RESEARCH PAPER

WAX INDUCER1 (HvWIN1) transcription factor regulates free fatty acid biosynthetic genes to reinforce cuticle to resist Fusarium head blight in barley spikelets

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Abstract

Fusarium head blight (FHB), caused by Fusarium graminearum, is one of the most devastating diseases of wheat and barley. Resistance to FHB is highly complex and quantitative in nature, and is most often classified as resistance to spikelet infection and resistance to spread of pathogen through the rachis. In the present study, a resistant (CI9831) and a susceptible (H106-371) two-row barley genotypes, with contrasting levels of spikelet resistance to FHB, pathogen or mock-inoculated, were profiled for metabolites based on liquid chromatography and high resolution mass spectrometry. The key resistance-related (RR) metabolites belonging to fatty acids, phenylpropanoids, flavonoids and terpenoid biosynthetic pathways were identified. The free fatty acids (FFAs) linoleic and palmitic acids were among the highest fold change RR induced (RRI) metabolites. These FFAs are deposited as cutin monomers and oligomers to reinforce the cuticle, which acts as a barrier to pathogen entry. Quantitative real-time PCR studies revealed higher expressions of KAS2, CYP86A2, CYP89A2, LACS2 and WAX INDUCER1 (HvWIN1) transcription factor in the pathogen-inoculated resistant genotype than in the susceptible genotype. Knockdown of HvWIN1 by virus-induced genes silencing (VIGS) in resistant genotype upon pathogen inoculation increased the disease severity and fungal biomass, and decreased the abundance of FFAs like linoleic and palmitic acids. Notably, the expression of CYP86A2, CYP89A2 and LAC2 genes was also suppressed, proving the link of HvWIN1 in regulating these genes in cuticle biosynthesis as a defense response.

Key words: Cuticle reinforcement, free fatty acids, Fusarium head blight, quantitative resistance, resistance-related metabolites, transcription factors.

Introduction

About two-thirds of the global barley crop is used as animal feed, while the remaining one-third is used for malting, brewing and distillation (Newton et al., 2011). Barley is not only critical for food and feed, but also has unparalleled impact on the social and economic development of several countries (McMullen et al., 2012). However, the crop is severely affected by the devastating disease Fusarium head blight (FHB) caused by a fungus, Fusarium graminearum (Trail,
Spores germinate and the hyphae enter through the space between the lemma and palea. Developing kernels are infected through the epicarp, which destroys layers of the seed coat and finally starch and protein in the endosperm (Jansen et al., 2005). FHB management requires implementation of a variety of cultural and management practices. However, plant resistance through genetic improvement is considered to be the most efficient, economic and ecofriendly approach to reduce disease intensity (Bai and Shaner, 2004).

Five different types of resistance have been documented in the Triticaceae, three of which are often used in breeding wheat and barley (Mesterházy, 1995). Since barley has high level of rachis resistance (type II), most research has focused on spikelet resistance (type I) and resistance to DON accumulation in grains (type III) (Zhu et al., 1999). Resistance in plants against pathogen stress has been defined as the degree of susceptibility, ranging from high susceptibility (low resistance) to hypersensitive response (high resistance). The resistance is controlled by hierarchies of R genes, including genes that regulate downstream genes, which biosynthesize resistance-related (RR) metabolites and proteins (Kushalappa et al. 2016). The RR metabolites and proteins can be constitutive (RRC) or induced (RRI) (Kushalappa and Gunnaiah, 2013). Semi-targeted metabolomics have identified hundreds of metabolites involved in FHB resistance, and also the deposition of hydroxy-dicinnamic acid amides (HCAA) and flavonoids that thicken cell walls to contain the pathogen to initial infection (Gunnaiah et al., 2012). Several metabolic pathways have been shown to be upregulated in barley against <i>F. graminearum</i> infection (Bollina et al., 2010; Kumaraswamy et al., 2011a, b). Further, gene expression and silencing studies have been used to confirm the upregulation of genes involved in the HCCA biosynthetic pathway (Yogendra et al., 2015a).

