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Convergent evidence for a role of WIR1 proteins during the interaction of barley with the powdery mildew fungus *Blumeria graminis*

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ABSTRACT

Pathogen attack triggers a multifaceted defence response in plants that includes the accumulation of pathogenesis-related proteins and their corresponding transcripts. One of these transcripts encodes for WIR1, a small glycine- and proline-rich protein of unknown function that appears to be specific to grass species. Here we describe members of the *HvWIR1* multigene family of barley with respect to phylogenetic relationship, transcript regulation, co-localization with quantitative trait loci for resistance to the barley powdery mildew fungus *Blumeria graminis* (DC.) E.O. Speer f.sp. *hordei*, the association of single nucleotide polymorphisms or gene haplotypes with resistance, as well as phenotypic effects of gene silencing by RNAi. HvWIR1 is encoded by a multigene family of moderate complexity that splits up into two major clades, one of those being also represented by previously described cDNA sequences from wheat. All analysed *WIR1* transcripts accumulated in response to powdery mildew attack in leaves and all mapped *WIR1* genes were associated with quantitative trait loci for resistance to *B. graminis*. Moreover, single nucleotide polymorphisms or haplotypes of *WIR1* members were associated with quantitative resistance of barley to *B. graminis,* and transient *WIR1* gene silencing affected the interaction of epidermal cells with the pathogen. The presented data provide convergent evidence for a role of the *HvWIR1a* gene and possibly other family members, during the interaction of barley with *B. graminis*.

Introduction

Upon attack by fungal pathogens plants mount a multifaceted defence response that may result in early abortion of appressorial penetration by local cell-wall reinforcement or in fungal arrest due to hypersensitive cell death of initially invaded cells (Collinge, 2009; Huckelhoven, 2007; Lipka et al., 2005). These defence responses are triggered by the perception of evolutionary conserved pathogen-associated molecular patterns (PAMPs) via plant receptor kinases or by the perception, via nucleotide-binding/leucine-rich-repeat (NB-LRR) proteins, of fungal effectors or effector–host-factor interactions (Jones and Dangl, 2006; van der Hoorn and Kamoun, 2008). PAMP perception triggers an evolutionary conserved defence-response known as PAMP-triggered immunity (PTI) whereas recognition of effectors by NB-LRR, which are encoded by resistance (R) genes, is assumed to be evolutionary more recent and is referred to as effector-triggered immunity (ETI). ETI represents a co-evolutionary arms race between specific plant and pathogen genotypes that is characterized by only short durability of R-gene efficacy due to rapid effector modifications by pathogen races, which is counterbalanced by the creation and accession of new R specificities. Impressive examples of plant-pathogen co-evolution with respect to ETI have been described in flax and barley interacting with the flax-rust and barley powdery-mildew fungus, respectively (Dodds et al., 2006; Yahiaoui et al., 2009). PTI on the other hand acts in a pathogen race-nonspecific manner and is assumed to be more durable than ETI. In recent years it has become evident that PTI although partially suppressed by fungal effectors is an important trait that can be tuned towards higher efficacy in modern crop cultivars (Miedaner and Flath, 2007). However, one of the major obstacles of efficiently using PTI in plant breeding is its complex genetic architecture based on several or many quantitative trait loci (QTL) contributing each only a minor proportion of the observed phenotypic variation.

Abbreviations: Bgh, *Blumeria graminis* f.sp. *hordei*; CC-NBS-LRR, coiled-coil, nucleotide-binding-site, leucine-rich-repeat; ETI, effector-triggered immunity; E/L, epidermis to leaf transcript signal ratio; HI, haustorial index; h.a.i., hours after inoculation; LD, linkage disequilibrium; PAMP, pathogen-associated molecular pattern; PTI, PAMP-triggered immunity; QTL, quantitative trait locus; SNP, single nucleotide polymorphism; SI, susceptibility index; TIGS, transient-induced gene silencing.

