How has Lr34/Yr18 conferred effective rust resistance in wheat for so long?

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Abbreviations: ABC transporter: ATP-binding cassette transporter, LR34: the protein encoded by the *Lr34* gene, LTN: Leaf tip necrosis, NBD: nucleotide-binding domain, TMD: transmembrane domain

Abstract

The *Lr34/Yr18* gene has been used in agriculture for more than 100 years. In contrast to many other resistance sources against leaf rust and stripe rust, it has remained effective and no virulence has been reported. This makes *Lr34* a unique and highly valuable resource for rust resistance breeding. The pleiotropic nature of the gene conferring partial resistance to different pathogen species, the associated leaf tip necrosis and its durability suggest a molecular mechanism that is different from major gene resistance. This is supported by the molecular nature of *Lr34* which was recently found to encode an ABC transporter. Interestingly, all tested wheat lines contain an allele of the *Lr34* gene on chromosome 7DS. In its susceptible form, the gene does not confer resistance. The difference between the encoded resistant and susceptible LR34 isoforms consists of only two amino acid changes, whereas the rest of the proteins are identical. These two changes must change the biochemical properties of the resistant LR34 transporter in such a way that the plant becomes resistant. We speculate that there is a slight conformational change in the resistant form of the protein, resulting either in modified specificity or kinetics of the transported molecule, or that the binding properties to an unknown second protein interacting with LR34 are changed, resulting in altered function. While the molecular nature of the molecule(s) transported by the LR34 protein remains unclear, it is likely that a physiological change related to *Lr34* activity is at the basis of resistance. We are currently establishing transgenic approaches in heterologous grass species to further investigate the molecular activity of *Lr34* and to better understand a physiological mechanisms resulting in disease resistance.

Introduction

The *Lr34/Yr18/Sr57/Pm38* gene is a globally important genetic resource for wheat resistance breeding against leaf rust and stripe rust (Fig. 1). It has also been shown to confer partial resistance to powdery mildew, and in some genetic backgrounds to stem rust. The locus has further been associated with tolerance to barley yellow dwarf virus. *Lr34* has been widely used in wheat cultivars worldwide and its resistance has remained effective over many years (possibly more than 100), making it one of the few known resistance genes with a generally large effect and durability despite large-scale, agricultural use. The gene was first described as *LrT2* (Dyck 1977, 1987) and later identified in several distinct groups of genetic material, e.g. CIMMYT lines, Chinese landraces and Eastern European winter wheat material (Kolmer et al. 2008; Krattinger et al. 2009). It is likely to have originated in Chinese landraces, but its use in the modern wheat breeding gene pool goes back to the Italian wheat cultivars Mentana and Ardito which were released at the beginning of the 20th century (Borghi 2001). These two lines, noted for their great adaptability and disease resistance, were also introduced to South America after the 1920s where they were widely cultivated and used in breeding (Vallega 1973). Based on lines developed there (e.g. Frontana in Brazil), the gene spread into additional lines, and was further distributed and later integrated into CIMMYT cultivars and breeding lines. Another use of the gene occurred in (Eastern) European winter wheat material, notably derivatives of the cultivar Bezostaya (Krattinger et al. 2009). The *Lr34/Yr18* gene acts quantitatively, i.e. it confers only partial resistance and generally has to be used in combination with other quantitatively acting resistance genes to provide sufficient resistance under heavy disease pressure. *Lr34* is active specifically in the adult plant stage and the flag leaf is usually evaluated in assays of plants containing the

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gene against leaf and stripe rust as well as powdery mildew (McIntosh 1992; Singh 1992; Spielmeyer et al. 2005). *Lr34/Yr18* activity is correlated with leaf tip necrosis (LTN) on the flag leaf which can be used as a phenotypic marker for *Lr34/Yr18* and which is also expressed in the absence of the pathogen (Fig. 1C; Dyck 1991; Lagudah et al. 2006). However, the expression of LTN is environmentally dependent and can vary greatly in different environmental conditions and genetic backgrounds. *Lr34/Yr18* was cloned by bi-parental mapping and positional cloning using integrated molecular marker information from several crosses as well as physical maps from the D genome of *Aegilops tauschii* and hexaploid wheat (Krattinger et al. 2009). Based on the knowledge of the *Lr34/Yr18* gene sequence, gene-specific markers were developed and have proven to be highly diagnostic for the *Lr34* gene (Lagudah et al. 2009; Dakouri et al. 2010). The analysis of eight independent mutants revealed that the same gene is in fact responsible for leaf and stripe rust resistances as well as LTN (Krattinger et al. 2009). Henceforth, we will refer only to *Lr34*, keeping in mind that it is a multi-pathogen resistance gene with a pleiotropic effect of leaf tip necrosis.

