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Barley putative hypersensitive induced reaction genes: genetic mapping, sequence analyses and differential expression in disease lesion mimic mutants

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Abstract The hypersensitive response (HR) is one of the most-efficient forms of plant defense against biotrophic pathogens, and results in localized cell death and the formation of necrotic lesions; however, the molecular components of pathways leading to HR remain largely unknown. Barley (*Hordeum vulgare* ssp. *vulgare* L.) cDNAs for putative hypersensitive-induced reaction (*HIR*) genes were isolated based on DNA and amino-acid homologies to maize *HIR* genes. Analyses of the cDNA and genomic sequences and genetic mapping found four distinct barley *HIR* genes, *Hv-hir1, Hv-hir2, Hv-hir3* and *Hv-hir4*, on chromosomes 4(4H) bin10, 7(5H) bin04, 7(5H) bin07 and 1(7H) bin03, respectively. *Hv-hir1, Hv-hir2* and *Hv-hir3* genes were highly homologous at both DNA and the deduced amino-acid level, but the *Hv-hir4* gene was similar to the other genes only at the amino-acid sequence level. Amino-acid sequence analyses of the barley HIR proteins indicated the presence of the SPFH protein-domain characteristic for the prohibitins and stomatins which are involved in control of the cell cycle and ion channels, as well as in other membrane-associated proteins from bacteria, plants and animals. *HIR* genes were expressed in all organs and developement stages analyzed, indicating a vital and non-redundant function. Barley fast-neutron mutants exhibiting spontaneous HR (disease lesion mimic mutants) showed up to a 35-fold increase in *Hv-hir3* expression, implicating *HIR* genes in the induction of HR.

Keywords Barley · Lesion-mimic mutants · Hypersensitive reaction · *HIR* genes · SPFH domain

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Introduction

Plant hypersensitive reaction (HR) is a defense response to pathogen infection involving rapid, localized cell death and the induction of many pathogenesis-related (PR) proteins, although not all plant–pathogen interactions evoke HR (Heath 2000; Lam et al. 2001). HR appears to share similar features with developmentally programmed cell death (PCD) (Heath 1998). Elicitors of HR cause intracellular influx of calcium, membrane dysfunction and increase in the generation of reactive oxygen species and salicylic acid (Heath 2000). HR results from signalling cascades started by the interaction of a pathogen-provided ligand with intra- or extra-cellular receptors encoded by plant disease resistance genes (Dangl and Jones 2001). Although there is a significant variety of resistance genes and pathogen ligands, the number of signal transduction pathways appears to be relatively small. Such pathways usually involving protein kinase cascades, e.g., MAP kinase and the WRKY transcription factor signalling-cascade in *Arabidopsis*, are in response to treatment with both bacterial and fungal pathogens (Asai et al. 2002).

Disease lesion mimic (necrotic) mutants show HR even in the absence of a pathogen and are important for understanding the disease resistance pathways and the HR in plants (Johal et al. 1995). Characterized lesion mutants include *Les22* from maize encoding uroporphyrinogen decarboxylase (Hu et al. 1998) and rice spotted-leaf gene *Spl7* encoding a heat stress transcription factor (Ya-

manouchi et al. 2002). Recently, a novel disease lesion mimic mutant *hlm1* was found in *Arabidopsis* which encodes the cyclic nucleotide-gated ion channel CNGC4 permeable to both sodium and potassium ions (Balague et al. 2003). The *hlm1* gene was also induced in response to pathogen infection. Ion fluxes, particularly calcium influx, are one of the earliest signalling events detected during the HR (Atkinson et al. 1990). Two other *Arabidopsis* lesion mimic mutants *dnd1* and *cpn1* encode a cyclic nucleotide-gated ion channel CNGC2 which allows passage of calcium and potassium ions (Clough et al. 2000) and a copine, calcium-dependent, phospholipid binding protein (Jambunathan et al. 2001).

Karrer et al. (1998) cloned tobacco cDNAs from the leaves challenged with tobacco mosaic virus and undergoing HR which were able to elicit formation of HR-like lesions in a transient expression assay. Some of the cDNAs encoded proteins linked to HR, such as ubiquitin and the Kunitz-type trypsin inhibitor, while nine others had no homology to known proteins. One of the cDNAs, NG1, was able to induce lesions and expression of a HR-specific PR2 protein, acidic β-glucanase (Karrer et al. 1998).

Recently, three maize hypersensitive-induced response (*HIR*) genes, *Zm-hir1, Zm-hir2* and *Zm-hir3*, were isolated based on the amino-acid homology to the tobacco cDNA NG1 (Nadimpalli et al. 2000). Studies of the *Zm-hir3* gene transcription indicated elevated expression in the maize partly dominant lesion mimic mutant *Les9* and reduced expression, when the mutant gene was crossed into the Mo20W background which suppressed the *Les9* lesion mimic phenotype. These data suggested participation of the maize *HIR* genes in HR.