In our previous studies on FHB disease resistance in both barley and wheat, free fatty acids (FFAs) showed a higher fold change (FC) in resistant than in susceptible (Kumaraswami et al., 2011a, b; Gunnaiah et al., 2012). The fatty acid metabolic pathway plays a significant role in plant defense against pathogen attack. For example, FFAs were implicated in their passive resistance mechanisms through the phytohormone jasmonic acid and cuticular components (Kachroo and Kachroo, 2009), which not only act as physical barriers but also as chemical antagonists against the invading pathogen. The cuticle is mainly made up of cutin monomers and oligomers, proposedly consisting of C16 and C18 fatty acids (Nawrath, 2002; Dominguez et al., 2015). Hydroxy and epoxy-hydroxy fatty acids of C16 and C18 fatty acids are inter-esterified to form cutin biopolyester. The cutin monomers also can form stable nanoparticles called cutinosomes which enable them to bind to each other to form layers (Dominguez et al., 2015). More than 190 candidate genes involved in the cuticle biosynthetic components have been isolated and characterized, mostly in the model plant Arabidopsis, including cytochrome P450 (CYPs), glycerol-3-phosphate: acyl-CoA acyltransferase (GPAT), long chain acyl-CoA synthetases (LACS) and members of ATP binding cassette (ABC) transporters (Xiao et al., 2004; Yang et al., 2014). The accumulated biochemical, physiological and genetic evidence of the cuticle in plant protection against biotic and abiotic stresses has stimulated search for gene networks underlying such protective responses (Borisjuk et al., 2014). Cuticle biosynthetic genes are regulated by transcription factors. A transcription factor, of the ethylene response factor (ERF) family, WAX INDUCER1/SHINE1 (WIN1/SHN1), has recently been shown to induce the production of epidermal waxes when overexpressed in Arabidopsis plants. Analysis of 3SS:WIN1 overexpressors suggested the role of <i>WIN1</i> in wax accumulation through direct or indirect regulation of metabolic pathway genes (Aharoni et al., 2004; Broun et al., 2004).

The aim of our study was to explore the biochemical and molecular basis of resistance in barley to FHB. Two barley genotypes with contrasting levels of resistance – a resistant (CI9831) and a susceptible (H106-371) – were mock- and pathogen-inoculated, and metabolites were profiled using liquid chromatography and high resolution mass spectrometry (LC-HRMS). The RR metabolites with high-fold change, especially the FFAs, were selected as candidate metabolites, mapped in metabolic pathways to identify the candidate genes, including a TF WAX INDUCER1 gene (designated herein as <i>HvWIN1</i>), which regulated them, based on genomic databases. The expression of these genes was confirmed based on quantitative real-time polymerase chain reaction (qPCR) studies. The RR genes in barley against FHB discovered here, based on metabolo-genomics, demonstrated the role of FFAs in cuticle biosynthesis in barley to resist <i>F. graminearum</i>. When the <i>HvWIN1</i> gene was silenced, based on virus-induced gene silencing (VIGS) in the resistant genotype, not only disease severity but also fungal biomass increased, confirming a shift from resistant to susceptible phenotype. Furthermore, it was associated with a decrease in abundance of several RR metabolites, whose biosynthetic genes were regulated by this gene, confirming its role in FHB resistance in barley. The potential application of this gene in breeding is discussed.

**Materials and methods**

**Plant production**

Two row barley genotypes varying in resistance to FHB, CI9831 (R, resistant) and H106-371 (S, susceptible), were selected based on field and greenhouse studies (Choo et al., 2004; Kumar et al., 2015). Five seeds of each genotype sown in pots filled with 50:50 pasteurized soil and 155 AgroMixR21 AF (Fafard, Quebec), thinned to three individuals following germination, were maintained in a greenhouse at 23 ± 2 °C, 70 ± 10% relative humidity and with a photoperiod of 16 h throughout the growing period (Chamarthi et al., 2014). Fertilizer solution (250ml), containing 0.3% of PlantProd (20:20:20 NPK) and 0.03% of trace elements was applied every 15 d to each pot.

**Fungal spore production and inoculation**

<i>F. graminearum</i> was grown in potato dextrose agar (PDA) plates and incubated at 26 °C for 4 d. For spore production, pathogen was further sub-cultured in Rye B agar plates and kept inverted for another 4 d by exposing the plates to 8 h dark and 16 h of near-UV light. Macroconidia were harvested from 7-day-old cultures, spore concentration was determined using a hemocytometer (American Scientific Products, USA) and finally adjusted to 1 × 10⁵ macroconidia ml⁻¹ (Bollina et al., 2010). Three alternate pairs of barley spikelets at 50% anthesis stage, were point inoculated with 10 μl of either macroconidial suspension.
in water or mock-solution using a syringe (GASTIGHT 1750 DAD, Reno, USA). After inoculation, plants were covered with polyethylene bags sprayed with water to maintain enough moisture inside. Bags were removed 48 h post inoculation (hpi).