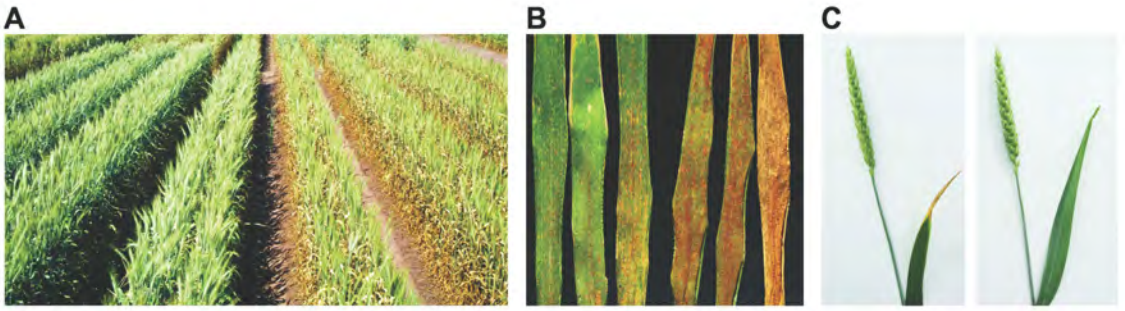

Figure 1 Phenotype of the *Lr34/Yr18* gene. (A) *Lr34* confers partial resistance. On the left there are rows of the resistant genotype Jupateco R and on the right of the susceptible near-isogenic genotype Jupateco S infected with leaf rust in Mexico. (B) Development of leaf rust on three successive leaves from the flag leaf of Jupateco R (left) and Jupateco S (right). (C) *Lr34* is associated with leaf tip necrosis shown here on a near-isogenic line 'Arina Lr34' (left) but not on the parental wheat cultivar 'Arina' which does not carry the *Lr34* gene (right). From Krattinger et al. (2009)

The molecular nature of *Lr34/Yr18*

The *Lr34* gene encodes a full-size ATP-binding cassette (ABC) transporter of the ABCG type (formerly called PDR transporter, for pleiotropic drug resistance). In contrast to half-size transporters (see Fig. 2), full-size transporters are thought to act as monomers in plant membranes. ABC transporters are integral membrane proteins that translocate molecularly diverse substrates across cell membranes. The substrates of many ABC transporters are still unknown. In addition, there is considerable mechanistic diversity of molecular transport functions within this large protein super-family (Lewinson et al. 2010). The typical eukaryotic ABC transporters are efflux transporters.

A common feature of ABC transporters is that they consist of two distinct domains, the transmembrane domain (TMD) and the nucleotide-binding domain (NBD). The TMD, also described as a membrane-spanning or integral membrane domain, consists of alpha helices embedded in the membrane bilayer. The sequences of TMDs are variable, and this variability is possibly one reason for the chemical diversity of substrates transported. The NBD or ATP-binding cassette (ABC) domain is cytoplasmic and more highly conserved. A characteristic of the NBD domain is its ATP binding. It is the hydrolysis of ATP that powers the substrate transport. In the LR34 protein the N-terminal NBD and the C-terminal transmembrane domains form a single polypeptide chain, arranged as NBD-TMD-NBD-TMD.

The three-dimensional structures of ABC transporters are predominantly derived from prokaryotic proteins so these are currently used as models for plant proteins. In prokaryotes, most exporters, such as the multidrug exporter Sav1866 (Dawson and Locher 2006) from *Staphylococcus aureus*, are made up of a homodimer (Fig. 2). There is no molecular structure yet for the plant/fungal specific ABCG/PDR type of transporters. Thus, molecular modeling is dependent on some of the bacterial ABC transporters for which the structure is known. For this reason such interpretations must be met with caution.