Nadimpalli et al. (2000) used PHI-BLAST searches in combination with multiple sequence alignments and neighbor-joining tree construction to show that maize HIR proteins, along with prohibitins and stomatins, form a protein superfamily. Both prohibitins and stomatins have a protein domain characteristic for membrane proteins, SPFH (Stomatins, Prohibitins, Flotillins, HflK/C; Tavernarakis et al. 1999). The SPFH domain is also found in murine caveolae-associated flotillins (Bickel et al. 1997) and in bacterial plasma-membrane proteins HflK and HflC (Noble et al. 1993). Prohibitins are involved in cell-cycle control, replicative senescence and tumor suppression, and are localized in mitochondrial inner membranes (McClung et al. 1995). Stomatins are integral membrane proteins of the red blood cells thought to be involved in the regulation of univalent cation permeability (Stewart 1997). Structural similarity of HIR proteins to prohibitins and stomatins suggests that they may be involved in regulation of ion channels (Nadimpalli et al. 2000).

Characterization of *HIR* genes from different sources should facilitate clarification of the function of these genes and their involvement in the plant hypersensitive response. We report the isolation of four *HIR* genes from barley *Hordeum vulgare* ssp. *vulgare* cultivar (cv) Morex, based on homology to the reported maize *HIR* genes (Nadimpalli et al. 2000) and associations of the barley *HIR* genes with the disease lesion phenotype in mutant barley plants.

Materials and methods

Identification and clustering of the cDNAs of barley putative *HIR* genes

The mRNA sequences of the maize *HIR* genes, *Zm-hir1* (AF236373), *Zm-hir2* (AF2363743) and *Zm-hir3* (AF236375) (Nadimpalli et al. 2000), were used in BLASTn and tBLASTx searches to screen barley EST database at http://www.ncbi.nlm.nih.gov/BLAST/. EST sequences with a BLAST similarity score higher than 100 were identified, aligned using the Clustal_X 1.81 multiple sequence alignment program (Thompson et al. 1997) and divided by manual inspection into four groups: *Hv-hir1, Hv-hir2, Hv-hir3* and *Hv-hir4*. This division was supported by EST clustering in the HarvEST database (Close and Wanamaker; (http://harvest.ucr.edu/). The most-complete cDNA clone for each group was isolated from the Clemson University Genomics Institute (CUGI) EST libraries (http://www.genome.clemson.edu/projects/barley/) and sequenced.

Genetic mapping populations

Steptoe × Morex and Harrington × Morex "minimapper" populations consisted of 35 selected doubled-haploid lines (DHL) allowing placement of markers to the barely Bin map (Kleinhofs and Graner 2001; Kleinhofs, unpublished). The Oregon Wolf Barley Dominant × Recessive population was also used (Costa et al. 2001).

Southern-blot analyses

Plant genomic DNA was extracted as previously described (Kleinhofs et al. 1993). BAC DNA was extracted by the standard alkaline-lysis procedure (Birnboim and Doly 1979). DNA probes were labeled with [α-^{32}P] dCTP (New England Nuclear) using the ALL-IN-ONE random prime kit (SIGMA, St. Louis, Mo., USA) and hybridized either to barley genomic DNA blots or to the arrayed barley Morex BAC library (Yu et al. 2000). DNA was digested with restriction enzymes following the manufacturer's recommendations and transferred to nylon membranes (New England Nuclear) by the alkaline-transfer procedure. Hybridizations were at 65 °C and a final wash at 65 °C with 0.5× SSC, 1% SDS.

Plant material and RNA extraction

RNA for 5′ RACE analysis was extracted from 3-week old Morex plants grown in the greenhouse. RNA for *HIR* gene expression in different plant organs was obtained from Morex plants grown in a greenhouse, except for etiolated seedlings that were grown in the growth chamber in the dark, and seedling roots were obtained from seed germinated on filter paper in Petri dishes. Plant organs were harvested at times indicated in Fig. 4. All tissue was harvested in liquid nitrogen and stored at –80 °C until processed. Total RNA for 5′ RACE and *HIR* gene-expression analysis was isolated using the Totally RNA kit from Ambion (Austin, TX, USA). A LiCI precipitation step or DNaseI treatment was included to facilitate removal of polysaccharides and contaminating genomic DNA.