Experimental design and metabolite analysis using LC-HRMS
The experiment was conducted as a completely randomized block design, with the two genotypes with contrasting levels of resistance to FHB (C19831 and H106-371), two inoculations (pathogen and mock), with five replicates over time at 3 d intervals. Each replicate, or the experimental unit, consisted of 48 pairs of spikelets, collected from eight spikes in three plants; from each spike three alternate pairs of spikelets inoculated with either macroconidial suspension or mock-solution and three alternate pairs of uninoculated spikelets were harvested. The samples were frozen immediately in liquid nitrogen and stored at −80 °C until use. Metabolite analysis was performed as described earlier (Gunnavah et al., 2012; Yogendra et al., 2014, 2015b). In brief, 100 mg of spikelets were ground in liquid nitrogen, and metabolites were extracted in 60% ice-cold methanol, to which 200 µg of genistin was added as internal control (Chamarthi et al., 2014). The extract was sonicated for 15 min at 25 °C using a water bath sonicator (Mesonix, USA) and 5 µl of resulting clear supernatant was used for metabolite analysis. These were analyzed in negative ionization mode using the LC-HRMS system (LC-ESI-LTQ Orbitrap, Thermo Fisher, USA), using a 5 cm kinetex column.

Identification of resistance-related (RR) metabolites
The data on intensity of peaks of monoisotopic masses (m/z = mass/charge ratio, subtracted with a proton mass because of negative ionization) were subjected to pairwise Student’s t-test analysis (SAS v 9.3). The treatment combinations tested were RM vs SM, RP vs RM, and SP vs SM, where R=resistant, S=susceptible, M=mock, and P=pathogen-inoculated. The peaks significant at P<0.05 (Kushalappa and Gunnavah, 2013), relative fold change of ≥2 and false discovery rate threshold of 0.05 (Vinaixa et al., 2012) were retained. False discovery rate of peaks depends mainly on the signal/noise (S/N) ratio; the lower the ratio, the higher are the false discovery rates. Therefore, the S/N ratio was kept high to avoid any false discovery. However, this was only used as initial selection criteria, to include as many metabolites as possible. The significant abundances of 756 metabolites, present in all five replicates, were subjected to canonical discriminant analysis using the CANDISC procedure (SAS v 9.3) to classify the observations. The data dimension was reduced by a nonsupervised principal component analysis, and the principal components were subjected to supervised discriminant analysis to classify the observations to treatments. The canonical discrimination analysis (CAN) scores were used to develop a scatterplot which discriminated the treatments and identified resistance functions (Hamezharghanni et al., 2005). The loadings of metabolites to CAN vectors were used to interpret the results.

The metabolites with significantly higher abundances in R than in S, based on a t-test, were considered as RR metabolites. These were further grouped into RR constitutive (RRC=RM>SM) and RR-induced (RRI=(RP>RM)>(SP>SM)) metabolites. For these RR metabolites, the fold change (FC) in abundance, relative to susceptible (R/S), was calculated. The RR metabolites were putatively identified based on two criteria: (i) the accurate mass match [accurate mass error (AME)<5 ppm] with metabolites reported in different databases: METLIN, KNAPSAck, Plant Metabolic Network (PMN), LIPIDMAPS and KEGG; (ii) the fragmentation pattern match with those in databases or in silico verification (Gunnavah et al., 2012). The metabolites were mapped on metabolic pathways using a pathway tool omics viewer searched against Arabidopsis and Hordeum vulgare metabolites (Bollina et al., 2010).

Prediction of genes involved in cuticle biosynthesis
Since the cuticle biosynthetic metabolites were highly accumulated in the resistant genotype, the reported gene sequences involved in cuticle biosynthesis in Arabidopsis and other plants were BLAST-searched using the assembly_WGSMorex IPK barley BLAST server (http://www.public.iastate.edu/~imagelpe/IBSC%20WebPage/IBSC%20Template-home.html). The contigs were downloaded and genes were predicted using the SoftBerry FGENESH program (http://linux1.softberry.com/berry.phtml?topic=fgenes&group=programs&subgroup=gfind).

RNA extraction, cDNA synthesis and quantitative real-time PCR analysis
Total ribonucleic acid (RNA) was extracted from the ground spikelets using the RNeasy Plant mini kit (Qiagen, Germany) and treated with DNase I as per the supplier’s recommendations. Purified RNA (500 ng from each sample) was reverse transcribed using the iScript cDNA synthesis kit (BioRad, ON, Canada). Two microliters of 40× diluted cDNA was used in a qPCR reaction using the iQ SYBR Green Supermix (BioRad) in an iQ CFX384™ Real-Time System (Bio-Rad, Mississauga, ON, Canada). Primer sequences and PCR conditions used for actin and other genes are given in Supplementary Table S1 at JXB online.

