34 transgenic lines (Figure 4a). No consistent
change in Cp-MIII expression occurred with the most resistant
line, 39-2, and line 36-4 showing no difference to the Stewart
control, while lines 17-1 and 41-2 showed a modest increase in
expression (two- to threefold) (Figure 4b). These data are not
consistent with elevated senescence gene expression in
Lr34 durum seedlings compared with nontransgenic
Stewart lines and suggest that this seedling resistance is not
associated with senescence induction.

An interesting feature of Lr34 expression in mature bread
wheat plants is the up-regulation of abiotic stress-responsive
genes, predominantly in the tips of flag leaves, in the absence of
pathogen infection (Hulbert et al., 2007). To determine whether
similar gene expression occurred in Lr34 durum seedlings,
expression of abiotic stress-responsive genes Rab15 (Kosova
et al., 2014; Tsuda et al., 2000) and HSP90 (Shi et al., 2012)
was quantified in leaf RNAs of uninfected seedlings grown at
22 °C (16-h light). A 20- to 150-fold induction of Rab15
occurred in Lr34 durum seedlings compared with nontransgenic
Stewart (Figure 4c), while a more modest four- to six-fold increase in HSP90 expression occurred (Figure 4d). The expres-
sion of Lr34 in durum seedlings therefore appears to induce an
apparent abiotic stress-related response in the absence of
pathogen infection.

Higher PR gene expression levels were reported in P. triticina-
infected flag leaves of Lr34 hexaploid plants compared with
control lines (Hulbert et al., 2007). Transgenic and control durum
seedlings were infected with P. triticina at the three- to four-leaf
stage (22 °C) and tissues harvested 12 dpi. Tissue samples were
quantified for both PR gene expression and relative fungal
biomass. Again, significantly less rust growth occurred in trans-
genic lines compared with Stewart seedlings (Figure 5a), which
correlated with Lr34 expression levels (Figure 1b). However, no
increased expression of PR1, PR2 or PR3 occurred in Lr34 durum
lines relative to control plants upon rust infection (Figure 5b and
Figure S7). These data, however, are complicated by rust infection
per se strongly inducing PR gene expression (Figure 5b and
Figure S7: compare uninfected and infected Stewart seedlings).
PR expression was therefore normalized relative to fungal
expression.
biomass. Relative to rust growth, strong induction of all three PR genes occurred in the most resistant transgenic, line 39-2, while the remaining more intermediate resistant lines showed no obvious increase in PR expression in response to rust infection compared with the control (Figure 5c).

To examine the influence of photoperiod on Lr34 resistance, transgenic seedlings were grown at 22 °C (16-h light) to the three- to four-leaf stage and then inoculated with P. triticina. Half of the infected plants were then grown under continuous light, while the remaining seedlings were maintained under a 16-h light/8-h dark photoperiod. All Lr34 lines had significantly less pathogen growth 10 dpi under continuous light compared with 16-h light (Figure 6a). Under both conditions, rust resistance levels again correlated with the transgene expression levels (Figure 1b). In contrast, control seedlings showed equivalent or increased rust growth under constant light compared with a 16-h light/8-h dark light regime (Figure 6a). Under these highly controlled growth cabinet conditions, increasing photoperiod therefore resulted in increased levels of Lr34 resistance. It is noteworthy that light has previously been speculated to promote Lr34 resistance in field-grown plants (Singh and Gupta, 1992).

Given the light association of L34 resistance and previous observation that Yr36 APR results in chloroplast H2O2 accumulation (Guo et al., 2015), H2O2 accumulation was also examined. Durum seedlings were grown at 22 °C (16-h light) and H2O2 content determined in infected and uninfected plants using Amplex Red (Invitrogen). While a clear increase in H2O2 content occurred upon P. triticina infection, no differences in accumulation of this reactive oxygen occurred in resistant and susceptible durum genotypes (Figure 6b). These observations were consistent with 3,3′ diamino benzidine (DAB) staining of H2O2 in rust-infected leaf tissue with no obvious difference in H2O2 accumulation apparent (Figure 2i, J).