Barley fast-neutron (FN) mutant lines were produced from Steptoe and Morex seed irradiated at 3.5 and 4.0 Gy at the FAO/IAEA Seibersdorf SNIF facility. M2 seed was planted in the field and lesion mimic mutants were selected by visual observation. Seed from the mutant lines was collected and planted in the greenhouse

Table 1 Primer sequences for the 5′ RACE and quantitative real-time PCR

Gene	Primer name	Sequence	Fragment size
5′ RACE primers			
Hv-hir1	HIR1_outer	5′-CCCTTGCTATTTCATTCTTC-3′	N/A
	HIR1_inner	5′-TGCTCCCTGGTGTTACTAAG-3′	
Hv-hir3	HIR3_outer	5′-CTTTGCCAGCCAGAGGTCGGTACT-3′	N/A
	HIR3_inner	5′-TTTTATCGATGGCCAACTACACGCTTCCCA-3′	
Quantitative real-time PCR primers			
Hv-hir1	Hir1_cw2	5′-TGAGCCAGATGAACATGTGAAGAG-3′	230 bp
	Hir1_ccw2	5′-AGTCCCAGGCACATTCTCAGAG-3′	
Hv-hir2	Hir2_cw1	5′-GGCAATGTCTATGTATGGGTATGAG-3′	201 bp
	Hir2_ccw1	5′-TCCCCACACCAGCCAAGTAC-3′	
Hv-hir3	Hir3_cw1	5′-TGGCTTTGAGATCGTGCAGAC-3′	230 bp
	Hir3_ccw1	5′-GTCCCTCAGGCCATCCACAA-3′	
Hv-hir4	Hir4_cw1	5′-TCCGCAGAGCAATGAACGAC-3′	202 bp
	Hir4_ccw1	5′-CCGAGTGCGAGAAGTTGAGG-3′	
Ubi 1	Ubi1_cw1	5′-GCCGCACCCTCGCCGACTAC-3′	219 bp
	Ubi1_ccw1	5′-CGGCGTTGGGGCACTCCTTC-3′	

to produce plant material for phenotype and RNA analysis. Leaves from lines FN044, FN211, FN227, FN303, FN338, FN364, FN367, FN370 and FN450, as well as parents, were harvested from 10-day old seedlings. Leaves from FN085, FN093, FN242, FN366 and Steptoe were harvested from 14-day old plants. Leaves from line FN451 and Steptoe were harvested from 24-day old plants. All tissue was harveted in liquid nitrogen and stored at –80 °C until processed. Total RNA for analysis of *HIR* gene expression in FN mutant lines was extraxted by the modified Trizol procedure (Invitrogen, Carlsbad, CA, USA) and treated with DNaseI from the Ambion DNA-free kit.

Rapid amplification of cDNA ends (5′ RACE)

Transcription start sites of the *Hv-hir1* and *Hv-hir3* genes were determined using the First Choice RLM-RACE kit from Ambion starting with approximately 10 μg of total RNA. Primer sequences are provided in Table 1. The primers were used in combination with RNA adapter-specific primers from the kit. PCR reactions were done in a PTC-100 thermocycler (MJ Research, Reno, NV, USA) with the following parameters: denaturing for 3 min, 30 to 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and final extension at 72 °C for 7 min. PCR products were either cloned in pGEM-T Easy vector (Promega, Madison, WI, USA) or sequenced directly.

Sequence analysis of the barley *HIR* genes

Pairwise BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) was used to determine the intron – exon structure of the barley *HIR* genes. Clustal_X 1.81 at default settings (Thompson et al. 1997) was used to produce initial barley EST alignment. The alignment for phylogenetic analysis was produced by Clustal_X 1.81 using the PAM series of the protein-weight matrix. Clustal_X 1.81 was also used to generate and bootstrap a neighbor-joining tree. The tree was visualized using TreeView 1.6.6. Rice HIR homologues P0700A11.21 or P0700A11.23 were used as an outgroup, because they appeared to be the most-distant HIR protein homologues. The presence of conserved domains in the predicted barley HIR amino-acid sequences was detected using the NCBI Conserved Domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

Quantitative real-time PCR and data analyses

Real-time PCR quantification of the *HIR* genes was performed as described by Horvath et al. (2003). *GAPDH* gene-primer sequences are described by Horvath et al. (2003). Primer sequences for *HIR* genes and the barley ubiquitin 1 (*Ubi1*) gene (GenBank accession M60175) are provided in Table 1. Real-time PCR data acquisition for the *Ubi1* gene was done at 81 °C. Quantification of the target and reference genes was achieved using standard curves obtained from serial 10-fold dilutions of the respective cDNA standards. Quantitative real-time PCR experiments were done in triplicate, and the *HIR* gene-transcript level was normalized to the average amount of *GAPDH* or *Ubi1* transcript by expressing it as a percentage. Normalized values of three replicate experiments were used to calculate the average amount of a *HIR* transcript and the standard deviation. The *HIR* gene-transcript in the FN mutant was expressed as a fold-difference to parent for each replicate of experiment and used to calculate an average and standard deviation.