Cloning, sequencing and bioinformatics analysis of the HvWIN1 transcription factor
Amplification of the full length sequence of HvWIN1 from barley cultivars CI9831 and H106-371 was carried out in a 25 µl volume using both genomic DNA and cDNA as template and gene specific forward (5′-ATGGCGGTCGAGTCTGGGAATTTGG-3′) and reverse primer pair (5′-CTATGGACGAGCTGCGTTCTGTAT-3′) designed from start and stop codon, respectively. Gene amplification was conducted using a thermal cycler (Bio-Rad, Mississauga, ON, Canada) with the following steps: heat denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, annealing at 58 °C for 40 s, extension at 72 °C for 2 min and final extension at 72 °C for 7 min. The amplified PCR product was cloned into the pGEM™-T Easy vector (Promega, USA) and sequenced using the ABI Automated DNA Sequencer. DNA sequences were translated to amino acid sequences using the ExPaSy Translate Tool (http://web.expasy.org/translate/). Multiple sequence alignments of nucleotide and amino acids were done using MultAlin (http://multalin.toulouse.inra.fr/multalin/) to identify polymorphisms in the sequences of resistant and susceptible genotypes. To check the polymorphic status, HvWIN1 was also cloned and sequenced from three other cultivars, namely Zhedar-2, AC-Metcalfe and CDC Copeland. All the sequences were submitted to the NCBI database. The sequences were aligned with barley reference sequence using multalin software (http://multalin.toulouse.inra.fr/multalin/). A phylogenetic tree was built using the à la carte function on the Phylogeny.fr server (http://www.phylogeny.fr/).

Construction of BSMV-derived vector, in vitro transcription of viral RNAs and plant inoculation for virus-induced gene silencing (VIGS) of HvWIN1 TF
For transient gene silencing, the target 250 bp HvWIN1 (GenBank ID: KT946819) fragment was amplified using forward (5′-TGGTTCTCCGAGATCGAC-3′) and reverse primers (5′-GAAGCTTGGCACTGAGGAC-3′). This region was chosen from the N-terminus region of the gene, which on BLAST N and BLAST X analysis on the NCBI server did not show homology with any other barley genes. Also, BLAST analysis on the IPK BLAST server (http://webblast.ipk-gatersleben.de/barley) revealed very low homology scores with other genes (Supplementary Table S2). The fragment was checked for genome-wide off-target siRNAs using the SGN VIGS Tool (http://vigs.solgenomics.net/) with 250 bp fragment size and n-mer of 21 bp using Brachypodium distachyon v3.1 as query database. Result of this analysis showed homology with the...
Bradi3g07450.1 gene, which on analysis was found to be the ethylene-responsive transcription factor WIN1-like gene. The fragment was cloned in pGEM® T-Easy vector (Promega, USA) followed by digestion of plasmid DNA with NotI enzyme, thereby producing NotI ends. The cDNA fragment was subsequently ligated to the pSL038-1 vector, a plasmid encoding a modified BSMV-modified γ genome segment with a cloning site downstream of the γ gene (Cakir and Scofield, 2008). The pSL038-1 vector carrying either the phytoene desaturase (PDS) gene or without any plant gene served as positive control and negative controls, respectively. The plasmids pP46 (BSMV α) and pYP038-1 were linearized with the MluI restriction enzyme, whereas pP42sp1 (BSMV β) was linearized by using the SpeI enzyme. All linearized plasmids were converted to capped in vitro transcripts using mMESSAGE mMACHINE® T7 in vitro transcription kit (Ambion, Inc., Austin, TX, USA) following the manufacturer’s protocol. A 20 µl reaction contained 10 µl of 2× dNTPs, 1 µg of linearized plasmid, 2 µl of 2× buffer, 2 µl of enzyme mix, and water to a final volume of 20 µl. The reaction setup was scaled up as per the requirements of the experiment. The reaction mix was incubated at 37°C for 2 h and in vitro transcription was confirmed by running 1 µl of transcript with 9 µl of RNase-free water on 1% agarose gel.

Flag leaf and spikelets of the resistant genotype CI9831 were rub-inoculated, at growth stage 50–55 (Zadoks et al., 1974), with all the three in vitro transcript reactions (α, β, and γ BSMV) in a 1:1:1 ratio (1 µl of each was used) along with 22.5 µl inoculation buffer (IB) that facilitated viral infection as it contained abrasive material (Scofield et al., 2005; Ma et al., 2012). The experimental units consisted of two plants, with a total of six spikes inoculated separately with test treatment (BSMV+HvWIN1) and negative control (BSMV:00), with five biological replicates over time. Barley spikelets were also rub-inoculated with positive controls (BSMV+PDS). Twelve days after silencing, three alternate pairs of spikelets in three spikes were inoculated with positive controls (BSMV+PDS). Twelve days after silencing, three alternate pairs of spikelets in three spikes were inoculated with 10 µl of either mock or macroconidial suspension (1 × 10⁸ macroconidia ml⁻¹) for both negative control and test treatment. Plants were covered with plastic bags to maintain high humidity. Spikelet samples for metabolite and gene expression studies were collected 3 dpi (days post-inoculation) whereas, for fungal biomass quantification, these were collected 6 dpi.