Discussion

Several rust resistance genes have been transferred from durum wheat into hexaploid wheat due to the relative simplicity of crossing these species and selecting fertile hexaploid lines or alternatively producing synthetic hexaploid wheat by crossing tetraploid wheat (AABB) with Aegilops tauschii (DD). Germplasm development in tetraploid wheat using interspecies crosses, while achievable (Huguet-Robert et al., 2001; Klindworth et al., 2012; Morris et al., 2011), is more difficult due to poor vigour and low fertility in tetraploid backgrounds (Ceoloni et al., 1996; Klindworth et al., 2012). Of particular difficulty is the introduction of D genome genes of hexaploid wheat or Ae. tauschii due to the absence of homologous chromosomes in AABB tetraploids and only a few examples have been reported (Ceoloni et al., 1996; Han et al., 2016; Joppa et al., 1998; Liu et al., 1996; Luo et al., 1996). Given the broad, multipathogen effectiveness and durability of Lr34, transfer of this gene to durum wheat by
transgenesis is potentially of use in controlling rust and mildew diseases in this crop species.

It was unexpected that this APR gene would also show high levels of seedling resistance in durum wheat which we attribute to transgene expression levels being five to ten times greater than the endogenous gene in hexaploid wheat seedlings, when uninfected seedlings grown at 22 °C were compared. A five- to 10-fold increase in Lr34 expression also occurs in resistant flag leaves of hexaploid Th+34 plants when compared with seedlings (Risk et al., 2012, 2013), consistent with this hypothesis. Importantly, elevated gene expression was not associated with negative pleiotropic effects in durum seedlings and no increased senescence was apparent either macroscopically or by quantification of senescence marker gene transcripts. This seedling resistance further enhances the agronomic potential of Lr34 in durum wheat cultivation. Seedling resistance is particularly important for protecting wheat from stripe rust disease because it occurs early in the growing season.

Previous introduction of Lr34 as a transgene into hexaploid wheat cultivar Bobwhite (BW26 AUS) also led to elevated expression levels, with some seedlings showing 10-fold higher expression; however, seedling resistance was not observed (Risk et al., 2012). These lines, however, did show typical Lr34 APR and minor leaf tip necrosis (Risk et al., 2012). Why elevated Lr34 expression does not provide seedling resistance in hexaploid wheat is unknown. In contrast, Lr34 transgenics made in a second wheat line, BW26SU, did show seedling resistance to P. triticina. This line, however, was not fully rust susceptible and resistance in these lines did not correlate with transgene expression levels. One highly resistant BWSUI line showed only a 2.4-fold increase in Lr34 expression relative to Th+34 seedlings. The authors concluded that background resistance present in BWSUI significantly enhanced Lr34 effects in this line (Risk et al., 2012). The Lr34 gene does show additive effects with minor resistance genes (Singh et al., 2011b) and several all-stage resistance genes (German and Kolmer, 1992).

We cannot exclude the possibility that the Lr34 resistance in Stewart durum seedlings may be associated with minor gene effects. However, we feel this is improbable. Rust assays showed that Stewart is moderately susceptible to the P. triticina (3C rating) and P. striiformis f. sp. tritici (3, 3+ C rating) isolates used in this study (Figure 2a, b), suggesting that minor resistance genes do exist in this background. However, Lr34 durum lines also had increased powdery mildew disease resistance, meaning minor background genes effective against all three pathogen species would be needed in the Stewart background that showed additive effects with Lr34. In addition, resistance levels in these durum lines directly correlated with Lr34 expression levels consistent with transgene expression being the predominant factor in this seedling resistance.

Elevated Lr34 expression in durum wheat is well tolerated with only mild leaf tip necrosis in mature plants, although higher expression levels could possibly be deleterious. Hexaploid wheat also tolerates elevated Lr34 expression, although cold-grown
necrosis and negative developmental effects were also seen in most Lr34 rice lines (Krattinger et al., 2016).