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The molecular analysis of the *Lr34* gene in different wheat varieties and grass species (for further evolutionary aspects also see below) has given some relevant insight into the molecular diversity. Importantly, wheat has an allohexaploid genome and we first analyzed the presence of homoeologous genes of *Lr34* on the A and B genomes (*Lr34* is located in the D genome). It was found that there is an active, expressed *Lr34*-related gene showing 97% homology at the amino acid level in the B genome, whereas the homoeolog on the A genome is inactive as it is disrupted by several transposon insertions (Krattinger et al. 2011). Thus, normal hexaploid wheat has two expressed and closely related *Lr34*-type of genes, whereas tetraploid wheat only has the B-genome copy. The most interesting finding came from the analysis of the *Lr34* gene in lines with known presence and activity of *Lr34* vs. lines lacking *Lr34*-conferred resistance. Lines without the *Lr34*-type of resistance had a closely related allelic form of *Lr34* which was called *Lr34sus* (for susceptible). In semi-quantitative comparative analysis, no expression differences were found for the two closely related alleles with largely identical promoter regions (Krattinger et al. 2009). In fact, in the complete protein with 1,401 amino acids, there are only two differences resulting in amino acid changes between the proteins encoded by *Lr34* and its susceptible allele. These are a deletion of three base pairs in exon 11 of the resistance allele resulting in the loss of a phenylalanine residue predicted to be in a transmembrane region (see Fig. 2), and a single base pair change in exon 12 converting a tyrosine to a histidine at a location that is probably localized at the cytoplasmic end of a transmembrane helix (Fig. 2).

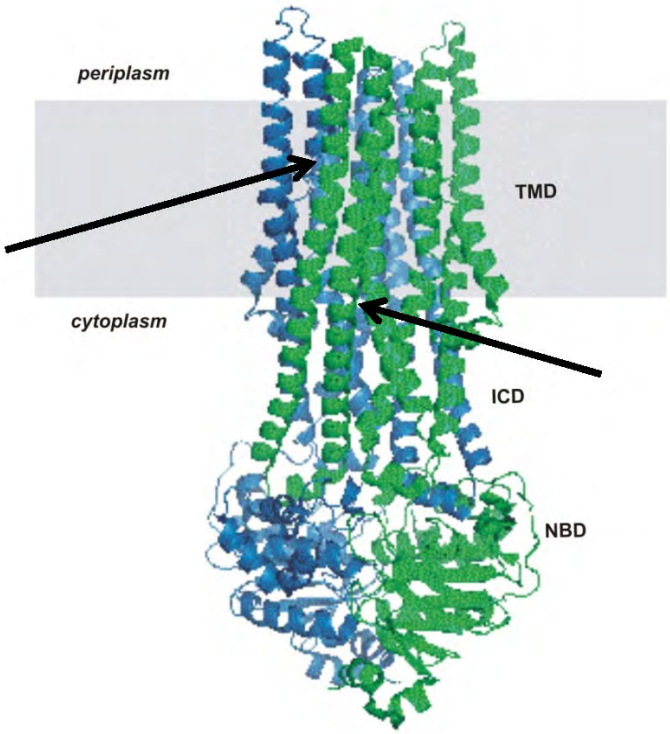

Figure 2 Structure of the ABC exporter Sav1866 from *Staphylococcus aureus* with bound nucleotide (Dawson and Locher 2006). Source: http://en.wikipedia.org/wiki/ATP-binding_cassette_transporter. Most exporters in prokaryotes, such as the multidrug exporter Sav1866, are made up of a homodimer consisting of two half-size transporters. The protein structure of the LR34 protein is unknown. However, as a full-size transporter (predicted to be active in a monomeric form), the LR34 protein certainly has a different conformation compared to the protein shown here. Nevertheless, the overall protein domain structure is expected to be similar, allowing us to derive hypotheses by protein modeling based on the protein structures of Sav1866 and related ABC transporters for which the molecular structures are available. Approximate positions of the two specific amino acid residues related to LR34

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function are indicated by arrows (deletion of phenylalanine, left arrow). TMD: transmembrane domain; ICD: intracellular domain; NBD: nucleotide-binding domain