Fungal biomass quantification

Genomic DNA was isolated from barley spikelets infected with F. graminearum (6 dpi) using a DNeasy Plant Mini Kit (Qiagen, Canada). Relative biomass of F. graminearum in the infected samples was quantified by quantitative real-time PCR (qPCR) as described by Kumar et al. (2015). PCR was performed in iQ CFX384® Real-Time System (Bio-Rad, Mississauga, ON, Canada) using primers for Tri6 as target gene and Actin as a housekeeping gene and an equal quantity of genomic DNA (20 ng) of each sample. The relative fungal biomass was estimated by normalizing the Ct values for Tri6 to that of barley Actin gene and calculating the relative gene copy number of Tri6 using the 2⁻ΔΔCt method (Livak and Schmittgen, 2001).

Results

Canonical discriminant analysis discriminated resistance and pathogenesis functions in plant-pathogen interaction

Canonical discriminant analysis was used to classify the observations, abundances of 1864 peaks consistent in all the five replicates and to explain resistance or pathogenicity functions. The five replicates of each variable were clustered in one group, meaning that the experimental error was minimal. The CAN1 vector explained 92% variance, discriminating the resistant and susceptible genotypes. The CAN2 vector explained only 4% variance, discriminating the inoculations, explaining the pathogenesis, meaning the CAN vectors mainly explained the resistance function (Supplementary Fig. S1).

Semi-targeted metabolomics analysis revealed FFAs as high-fold resistance-related induced metabolites

Metabolites were profiled in the spikelets of resistant and susceptible genotypes at 3 dpi, inoculated with F. graminearum or with water and analyzed based on LC-HRMS. A total of 1864 monoisotopic peaks were detected and the significant ones were categorized into RRC and RRI metabolites. Data was reduced by considering only those metabolites that had abundances FC>2. Out of these 47 high FC metabolites, 24 were identified as RRC metabolites and 21 as RRI metabolites (Table 1; Fig. 1A, B; Supplementary Table S3; Supplementary Fig. S2). The majority of the high FC RRI metabolites belonged to the fatty acid pathway. Among these, five FFAs were induced with high-fold change: linoleate (FC=39.00), palmitic acid (FC=6.56), sebacic acid (FC=2.79), arachidonoyl m-Nitroaniline (FC=2.60), auricolic acid (FC=2.05).

Transcript expression of barley genes involved in cuticle biosynthesis

Based on literature, some of the important genes involved in fatty acid and cuticle biosynthesis were selected for transcript expression analysis. These genes were KAS2, CYP86A2, CYP89A2, LACS2, GPAT6 and CER5; which were considered to be involved in the production of ω-hydroxy fatty acid components that formed the cutin polymer (Smith et al., 2000; Kannangara et al., 2007). Detailed information on the predicted gene sequences in the barley genome – including gene size, chromosomal localization and expression primers used for qPCR – is given in Supplementary Table S4 and Supplementary Table S1, respectively.

β-ketoacyl-(acyl carrier protein) synthase 2 (KAS2) is a key gene involved in the biosynthesis of FFAs, which catalyzes the biosynthesis of malonyl and stearate (utilizing palmitoyl-ACP or myristoyl-ACP for condensation with malonyl-ACP and controlling the final ratio of C16/C18 products). KAS2 showed enhanced gene expression in pathogen-inoculated compared to mock-inoculated genotypes (Fig. 2). The expression of CYP86A2/CYP89A2 that catalyzes the oxidation of fatty acids and diverts metabolic flux toward cutin biosynthesis, was significantly (P<0.05) increased in the resistant genotype, following pathogen inoculation, compared to mock inoculation. No significant difference in gene expression was observed between mock and pathogen-susceptible genotype (Fig. 2) for CYP86A2. LACS2 is involved in the conversion of ω-hydroxy fatty acids to their CoA thioesters. Whereas the expression of LACS2 was significantly (P<0.05) increased in the pathogen-inoculated resistant (CI9831) genotype, as compared to the susceptible genotype H106-371, in which it was actually decreased. In parallel, the GPAT6 that catalyzes the conversion of FFAs to ω-OH-acylglycerol, was also higher in both CI9831 and...
**Table 1.** Resistance-related metabolites detected in the spikelets of barley genotypes inoculated with water or spores of *F. graminearum*. RRI, resistance-related induced metabolites; RRC, resistance-related constitutive metabolites.