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Barley interacting with the powdery mildew fungus *Blumeria graminis* (DC.) E.O. Speer f.sp. *hordei* (*Bgh*) is one of the well-studied examples of PTI as well as ETI, with several key loci and genes that mediate either response being known or cloned (Azevedo et al., 2002; Buschges et al., 1997; Shen et al., 2007; Shirasu et al., 1999; Zhou et al., 2001). The knowledge of the barley-powdery mildew interaction ranges from well-defined cytological interaction phenotypes including re-orientation of the cytoskeleton, membrane modifications, cell-wall appositions over a large number of race-specific major powdery mildew resistance (R) genes to the localization of a considerable number of QTL for race-nonspecific resistance to *Bgh* (Collinge et al., 2008; Thordal-Christensen et al., 2000). The best studied major R gene is *Mla* (for *Mildew resistance locus a*) encoding a coiled-coil, nucleotide-binding-site, leucine-rich-repeat (CC-NBS-LRR) protein, of which over 30 alleles have been described until present (Wei et al., 2002). A number of components of the *Mla*-mediated resistance pathway have been molecularly described including Mla-interacting proteins Rar1, SGT1, HSP90, WRKY2 as well as the recognized *Bgh* effector protein Avra10 (Azevedo et al., 2002; Eckey et al., 2004; Ridout et al., 2006; Shen et al., 2007; Shirasu et al., 1999; Zhou et al., 2001). Recessive loss-of-function alleles of *Mlo* (for *Mildew resistance locus o*), a putative co-opted susceptibility factor that might be normally involved in cell-death control during plant development and abiotic stress, appears to trigger strong PTI (Buschges et al., 1997; Piffanelli et al., 2002). Interestingly, the function of *Mlo* in powdery mildew susceptibility has recently been found to be conserved among cereals and several dicotyledonous species (Bai et al., 2008; Consonni et al., 2006; Feechan et al., 2008). The *mlo5* resistance allele of barley has been described to mediate more pronounced transcriptional responses and defence reactions to *Bgh* that also resemble nonhost-resistance responses to non-adapted powdery mildew fungi such as the wheat powdery mildew *B. graminis* (DC.) E.O. Speer f.sp. *tritici* (*Bgt*) or to a non-adapted blast fungus *Magnaporte grisea* isolate (Zierold et al., 2005; Zellerhoff and Himmelbach et al., 2010).

Assuming that QTL-mediated, *mlo*-mediated as well as nonhost resistance are likely to represent different flavours of PTI in barley renders a better understanding of the underlying mechanisms a research goal of high priority (Wise et al., 2009). However, the genetic setup of PTI is complex and may comprise many defence-related genes underlying QTL for seedling- or adult-plant resistance. The recent availability of transient gene expression and silencing assays in barley has allowed to test first candidate gene (families) such as peroxidases, germin-like proteins, RACB proteins, Bax-inhibitor, WRKY transcription factors, ubiquitin and Bluefensin for their role in powdery mildew interactions (Dong et al., 2006; Huckelhoven et al., 2003; Johrde and Schweizer, 2008; Meng et al., 2009; Schultheiss et al., 2003; Shen et al., 2007; Zimmermann et al., 2006).

A transcript that strongly accumulates in pathogen-attacked cereals encodes a small, basic, glycine- and proline-rich protein of unknown function designated as WIR1 (for Wheat Induced Resistance 1) (Bull et al., 1992; Schweizer et al., 1989). WIR1 was first described in wheat and, despite its discovery over 20 years ago, very little is known about its relevance for PTI or ETI and its molecular function. WIR1 transcripts have been found not only in wheat but also in rice and barley (Mauch et al., 1998; Yuan et al., 2004; Zierold et al., 2005). All sequences share an N-terminal secretion signal, however, with different cleavage probability suggesting that some WIR1 proteins may be membrane anchored. The positive charge of the basic WIR1 proteins in the acidic apoplast, together with the high abundance of glycine- and proline residues that might provide cell-wall contact, led to the speculation that WIR1 might be involved in linking the plasmalemma to the cell wall (Bull et al., 1992). Until present only RIR1b, which is one of the WIR1-like rice proteins, has been experimentally demonstrated to be secreted (Mauch et al., 1998). Recently, *HvWIR1* mRNA was identified in barley phloem sap and the authors of this study speculated about a role of the transcript in systemic defence responses, similar to previously described phloem-located transcripts that led to observable phenotypic alterations upon grafting (Gaupels et al., 2008). First direct evidence for a role in WIR1 proteins in plant–pathogen interactions comes from transient and stable overexpression in wheat and rice, respectively, that significantly altered the interaction phenotypes (Schaffrath et al., 2000; Schweizer et al., 1999).