Using transgenic approaches we are currently determining whether both polymorphisms are necessary for the resistance activity or whether one of the two changes is sufficient for the observed gain-of-function in the LR34 protein. Dakouri et al. (2010) studied the genetic diversity of the *Lr34* gene in a broad set of wheat germplasm. Among 700 wheat accessions, two lines were particularly of interest as they both had an allelic version of *Lr34* which only had the polymorphism resulting in the loss of a phenylalanine residue (characteristic for the *Lr34* gene), but not the second polymorphisms. Both these lines were described to have a susceptible phenotype after infection with leaf rust. Thus, these observations based on genetic diversity indicate that either the His to Tyr polymorphism is sufficient for *Lr34* resistance activity, or that both polymorphisms are required for resistance activity. However, the phenotyping of these two critical cultivars needs to be confirmed. In addition, it has to be established if the *Lr34* genes in these two lines are expressed and each encodes a full-length functional protein. Thus, it is important to determine if there are other polymorphisms in the *Lr34* genes of these lines, possibly resulting in an inactive gene as, for example, was found in cultivar Jagger (Lagudah et al. 2009; Cao et al. 2010) before any conclusions on protein structure – function can be made. Transgenic forms of the gene with the individual mutations will further contribute to clarification of this issue and will help to characterize the functional differences between the two allelic forms of *Lr34*. We conclude that the crucial change of function from a normal, susceptible allele of *Lr34sus* to the *Lr34* resistance gene is based on maximally two amino acid changes. These changes must result either in a change of molecular specificity of the transporter protein, a modification of the binding affinity for the substrate, or a change in the binding activity of LR34 to an unknown interacting protein, resulting in the observed effects in resistance as well as LTN (Spielmeyer et al. 2008; Krattinger et al. 2009).

Lr34/Yr18 compared to other cloned leaf rust or stripe rust resistance (*R*) genes

Three of the major leaf rust resistance genes described in the wheat gene pool have been cloned: *Lr1* (Cloutier et al. 2007), *Lr10* (Feuillet et al. 2003) and *Lr21* (Huang et al. 2003). They all encode coiled-coil, nucleotide-binding site, leucine-rich repeat (CC-NBS-LRR) proteins, a well-known class of plant resistance proteins. Their molecular modes of action resulting in leaf rust resistance are not yet known. However, based on knowledge mostly gained from molecular studies in Arabidopsis and flax resistance proteins, it is likely that they directly or indirectly recognize effectors of the fungal pathogen. As *Lr34* encodes a protein of a completely different class to the other cloned *Lr* genes, it is likely that it also has a completely different function. The leaf rust pathogen can overcome the classical *Lr* genes (such as *Lr1*, *Lr10* and *Lr21*), the different mechanism of action of *Lr34* might account for its durable resistance. No major gene against stripe rust (*Yr* genes) has yet been isolated. The cloned *Yr36* is a temperature-dependent resistance gene with no race specificity detected to date (Fu et al. 2009). *Yr36* encodes a protein with an N-terminal kinase domain a C-terminal lipid transfer (START) domain. Thus, the protein structure and possibly molecular mode of action is very different from *Lr34*.

The physiology of *Lr34* action

As described above, we assume that the LR34 protein transports a molecule which results in a defense response against multiple pathogens, as well as in leaf tip necrosis. The expression of the *Lr34* gene is regulated during development and is higher at later stages of plant growth which is in agreement with the adult plant resistance conferred by *Lr34* (Krattinger et al. 2009; Risk et al. 2012). Gene regulation under developmental control was also observed in wheat lines with a transgenic *Lr34* gene under control of its native promoter (Risk et al. 2012). The work on transgenic wheat lines was done in the genetic background of the easily transformable cultivar Bobwhite. Transformation of the genomic *Lr34* fragment (containing native promoter and terminator sequences) has confirmed that this sequence is sufficient to confer leaf rust resistance as well as LTN. Interestingly, it was observed that the transgenic *Lr34* resistance gene can result in a broader developmental range of resistance activity. In one particular genetic background, resistance was already observed at the seedling stage (Risk et al. 2012). It is not clear if this enhanced resistance is based on a slightly altered expression level and/or developmental stage of gene expression, or if it is simply an additive effect of *Lr34* with other genes present in the particular genetic background of the transformed line. In any case, this is a clear demonstration

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that the transgenic use of *Lr34* might be of practical breeding interest at least in some genetic backgrounds. It will be interesting to define in more detail the genetic components that are responsible for the additive effect of transgenic *Lr34*.