| Observed mass (Da) | Exact mass (Da) | Name                                                                 | Relative fold change (FC) |
|--------------------|---------------|----------------------------------------------------------------------|---------------------------|
| 326.2005           | 326.1994      | Ajmaline                                                             | 2.44 RRI**, 2.03 RRC**    |
| 340.2158           | 340.2150      | (+)-Sandviciclidine                                                  | 2.10 RRI*                 |
| 449.2769           | 449.2777      | Condelphine                                                          | 23.00 RRI***              |
| 596.3533           | 596.3515      | C-Curarine                                                           | 3.26 RRI*, 2.03 RRC**     |
| 202.1210           | 202.1205      | Sebacic acid                                                         | 2.79 RRI*                 |
| 256.2405           | 256.2402      | Palmitic acid                                                        | 6.56 RRI**                |
| 280.2404           | 280.2402      | Linoleate                                                            | 39.00 RRI***              |
| 324.2674           | 324.2664      | Aruricilic acid                                                      | 2.05 RRI*                 |
| 390.1893           | 390.1890      | (-)-11-hydroxy-9,10-dihydrojasmonic acid 11-beta-D-glucoside         | 2.75 RRC**                |
| 424.2723           | 424.2726      | Arachidonoyl m-Nitroaniline                                           | 2.60 RRI**                |
| 410.2435           | 410.2433      | 1-Palmitoylglycerol 3-phosphate                                       | 2.43 RRI*                 |
| 572.2961           | 572.2962      | PI (16:0/0:0) or 1-hexadecanoyl-sn-glycero-3-phospho-[1'-myo-inositol]| 4.21 RRI*                 |
| 610.3843           | 610.3846      | PG (12:0/12:0) or 1,2-didodecanoyl-sn-glycero-3-phospho-[1'-sn-glycerol]| 2.73 RRC**                |
| 740.4631           | 740.4628      | PG (14:1(9Z)/20:4(5Z,8Z,11Z,14Z))                                    | 5.44 RRC**                |
| 754.4414           |               | PC (5:0/30:11)                                                       | 3.32 RRC*                 |
| 772.4521           |               | PS (0:0/37:10)                                                       | 2.67 RRC*                 |
| 788.4469           | 788.4476      | PI (13:0/18:4(6Z,9Z,12Z,15Z))                                        | 2.04 RRC**                |
| 790.4656           | 790.4632      | PI (13:0/18:3(6Z,9Z,12Z))                                            | 2.07 RRC*                 |
| 956.8783           | 956.8772      | TG (19:0/20:1(11Z)/20:1(11Z)[iso3]                                    | 2.84 RRC*                 |
| 650.3218           | 450.3223      | C17 sphingosine-1-phosphocholine                                      | 2.69 RRI*                 |
| 476.2177           | 476.2198      | Fuscaxanthone A                                                      | 2.08 RRC*                 |
| 380.1057           | 360.1056      | Syringic acid beta-glucopyranosyl ester                               | 3.50 RRC*                 |
| 270.0531           | 270.0528      | Apigenin                                                             | 2.46 RRC**                |
| 338.1164           | 338.1154      | (-)-Glyceollin I                                                     | 5.50 RRC*                 |
| 372.1211           | 372.1209      | Sinensetin                                                           | 2.20 RRI*                 |
| 432.1061           | 432.1056      | Petargonidin 3-O-glucoside                                           | 2.91 RRC*                 |
| 506.1062           | 506.1060      | Quercetin 7-(6''-acyetylglucoside)                                    | 2.96 RRC**                |
| 578.1642           | 578.1636      | Rhoifolin                                                            | 12.12 RRC**               |
| 596.1728           | 596.1741      | Isobutrin                                                            | 2.05 RRC*                 |
| 608.2114           | 608.2105      | Matteucinol 7-O-beta-D-apiofuranolysyl[1->6]-beta-D-glucopyranoside   | 2.22 RRC*                 |
| 740.2162           | 740.2164      | Robinin (kaempferol-3-D-robinoside-7-O-rhamnoside)                    | 4.84 RRC**                |
| 386.2868           | 388.2668      | 6-Deoxerythronolide B                                               | 2.51 RRI*                 |
| 418.1631           | 418.1628      | Lirioreinosol A, (+)-Syringaresinol                                  | 2.16 RRC*                 |
| 596.2439           | 596.2410      | Magnoligman G                                                        | 2.29 RRI*                 |
| 748.2949           | 748.2942      | Alangisesquin C                                                     | 4.53 RRC**                |
| 740.4347           | 740.4347      | Timosaponin A-III                                                   | 2.08 RRI*                 |
| 868.5170           | 868.5184      | Nephelioside I                                                       | 2.04 RRI*                 |
| 870.4979           | 870.4976      | Racemoside C                                                         | 3.12 RRI*                 |
| 446.2294           | 446.2305      | Glycineocladin A                                                     | 7.73 RRI**                |
| 596.3864           | 596.3865      | Astaxanthin                                                          | 2.14 RRC*                 |
| 732.4579           | 732.4601      | 3-(E)-Coumaroylbetulin-28-yl-ethyl (2R)-2-hydroxysuccinate           | 2.68 RRC*                 |