While Lr34 resistance usually coincides with accelerated senescence in most plant tissues, including transgenic durum flag leaves, the absence of visible senescence or senescence gene up-regulation in Lr34 durum seedlings suggests that senescence is not required for resistance. Consistent with this hypothesis, in rice the amount of leaf tip necrosis in Lr34 lines did not necessarily correlate with resistance levels (Krattinger et al., 2016), implying these two phenotypes are not directly correlated. Ltn is not always apparent in field-grown, adult Lr34 plants being dependent on both the environment and genetic background. It is unknown, however, whether plants under these circumstances show senescence up-regulation without visible necrosis or, alternatively, show no altered senescence response. Hexaploid Lr34 seedlings grown at 10 °C during rust infection also show resistance, presumably due to elevated Lr34 expression, without visible accelerated senescence.

This Lr34 durum seedling resistance, however, is associated with up-regulation of abiotic stress genes such as rab15 and HSP90, suggesting that this response is required for resistance. Only some of the responses induced by Lr34 may provide disease resistance and additional effects such as accelerated senescence may be pleiotropic. Common signalling pathways between senescence and abiotic stress responses are well established (Gepstein and Glick, 2013). Some evidence of modest up-regulated of PR expression was observed in rust-infected durum seedlings although it was confounded by rust infection per se causing PR induction. Other analyses have shown limited PR induction in Lr34 hexaploid wheat (Risk et al., 2012), which differs from the observations of Hulbert et al. (2007).

These data raise several possible models for Lr34 function in durum wheat. In the first scenario, Lr34 resistance and senescence are mechanically related, but additional factors, regulated by plant development and the environment, are required for the latter response to occur (Figure 7a). In the case of barley, which does not have an Lr34 ortholog and may therefore lack appropriate regulatory control of Lr34-mediated processes, these additional factors are inappropriately produced or recognized during initiation of age-dependent seedling leaf senescence resulting in accelerated leaf necrosis. In hexaploid wheat, a minimum transcriptional threshold needed for Lr34 resistance is not reached until later in plant development at which time both resistance and senescence occur concomitantly. Consistent with this model, elevated expression of Lr34 in durum seedlings and in cold-grown, infected, hexaploid seedlings results in resistance without leaf senescence (Figure 7a). In the second scenario (Figure 7b), the resistance mechanism conferred by Lr34 is independent of an Lr34-mediated senescence response, which only occurs in tissues of mature plants after reaching a specific developmental age.

Lr34 durum lines that show robust seedling resistance when grown under field-like conditions will be a valuable resource to further investigate the mechanistic basis underlying resistance. These transgenic lines demonstrate a clear photoperiod effect on Lr34 resistance. These observations were of particular interest given the proposed mechanism of the Yr36 START-kinase protein that phosphorylates a chloroplast thylakoid-associated ascorbate peroxidise (Guo et al., 2015). Elevated H$_2$O$_2$ levels occur in Yr36 transgenic plants and accelerated leaf senescence of older leaves (Guo et al., 2015). However, no increased H$_2$O$_2$
accumulation occurred in either uninfected or rust-infected Lr34 durum seedlings, suggesting a potentially different mode of action.

In summary, Lr34 resistance in durum seedlings is not associated with necrosis or accelerated senescence. In contrast, induction of abiotic stress-response genes occurred in the absence of pathogen infection in these seedlings as previously observed in adult hexaploid wheat plants (Hulbert et al., 2007). Photoperiod had a significant effect on Lr34 phenotypes by as yet undefined mechanisms. Manipulation of APR gene expression can enhance disease resistance without associated negative pleiotropic effects in some instances, which is of potential agronomic benefit.

**Experimental procedures**

**Plant and pathogen growth conditions**

Wheat plants were grown in growth cabinets at 22 °C, 16-h light/8-h dark unless otherwise specified. Wheat seedlings were infected with *Puccinia striiformis* f. sp. *tritici* isolate accession number 821559 (pathotype 104 E137 A-) and *Puccinia triticina* isolate accession number 020281 (pathotype 104-1,2,3,(6), (7),11 + Lr37) obtained from the Plant Breeding Institute, NSW, Australia. Plants were inoculated with *P. striiformis* and *P. triticina* urediospores and incubated in a humid chamber overnight at 10 or 22 °C, respectively. Infected plants were then transferred...
to growth cabinets (22 °C, 16-h light/8-h dark) for growth of rust pathogens. Extended growth times were used for experiments at 10 °C due to significantly slower rust growth (Risk et al., 2012). Rust inoculum was propagated on wheat cultivar Morocco and urediospores collected from infected plants by shaking plants over aluminium foil. For infection with B. graminis, durum leaves were harvested from 26-day-old seedlings and placed on MS salt media and infected with B. graminis isolate IR208; 7 dpi B. graminis growth was measured by chitin assay (Ayliffe et al., 2013) using 14 biological replicates per genotype.