Biochemical and cytological approaches were used to study the transgenic *Lr34*-based resistance. These observations were compared with the endogenous resistance based on *Lr34*. In both the near-isogenic Thatcher line with *Lr34* as well as the transgenic lines with *Lr34*, the presence of *Lr34* did not increase pathogenesis-related (*PR*) gene induction in flag leaves to a significantly higher level (although there was a trend in that direction) after pathogen infection (Risk et al. 2012). Furthermore, callose induction which is associated with infection sites in rust-infected flag leaves was studied. However, even in the transgenic lines with increased resistance, there was no apparent alteration of callose deposition in flag leaves seven days after infection. It was concluded that all the lines had an equivalent capacity to deposit callose. Finally, no increased production of reactive oxygen species was found in *Lr34* transgenic lines. These findings are in accordance with work done by Rubiales and Niks (1995) who concluded that *Lr34* based resistance is not conferred by a hypersensitive response. All these data provide evidence that resistance in *Lr34* lines is not based on hypersensitive-response like defense mechanisms which are characteristic of the reactions of major resistance genes. Rather, a completely different type of resistance must underlie *Lr34*-based resistance.

Several intriguing observations were made concerning the molecular basis of LTN, either in lines with the endogenous *Lr34* gene and/or in lines transgenic for *Lr34*. First, it was found that the senescence-associated gene *HvS40* was induced in flag leaves of *Lr34*-containing lines when compared to lines without the gene. Further evidence for senescence-like processes involved in leaf tip necrosis came from the observation of non-fluorescent chlorophyll catabolites (NCCs) specifically in lines with *Lr34* (Krattinger et al. 2009; Risk et al. 2012). It is known that the production of NCCs is a highly controlled process in senescence, and it seems that *Lr34* can induce such processes prematurely. Importantly, this would not interfere negatively with the normal growth process relevant for agronomic parameters such as time to maturity and yield, at least in relevant agricultural environments.

Two large-scale transcriptomic studies analysed the global gene expression patterns in lines with *Lr34* compared with lines without *Lr34*, both when non-infected and after leaf rust infection (Hulbert et al. 2007; Bolton et al. 2008). Microarrays, based on 55,052 transcripts, were used for these studies. In mock-inoculated leaf tips of flag leaves with *Lr34* a total of 57 transcripts were consistently up-regulated in two different cultivars containing *Lr34*. The genes with higher expression levels in the *Lr34* genotypes are known to be ABA-inducible and related to responses to osmotic stress, cold stress and/or seed maturation. These genes are typical for plant reactions to abiotic stress. No *PR* gene induction was found in mock-inoculated leaves. After inoculation, *PR* genes were induced in both resistant and susceptible flag leaves, but expression was generally higher in resistant plants (Hulbert et al. 2007). Based on these data, the authors suggested that *Lr34* might in some way "prime" the plant for a stronger induction of defense responses. In the second study (Bolton et al. 2008), the authors identified a coordinated upregulation of key genes in several metabolic pathways. They speculated that there is an increased carbon flux particularly through the tricarboxylic cycle, resulting in a high energetic demand for the *Lr34*-based resistance in the plant.

Lr34 resistance probably does not depend on recognition of fungal effectors

One of the characteristics of *Lr34* is the improved resistance against several pathogens (leaf rust, stripe rust, powdery mildew, BYDV, and at least in certain genetic backgrounds, stem rust). *Lr34* can therefore be described as a multi-pathogen resistance gene. There are several other, cloned plant genes that have been shown to confer resistance against several diseases/pests. One of them is the *Mi* gene from tomato, which confers resistance to root-knot nematodes (Milligan et al. 1998), whitefly (Nombela et al. 2003) and aphids (Rossi et al. 1998). *Mi* encodes an NBS-LRR protein and likely guards a plant protein target that is modified by effectors of all three different organisms, similar to other proteins which confer resistance to several different pathogen species. Given the protein structure of LR34 it is unlikely to be a protein guarding a common effector target. Rather, we can assume that the molecular mechanism of resistance is completely different from other multi-pathogen resistance genes such as *Mi*.