Detailed compound identification is presented in Supplementary Table S3. Fold change calculation was based on relative intensity of metabolites: RRC=R/M/S and RRI=(R/P/R)/(S/P)(SM). Significance (t-test): *, P<0.05; **, P<0.01; ***, P<0.001.
H106-371, inoculated with pathogen, but was not significant. Similarly, expression of CER5, an ABC transporter, considered to be involved in cuticular lipid export was also higher in pathogen-inoculated compared to mock-inoculated resistant genotype CI9831. Conversely, the expression of CER5 was reduced in pathogen-inoculated susceptible H106-371 genotype, as compared to mock-treated samples (Fig. 2).

**Differential transcript expression of HvWIN1: a potential transcriptional regulator of cuticle biosynthetic genes in barley**

Previous studies in Arabidopsis have shown WIN1 as a transcriptional regulator of genes involved in cutin biosynthesis (Aharoni et al., 2004; Broun et al., 2004; Kannangara et al., 2007). Further, we studied the potential interacting partners of WIN1 using WIN1/SHN1 as query and Arabidopsis as search organism in STITCH 4.0 software (http://stitch.embl.de). STITCH is a resource used to explore known and predicted interactions of chemicals and proteins. Chemicals are linked to other chemicals and proteins by evidence derived from experiments, databases and the literature (Kuhn et al., 2014). The resulting network clearly showed direct interaction of WIN1 TF with downstream targets like LACS2, GPAT4, CYP86A4 and CER5 (Supplementary Fig. S3) and also with palmitate, with high FC observed in our study. Therefore, WIN1 was considered as a potential TF to explore further.

**Silencing of HvWIN1 gene in the resistant genotype rendered it susceptibility to FHB**

To prove the resistance function of HvWIN1 in barley against FHB, the HvWIN1 gene in the resistant plant was knocked down by VIGS. The schematic representation of the VIGS experiment is shown in Fig. 5A. Plants expressing the pSL08-1 vector carrying the PDS gene (involved in the carotenoid metabolic pathway) exhibited photobleaching after 12 d of rub inoculation, which served as positive controls confirming the success of the silencing protocol (Fig. 5B). Complete photobleaching was observed at and after 16 dpi (Fig. 5B). To assess the effect of HvWIN1 in FHB resistance, the F. graminearum biomass was quantified in silenced and non-silenced resistant plants. Silencing of
**HvWIN1** in CI9131 led to a significant increase (\( P<0.005 \)) in copy number of the *Tri6* gene (3-fold), depicting the amount of fungal biomass, compared to non-silenced control plants (Fig. 5C). Transcript abundance analysis of **HvWIN1** using qPCR showed substantial reduction in copy number due to knockdown of the gene (Fig. 5D). These results suggested that the observed phenotypic difference in FHB resistance was due to **HvWIN1** silencing.

Silencing of HvWIN1 reduced fatty acid RR metabolites and led to reduced transcript abundance of genes involved in cutin biosynthesis