**Generation of homozygous Lr34 transgenic durum wheat plants**

Transgenic Stewart durum wheat plants were generated by Agrobacterium-mediated transformation of cultivar Stewart (Ishida et al., 2013; Richardson et al., 2014). The Lr34 transgene (Figure S1A) was cloned into binary plasmid pWBVec8 (Murray et al., 2004; Wang et al., 1998), which encodes a hygromycin phosphotransferase gene. Transgenic plants were selected using 30–50 μg/ml of hygromycin. To identify Stewart plants containing at least one complete Lr34 transgene, DNA blot analysis was undertaken on T0 plants as previously described (Ayliffe et al., 2000). DNAs were restricted with NotI and hybridized with a probe encoding 2 kb of the 3′ terminus of the Lr34 ORF (Table S1). A predicted 16-kb fragment with homology was identified in lines containing a complete transgene (Figure S1B). T1 plants were screened for potential Lr34 transgene homozygosity by PCR analysis of 20 individual T2 seeds using Lr34-specific primers (Table S1, ABCTF4N and Lr34plusR). Transgene copy number in each family was then determined, and homozygosity confirmed, by DNA blot analysis of 25 Dral-restricted T2 plant DNAs hybridized with a probe complementary to a 481-bp fragment of the Lr34 3′ untranslated region (3′UTR) (Table S1, Figure S3). Homozygous families were identified for four independent, single-copy Lr34 transgenic events, that is 17-1, 36-4, 39-2 and 41-2.

**Fungal biomass assays**

Seedlings (10–15) at the three- to four-leaf stage from either homozygous Lr34 transgenic lines or nontransgenic control lines were infected with rust urediospores. Rust-infected leaves were harvested 10–14 days postinoculation, and relative chitin biomass per mg fresh weight of harvested tissue was determined by pooling seedling leaves of the same genotype and measuring the binding of wheat germ agglutinin–fluorescein isothiocyanate (Sigma-Aldrich, St. Louis) and visualized under blue light (Ayliffe et al., 2011). The wheat resistance gene identifies homologues in barley that have been subjected to diversifying selection. (2011) The wheat resistance gene identifies homologues in barley that have been subjected to diversifying selection. Theor. Appl. Genet. 100, 1144–1154.

**Microscopy and H2O2 quantification**

Fungal-infected tissue was stained with wheat germ agglutinin–fluorescein isothiocyanate (Sigma-Aldrich, St. Louis) and visualized under blue light (Ayliffe et al., 2011). Histochemical detection of H2O2 in infected leaf tissue was undertaken by 3,3′-diaminobenzidine (DAB) staining (Sigma-Aldrich, St. Louis) (Thordal-Christensen et al., 1997). H2O2 levels were determined in ground leaf tissues using an Amplex Red H2O2 assay kit as described by the manufacturer (ThermoFisher).

**Data analysis**

All data were analysed by ANOVA using an online calculator (http://statistica.mooo.com/) unless otherwise stated in figure legends. Significantly different data points had a P < 0.05 unless indicated otherwise in the text. Standard deviations are indicated on graphs. Graph columns with common annotation were not statistically different (ANOVA, P < 0.05), throughout.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

AR undertook experiments described in Figures 1a, c, 2, 3c, d, 4, 5, 6a. BG undertook experiments in Figures 1c, 2c. RB and SK produced data in Figure 3a. DS and RP screened rust collections to identify and provide a P. triticina isolate virulent on durum cultivar Stewart. EL undertook field trial results in Figure 3d and provided experimental design planning. MA contributed images in Figure 2, undertook experiments in Figures 2l–o and 6b in addition to providing experimental design.

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