Results

Identification of the barley *HIR* gene cDNAs

Three highly homologous groups of barley cDNAs were detected based on BLASTn homology to the maize *HIR* cDNAs (similarity score >100). Clones containing apparently complete coding sequences were found for the *Hv-hir1* and *Hv-hir3* genes (Table 2). A partial cDNA clone for the *Hv-hir2* gene was found in the cDNA libraries available to us. Other barely EST sequences covering the entire *Hv-hir2* coding sequence were available from the NCBI.

The three cDNA sequences were used in tBLASTx search against barley EST database and yielded another group of ESTs with significant amino-acid homology, but low DNA homology to the *Hv-hir1, Hv-hir2* and *Hv-hir3* genes (BLASTn S $\leq$ 75). We concluded that this fourth group represented a transcript of yet-another barley *HIR* gene, *Hv-hir4* (Table 2).

Table 2 Characteristics of cDNA, BAC and plasmid clones used for genetic mapping and sequencing of the barley *HIR* genes

Barely *HIR* gene	cDNA clone[a]	BAC clone	Plasmid subclone	Clone used for mapping	GenBank accession of genomic sequence
Hv-hir1	HV_CEb009G17 (AY137511)	305h04	NRG041 4.6 kb/*Eco*RI NRG044 2.9 kb/*Eco*RI	HV_CEb0009G17 (AY137511)	AY137518[b]
Hv-hir2	HVSMEi0003M10 (AY137512)	468a10	NRG045 3.4 kb/*Pst*I	NRG045	AY137516
Hv-hir3	HVSMEh0023E24 (AY137513)	150i13	NRG024 2.7 kb/*Hin*dIII	HVSMEh0023E24 (AY137513)	AY137517[c]
Hv-hir4	HVSMEc0013M02 (AY137514)	707d02	None[d]	HVSMEc0013M02 (AY137514)	AY137515[d]

[a] cDNA clone inserts were sequenced at Washington State University. EST sequence in GenBank, BE216098, BG365696 and BF616914, have been obtained from the clones HV_CEb009G17, HVSMEi0003M10 and HVSMEc0013M02, respectively
[b] NRG041 and NRG044 represent a contiguous stretch of genomic DNA cleaved by the *Eco*RI restriction enzyme as confirmed by direct sequencing of the BAC clone
[c] The 2.7-kb *Hin*dIII fragment covered only the 3′ part of the gene. The rest of the gene and its promoter region were sequenced directly from the BAC clone using the primer walk
[d] Primers were designed from the corresponding cDNA sequence and used to obtain genomic DNA sequence directly from the BAC clone. Primer walk was employed as necessary to complete the sequence

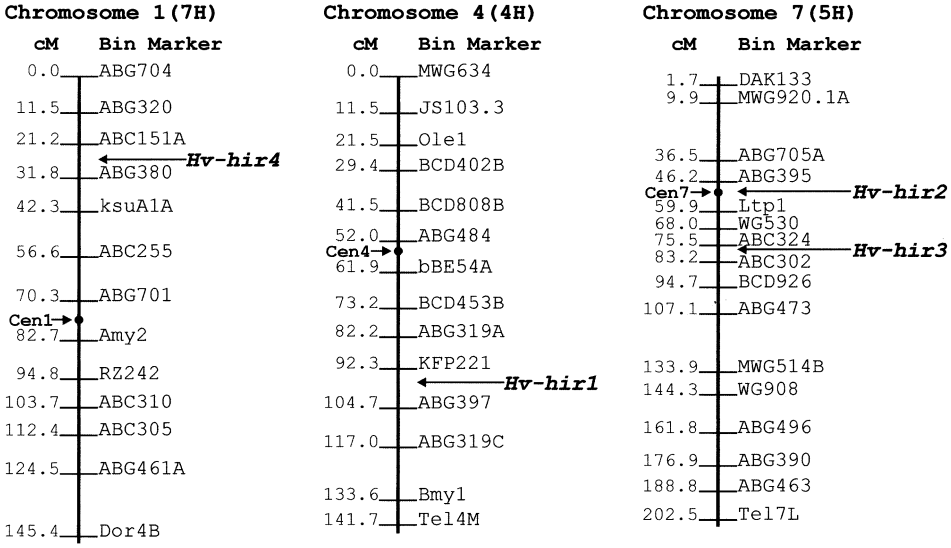

Fig. 1 Chromosome locations of the barley putative hypersensitive-induced reaction genes (*Hv-hir*) on the barley consensus bin map

Genetic mapping of the barley *HIR* genes

The probes for *Hv-hir1, Hv-hir2, Hv-hir3* and *Hv-hir4* genes (Table 2) mapped to four distinct loci on chromosomes 4(4H) bin10, 7(5H) bin04, 7(5H) bin07 and 1(7H) bin03, respectively (Fig. 1).