Here, we provide convergent evidence for a role of members of the *HvWIR1* multigene family of barley in QTL-mediated (basal) resistance to *Bgh*. For this purpose, transcript profiling, genetic mapping, association genetics and transient gene silencing experiments have been performed in susceptible or quantitatively resistant barley genotypes.

Materials and methods

Plants and fungi

Barley plants cv. “Golden Promise” (used for TIGS experiments) or cv “Ingrid” (used for transcript profiling) were grown in pots of 14 cm diameter containing compost soil from IPK nursery without fertilization in a growth chamber at 20 °C with 60–70% relative humidity and a 16 h photoperiod (216 μmol m^{-2} s^{-1}). Barley plants used for association mapping were grown in 7 × 11 multipot trays containing compost soil from IPK nursery without fertilization in a greenhouse at 17–20 °C with supplemental light from sodium halogen lamps to reach a photoperiod of 16 h. *Blumeria graminis* (DC.) E.O. Speer f.sp. *hordei*, strain CH4.8, was used for TIGS and transcript-profiling experiments and cultivated by weekly inoculation of 7-day-old seedlings of barley cv “Golden Promise”. Polyvirulent German *Bgh* isolates 78P and D12-12 were used for association genetic analysis and cultivated as described for CH4.8. Together, they carry virulence against resistance genes He, Ml(La), Ml(AB), Ml(Ab), Ml(Ba), Ml(BW1, Ml(CP), Ml(Dr), Ml(Du2), Ml(Hu4), Ml(Im9), Ml(Kr), Ml(LG2), Ml(Ru2), Ml(Ru3), Ml(St1), Ml(Tu2), Mla01, Mla03, Mla06, Mla07, Mla09, Mla10, Mla12, Mla13, Mla14, Mla17, Mla22, Mla27, Mla28, Mlat, Mlg, MlH, Mlj, Mlk, MlLa, Mlnn, Mlp, Mlra, and U. *B. graminis* DC Speer f.sp. *tritici* Em Marchal (*Bgt*) (swiss field isolate FAL 92315) was also used for transcript profiling and maintained at 20 °C and 16 h light by weekly transfer to fresh wheat cv. Kanzler.

For TIGS and transcript-profiling experiments, 7-day-old seedlings were inoculated with *Bgh* or *Bgt* as described (Douchkov et al., 2005, Zellerhoff and Himmelbach et al., 2010). For association-genetic experiments, second leaves of 2-week-old seedlings were inoculated with *Bgh* 78P or D12-12 in a detached leaf assay, and powdery mildew symptoms were rated 7 days after inoculation as described (Altpeter et al., 2005).

Sequence analysis

HvWIR1 cDNA sequences were obtained by a keyword search in HarvEST database, barley assembly Nr. 35 (http://www.harvest-web.org/). The following unigene numbers correspond to individual *HvWIR1* transcripts: 2397 (*HvWIR1a*), 2396 (*HvWIR1b*), 11993 (*HvWIR1c*), 20185 (*HvWIR1d*), 17715 (*HvWIR1e*), 17716 (*HvWIR1g*), 603 (*HvWIR1h*), 606 (*HvWIR1i*), 609 (*HvWIR1j*), 150 (*HvWIR1k*), 487 (*HvWIR1l*), and 5984 (*HvWIR1m*). Transcript *HvWIR1f* is represented by cDNA clone HO09M20. Sequence comparisons included the two prototype *WIR1* sequences from wheat *TaWIR1a* (Acc. M94959) and *TaWIR1b* (Acc. X87686).