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Evolutionary aspects and origin of *Lr34* in the grass gene pool

Evolutionary studies on the origin and divergence of genes can assist the formulation of hypotheses on gene function in different species. The *Lr34* gene seems to be specific to the grass family, as it has not been identified in a clearly orthologous form in other plant or fungal species. *Lr34* orthologs were identified in rice and sorghum (Krattinger et al. 2011). The rice ortholog has an identity of more than 80% at the amino acid level compared to LR34. Interestingly, barley is a species closely related to wheat but lacks an *Lr34* ortholog, as do maize and Brachypodium (Krattinger et al. 2011). It seems likely that all these species have independently lost the *Lr34* ortholog, whereas wheat, rice and sorghum have retained the gene. This indicates that *Lr34* can give a selective advantage under certain conditions, but that the loss of the gene is not detrimental and species can further evolve without it. A closer inspection of the rice and sorghum *Lr34* orthologs revealed that the two critical polymorphic amino acid residues for a functional resistance protein were identical to the susceptible isoform of LR34. In addition, the LR34 protein encoded by the B-genome homoeolog in wheat (see above) also carries the amino acids of the susceptible form of LR34. We conclude that the functional *Lr34* resistance gene only evolved on wheat chromosome 7D and that all the characterized orthologs in grass species (including those from the D-genome donor *Aegilops tauschii*) have the "susceptible" haplotype (no deletion of phenylalanine 546 and a tyrosine at position 634). This indicates that the critical mutations resulting in the gain-of-resistance-function in LR34 occurred after domestication and formation of hexaploid wheat (Krattinger et al. 2011).

Conclusions and next steps towards understanding *Lr34/Yr18* durable resistance

At this stage there are two essential questions on *Lr34*-based resistance which need to be answered: first, what is the molecular nature of the molecule transported by LR34 and how does the resistant form of the transporter differ in structural and biochemical properties from the susceptible form? Second, what are the molecular consequences of this transport/the transported molecule that result in durable, multipathogen resistance?

As indicated by LTN which is independent of pathogen infection, the *Lr34* gene also has an effect on plant physiology in the absence of the pathogen. Thus, it is possible that the senescence-like processes, induced by *Lr34*, simply make the tissue less conducive for a biotrophic pathogen. This might be caused by a limited availability of nutrients and/or by an accumulation of growth-inhibiting substances. Both effects would result in a quantitative limitation of fungal growth, explaining the partial and limited resistance effect in wheat. These metabolic changes would not result (at least in some relevant environments) in a yield loss or any other drastic negative consequences for growth of the wheat plant. Such a metabolic change would be deeply rooted in the physiology of the plant and could not be overcome easily by the pathogen, possibly explaining the durability of the *Lr34* resistance. Such resistance does not fit into the current models of effector and PAMP-triggered immunity and would thus represent a new type of molecular mechanism.

In an alternative model, based on transcriptomics studies, it can be envisaged that the activity of *Lr34* results in some form of priming of the plant revealed by a quantitatively higher, but not qualitatively different, induction of defense genes after infection in wheat genotypes containing the *Lr34* allele. This priming might include components from systemic acquired resistance or more likely from induced systemic resistance (ISR, Hulbert et al. 2007). It is known that ISR can be stimulated in Arabidopsis by priming, e.g. by the application of β-amino-butyric acid (Ton et al. 2005), demonstrating that a relatively simple chemical compound can result in such a primed state. However, for grasses in general or wheat specifically, no such mechanisms or phenomena have yet been described. Chemically induced resistance is known in wheat, but the genes induced there were not found to be induced in *Lr34*-containing genotypes (Hulbert et al. 2007). There are some testable consequences from the priming hypothesis, and this will represent a major research area for future experiments to understand *Lr34* gene function.

Based on the two mutations in *Lr34* resulting in an active resistance gene we propose that the evolution of the specific resistant haplotype was a unique event in evolution. This raises the question of whether such an event could be experimentally designed to enable additional durable resistance sources. However, based on the discussion above our current understanding of the underlying molecular mechanisms is still too meager to

rationally design new forms of resistance similar to *Lr34*. Thus, it is highly important and urgent to improve our knowledge on the molecular mechanisms of quantitative resistance. There are at least three areas of research which should be pursued with high priority and which will be highly relevant to reach this goal: first, there is a need to isolate additional durable resistance genes/QTL against rust diseases from wheat lines known to carry such resistances. Second, at CSIRO and UZH we are using heterologous systems transgenic for *Lr34* to study the effects of this gene in other species and to identify biological activities of *Lr34* in those genetic backgrounds. Third, it will be essential to genetically identify components of *Lr34*-based resistance to understand the molecular pathways resulting in resistance. All these approaches should finally allow us to make a big step forward in understanding quantitative, durable resistance.

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