Isolation and sequencing of barley *HIR* genomic clones

Positive BAC clones were isolated from the Morex library and confirmed to contain all four *HIR* genes by Southern hybridization (Fig. 1 in electronic suplementary material). BAC clones hybridizing with barley *HIR* cDNA probes are listed in Table 2 in electronic supplementry material. Representative BAC clones for *Hv-hir1, Hv-hir2, Hv-hir3* and *Hv-hir4* genes were sequenced by subcloning or by direct sequencing of the appropritate BAC clones using the primer walk. cDNA probes used to screen BAC library, BAC clones chosen for subcloning, plasmid subclones used for sequencing and GenBank accession numbers of the sequences are given in Table 2.

Structure of the barley *HIR* genes

Alignment of available cDNA and EST sequences with the genomic DNA sequences was employed to predict the intron–exon structure of the barley *HIR* genes (Fig. 2). In addition, 5′ RACE was used to determine the 5′ end of the *Hv-hir1* and *Hv-hir3* genes. All four genes showed highly conserved coding region structure. Coding sequences were covered by five exons, except in the *Hv-hir3* gene where intron 4 was deleted merging exons 4 and 5. Sizes of the exons were highly conserved as well, even in the case of the more-distantly related *Hv-hir4* gene. Introns were not conserved in size or sequence.

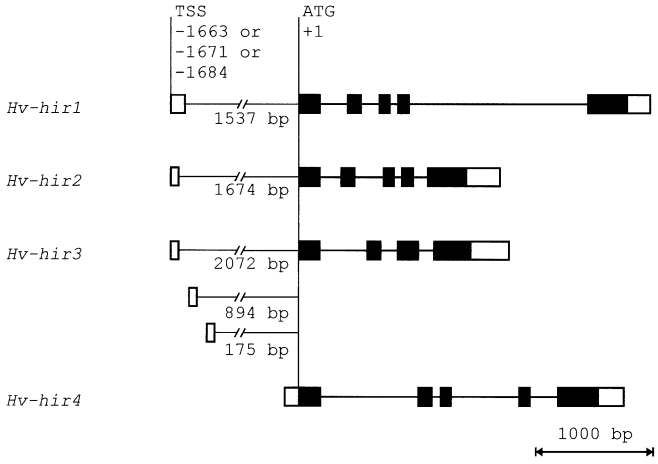

Fig. 2 Structure of the barley *HIR* genes. Coding parts of the exons are shown as *black boxes* and 5′ and 3′ non-coding regions are shown as *open boxes*. Introns are shown as *lines*. Genes are aligned with respect to the start of the coding sequence (ATG) and their structure is shown to scale. The transcription start site (*TSS*) was determined for the *Hv-hir1* and *Hv-hir3* genes, while the 5′ end of the other barley *HIR* genes was inferred from the available EST and cDNA sequences. In the case of the *Hv-hir3* gene, intron 4 is deleted

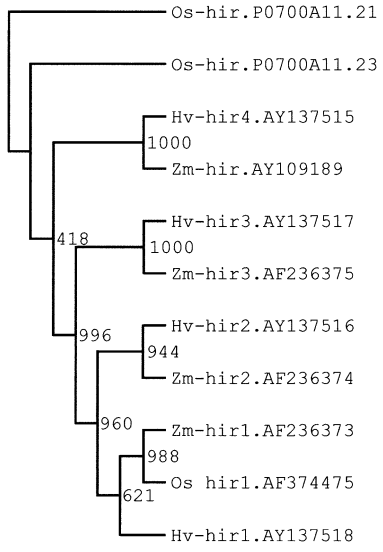

Fig. 3 Phylogenetic relationship of barley, maize and rice HIR proteins. Branches of the neighbor-joining phylogenetic tree are labeled with species name (*Hv – Hordeum vulgare*, *Os – Oryza sativa* and *Zm – Zea mays*) followed by the gene name and GenBank or the protein data-bank accession number. *Numbers* at the nodes of branches indicate bootstrap values

A common feature of the *Hv-hir1, Hv-hir2* and *Hv-hir3* genes was a 5′ non-coding exon separated from coding exons by a large intron. The 5′ non-coding sequence of the *Hv-hir4* gene was part of the first exon. The 5′ non-coding exon of the *Hv-hir3* gene showed significant variation in structure. Alignment with the barley cDNA AY137513 predicted the first exon more than 2-kb upstream of the first coding exon. Five other barley EST sequences predicted a different exon located 894-bp upstream of the first coding exon. Six independent sequences of the 5′ RACE products suggested a different exon located 175-bp upstream of the first coding exon with transcription start site at position -244 with respect to the ATG codon (Fig. 2). The beginning of the first coding exon, however, was identical in all three different cDNA types and these putative alternative mRNA forms did not affect the coding sequence. No similar variability in the first exon was observed for the *Hv-hir1* and *Hv-hir2* genes, although the *Hv-hir1* gene showed three different transcription start sites as evidenced by 5′ RACE products and ESTs (Fig. 2). No homology was detected among non-coding exons of the *Hv-hir1, Hv-hir2* and *Hv-hir3* genes, and there was no homology among the putative promoter regions.

Analysis of the deduced amino-acid sequences of the barley *HIR* genes

The neighbour-joining tree derived from the phylogenetic analysis of barley, maize and rice HIR predicted the amino-acid sequences shown in Fig 3. The protein-conserved domain search at the NCBI web page indicated that all four barely HIR sequences contain the signature SPFH domain (pfma01145) at the N-terminus of the proteins. All four amino-acid sequences were aligned with the SPFH domain sequence over its entire length, with similarity scores between 119 and 113 and E values between $1e^{-28}$ and $7e^{-27}$.

Expression of the *HIR* genes in barley organs

All four *HIR* genes were expressed in all organs and developmental stages analyzed (Fig. 4). Normalization to *GAPDH* and *Ubi1* resulted in somewhat different expression patterns indicating that detected differences may be caused by variation in reference gene-transcription depending on the tissue type.

Expression of the *HIR* genes in barley fast neutron (FN) lesion mimic-mutant lines

Fourteen of 31 field-selected FN mutuants displayed a disease lesion phenotype in the greenhouse (Fig. 2 in the electronic supplementary material). Most of the lines expressed the phenotype at the seedling stage (7–14 days old), except for the line FN451 that showed the phenotype when it was about 20-days old. Disease lesion-like spots initially appeared on the leaf tips, but later also on the other parts of the leaf. In general, older leaves exhibited more severe symptoms. Some lines, e.g., FN093 and FN366, initially showed symptoms more similar to senescence than to disease lesions, although later they developed necrotic spots. Lines FN044, FN093, FN211,

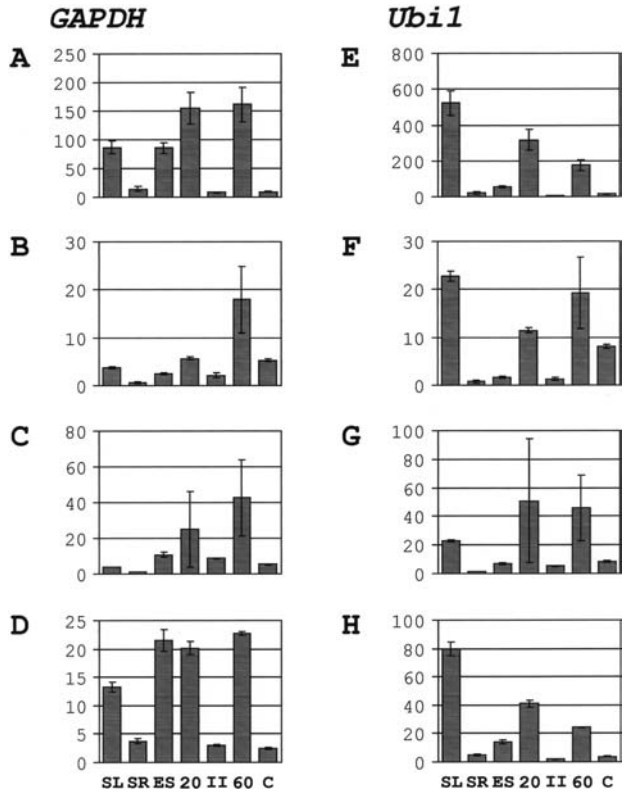

Fig. 4 Real-time PCR quantification of *HIR* gene transcripts in barley organs. The amount of transcript was normalized to *GAPDH* (*left column*) or *Ubi1* (*right column*) genes and was expressed as the percentage of the reference gene transcript. *Panels A and E – Hv-hir1* gene, *panels B and F – Hv-hir2* gene, *panels C and G – Hv-hir3* gene, *panels D and H – Hv-hir4* gene. *SL* – seven-day old seedling leaves, *SR* – three-day old seedling roots, *ES* – eight-day old etiolated seedlings, *20* – leaves from 20-day old plants, *II* – immature inflorescences from 40-day old plants, *60* – leaves from 60-day old plants, *C* – young caryopses from 61-day old plants. *Vertical bars* represent the standard deviation

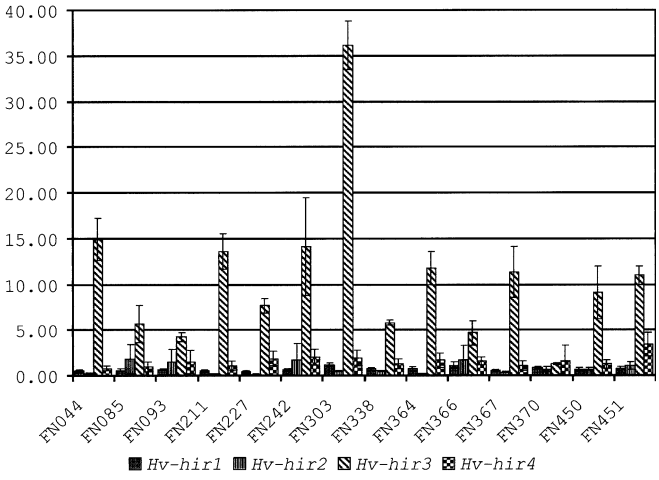

Fig. 5 Real-time PCR quantification of the *HIR* gene transcripts in FN mutant lines. The Y-axis indicates the fold-difference in the amount of transcript compared to the parent cultivar. *Vertical bars* represent the standard deviation

FN227, FN242, FN303, FN338, FN366, FN367 and FN370 displayed symptoms on all the leaves, while other lines, FN085, FN364, FN450 and FN451, had necrotic spots on several, but not all, leaves. *Bam*HI-digested genomic DNA from the mutant lines did not show detectable changes in the banding pattern, when hybridized with the *HIR* gene-probes and compared to the parent cultivar (data not shown) indicating that *HIR* genes were present and probably intact. Quantitative real-time PCR confirmed that all *HIR* genes were expressed (Fig. 5). Expression of the *Hv-hir1* and *Hv-hir2* genes appeared to be little affected in all the lines analyzed, although some lines showed a slight decrease in expression, e.g., FN044, FN211, FN364 and FN367. The *Hv-hir4* gene was induced slightly (approximately 3-fold) only in line FN451. The amount of the *Hv-hir3* transcript was strongly induced in most of the lines, except FN370. Line FN303 showed the highest level of *Hv-hir3* transcript which increased by approximately 35-fold compared to Steptoe, while other lines showed a moderate 4- to 15-fold increase. The FN303 line also exhibited significantly slower growth and, along with FN044 and FN211, the most-severe necrotic symptoms.

Discussion

The barley hypersensitive-induced-reaction (*HIR*) genes cDNAs formed four groups that represented four different genes, *Hv-hir1, Hv-hir2, Hv-hir3* and *Hv-hir4*, based on sequence analyses and genetic mapping (Fig. 1). Since the tobacco homologue of *HIR* genes, NG1, has been shown to induce the formation of HR-like lesions (Karrer et al. 1998), we compared the map locations of the *Hv-hir* genes with map locations of known barley necrotic (*nec*) mutants (reviewed by Lundqvist et al. 1997). None of the four barley *Hv-hir* genes mapped in the vicinity of the previously mapped barley *nec* loci.

Analyses of the genomic sequences of the barley *HIR* genes showed a very similar arrangement of exons, even for the *Hv-hir4* gene, which had the lowest homology to the other *HIR* genes. The intron sequences, however, had no homology to each other and varied widely in size, suggesting ancient duplication events as a source for the gene-family expansion (Mitchell-Olds and Clauss 2002). The *Hv-hir1, Hv-hir2* and *Hv-hir3* genes had a 5′ untranslated region as the first exon. These non-coding exons exhibited no homology among the three genes and were located at a considerable distance from the first coding exon (Fig. 2). 5′ RACE results and alignment with available EST sequences indicated that *Hv-hir1* had three different transcription start sites within a 22-bp region (Fig. 2). Accordingly, at least two TATA box-like sequences were identified upstream of the transcription start sites, although none of them matched the consensus sequence very well (data not shown). Interestingly, three alternative non-coding exons were identified in the 5′ end of the *Hv-hir3* gene (Fig. 2). Alignment of EST and 5′

RACE-product sequences indicated that these alternative exons all were spliced to the same second exon containing the translation start site. Two of these alternative exons had non-canonical splice sites AG/GC and GG/GC at the exon–intron boundary, which all were spliced to the exon 2 containing canonical AG/G intron–exon boundary (Schuler 1998). The functional significance, if any, of the alternative non-coding exons remains to be determined.

There are no maize genomic *HIR* gene sequences available and the map locations of the maize *HIR* genes have not been reported; thus we could not determine syntenic relationships between barley and the maize *HIR* genes. There are two rice *HIR* genes located within a 10-kb region on the chromosome 1 PAC clone AP003300 which is linked to the genetic marker Y2820L. These two proteins, P0700A11.21 and P0700A11.23, are homologous to the barley *HIR* genes only at 40–54% amino-acid identity suggesting that they represent a different *HIR* gene group. Rice chromosome 1 is syntenic to barley chromosome 3(3H) (Ahn et al. 1993; Van Deynze et al. 1995), but none of the genes mapped in this study was on chromosome 3(3H). Homologues of these two rice genes may be absent from the barley genome or expressed at very low levels, since we detected only barley *Hv-hir1, Hv-hir2* and *Hv-hir3* gene ESTs in a tBLASTn search using P0700A11.21 and P0700A11.23 amino-acid sequences as probes. The only other rice sequence encoding a HIR-like protein was AF374475 whose product was highly similar to the *Hv-hir1* protein. AF374475 is a mRNA sequence of the *RHIR1* gene without a reported map location.

The neighbor-joining tree of all available HIR sequences (March 25, 2003; Fig. 3) showed a high degree of relatedness of the *HIR1, HIR2* and *HIR3* groups across the species analyzed. The deduced amino-acid sequence from the maize cDNA AY109189 grouped together with the *Hv-hir4* protein. These results suggested that the four groups of *HIR* genes were already present in the ancestor of barley and maize. Rice proteins P0700A11.21 and P0700A11.23 formed independent branches and may represent two groups of *HIR* genes absent or not expressed in barley.

Amino-acid sequence analyses of the barley proteins indicated the presence of the SPFH protein domain characteristic for the HIR proteins, prohibitins and stomatins, as well as other proteins from bacteria, plants and animals that are associated with membrane functions (Tavernarakis et al. 1999). Most of the characterized SPFH domain-containing proteins are integral membrane proteins with the domain positioned in the cytoplasm (Tavernarakis et al. 1999). Nadimpalli et al. (2000) postulated that HIR proteins, similar to other members of this protein superfamily, are involved in control of the membrane ion channels. Several conserved motifs were identified within the SPFH domain, as well as two invariant amino-acid positions (Nadimpalli et al. 2000). These motifs were conserved also in the barley HIR proteins. Of the two invariant amino acids, the aspartic acid residue in position 64 was conserved in all four barley HIR proteins, while the alanine residue in position 167 was substituted with glycine in the case of the *Hv-hir2* protein. Only the *Hv-hir4* protein was predicted to have a putative transmembrane helix at its N-terminus using the TMpred tool at http://www.ch.embnet.org/software/TMPRED_form.html (data not shown). The other three HIR proteins did not have apparent transmembrane regions. However, the Prosite search indicated that the N-termini of these proteins had a N-myristoylation motif and thus could be anchored to membranes.

Barley *HIR* genes were expressed in all the organs tested at various developmental stages (Fig. 4). Normalization to the *GAPDH* and *Ubi1* genes provided slightly different results which could be accounted for by the differential expression of the reference genes in different organs. In general, the expression of all *HIR* genes appeared to be higher in the leaves at all stages and lower in immature inflorescences and seedling roots. The ubiquitous presence of all barley *HIR* genes suggested that they may have vital, non-redundant functions.

The finding of Nadimpalli et al. (2000) that expression of the *Zm-hir3* gene was elevated in the maize *Les9* lesion mimic mutant, prompted us to investigate the expression levels of the barley putative *HIR* genes in our barley fast neutron (FN)-lesion mimic-mutant collection (Fig. 2 in the electronic supplementary material). The barley *Hv-hir3* gene, possibly orthologous to the *Zm-hir3*, was found to be overexpressed in most of the FN lines by as much as 35-fold (Fig. 5). In addition, the *Hv-hir4* gene showed slightly elevated expression in the FN451 line, while some other lines showed moderately decreased expression of the *Hv-hir1* and *Hv-hir2* genes. These results provided a strong link between the *HIR* genes and expression of the disease mimic lesions in the independently obtained, phenotypically different, mutant lines. However, the FN370 line showed no changes in the expression of the *HIR* genes. Thus, *Hv-hir3* may be associated with, but not required for, expression of the disease-lesion phenotype. Nadimpalli et al. (2000) implicated *HIR* genes in ion-channel regulation based on their structural similarity to known membrane proteins with an established function. Two of the *Arabidopsis* lesion mimic mutants, *hlm1* and *dnd1*, encode cyclic nucleotide-gated ion channels CNGC4 and CNGC2, respectively (Clough et al. 2000; Balague et al. 2003). We propose that in most of the barley mutants the over-expression of *Hv-hir3* (caused by the uncharacterized mutation) may lead to aberrant regulation of ion channels and, subsequently, to the disease-lesion phenotype. This is consistent with results of Karrer et al. (1998) who showed that the tobacco homologue of *HIR* genes, NG1, could induce HR, when transiently overexpressed from the tobacco mosaic-virus coat promoter. The phenotype of the FN370 line may be caused by a different mutation which does not affect the expression of the *HIR* genes, but may disrupt other functions.

In summary, four barley genes homologous to the hypersensitive-induced reaction genes from maize were

cloned. The genetic-map locations, sequence features and expression patterns of the barley *HIR* genes should facilitate further characterization of the molecular mechanism of the hypersensitive response in plants.

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