As discussed in the previous section, **HvWIN1**-silenced barley spikes exhibited lower levels of resistance to FHB; accordingly, the plausible biochemical and molecular mechanisms of involvement of **HvWIN1** to resist FHB were further explored. Semi-targeted metabolomic analysis was carried out to confirm the effect of **HvWIN1** silencing on secondary metabolite production. The FFA RRI metabolites palmitic acid (FC=6.56–1.3) and linoleate (FC=39.00–3.09) were significantly reduced (\( P<0.05 \)) after **HvWIN1** silencing (Table 2), suggesting the potential involvement of **HvWIN1** in the FFA biosynthetic pathway. Other FFAs such as sebacic acid (FC=1.08), arachidonoyl m-Nitroaniline (FC=1.20) and auricolic acid (FC=1.10) were also detected in less abundance (Table 2). To further study the downstream targets of **HvWIN1**, we carried out promoter analysis of genes for which promoter sequences were available. One thousand bases above the transcription start site were considered for analysis using the bioinformatics tool PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Database sequences were available for CYP86A2, CYP89A2, LACS2 and GPAT6. However, for CYP89A2 only 400 bp were available in the contig. Promoter analysis of these genes, however, failed to reveal any GCC box necessary for **HvWIN1** to bind to these promoters (Supplementary Table S6). Since KAS2, CYP86A2 and CYP89A2, and LACS2 had the highest expression upon pathogen inoculation, these were studied for gene expression after **HvWIN1** silencing. There was no significant difference in expression of KAS2 upon **HvWIN1** silencing (Fig. 6). However, transcript abundance was significantly reduced for CYP86A2 and CYP89A2, and LACS2 upon pathogen treatment (Fig. 6), suggesting these as potential targets of **HvWIN1**.
Plants grown in the field have to face several environmental stresses. One of the mechanisms that protect the plants from biotic stresses is the formation of lipophilic barriers in the form of cutin or suberin (Yang et al., 2012). Cutin and suberin are polymers of fatty acid derivatives linked by ester bonds, i.e., polyesters (Beisson et al., 2012). These insoluble polymers, and associated waxes, function to control water loss, gas and ion fluxes, and also function as physical barriers to protect plants from pathogen invasion (Schreiber, 2010). These are mainly composed of ω-oxidized fatty acids (ω-OHFA), namely α,ω-dicarboxylic acids (DCAs), with a varying range of glycerol (Yang et al., 2010). Cutin is mostly composed of C16 and C18 ω-hydroxy acids, polyhydroxy acids, epoxyacids and dicarboxylic acid (DCA) (Domínguez et al., 2015). Although these are among the most abundant lipid monomers in plants, the genes involved in cutin biosynthesis are largely unknown.

F. graminearum enters through the space between lemma and palea, then infects cereal florets through natural openings, such as stomata, or by direct penetration (Bushnell, 2001). After penetrating the cuticle, the fungal hyphae grow subcuticularly in

**Discussion**

In silico analysis of HvWIN1. (A) Catalytic domain prediction by NCBI Conserved Domain Database Search. (B) Multiple sequence alignment of HvWIN1 from closely related plant species showing AP2/ERF DNA binding domain. (C) Hydropathy plot analysis at a window size of 19. Peaks with scores greater than 1.8 (horizontal line) indicate possible transmembrane regions. (D) Phylogenetic relationships of HvWIN1 with other plant WIN1 sequences retrieved from the NCBI database. The phylogenetic tree was built using the ‘à la carte’ function on the Phylogeny.fr server. A colour version of this figure is available at JXB online.

![Fig. 3](image-url)
HvWIN1 regulates cutin biosynthetic genes to resist FHB in barley

the tissues of glume, palea and lemma (Kang and Buchenauer, 2000; Jansen et al., 2005). Fungal cutinases along with other cell wall degrading enzymes such as lipases are overexpressed during the infection process. Plants can defend against fungus by orchestrating a battery of genes to biosynthesize metabolites with either antimicrobial functions or by channelizing its resources to form structures that can confine the pathogen to the initial infection site. The epidermal cuticle is the first level of resistance the fungal pathogen has to overcome (Wanjiru et al., 2002). A number of genes have been reported to be involved in biosynthesis of the cuticle in Arabidopsis (Yeats and Rose, 2013). However, in barley the specific metabolites or the genes involved in biosynthesis of the cuticle, especially its reinforcement to contain pathogens, have not been reported.

In the present study, an integrated metabolomics and functional genomics approach was used to explore mechanisms of resistance in barley against FHB, using two two-row barley genotypes, CI9831 and H106-371, with contrasting levels of resistance. A semi-comprehensive metabolomics analysis revealed a high-fold accumulation of FFAs, which were not only constitutively present but also induced following pathogen invasion (Table 1; Supplementary Table S3). The FFAs

Table 2. Effect of HvWIN1 silencing on resistance-related fatty acid metabolites upon F. graminearum or mock-solution inoculation.

| Observed mass (Da) | Exact mass (Da) | Name                                  | Fold changes before silencing | Fold changes after silencing |
|--------------------|----------------|---------------------------------------|-------------------------------|-----------------------------|
| 280.2404           | 280.2402       | Linoleate                             | 39.00 RRI***                  | 3.09*                       |
| 256.2405           | 256.2402       | Palmitic acid                         | 6.56 RRI**                   | 1.30*                       |
| 202.1210           | 202.1205       | Sebacic acid                          | 2.79 RRI*                    | 1.08*                       |
| 424.2723           | 424.2726       | Arachidonoyl m-Nitroaniline           | 2.60 RRI**                   | 1.20*                       |
| 324.2674           | 324.2664       | Auricular acid                        | 2.05 RRI*                    | 1.10*                       |

Fold change calculation was based on relative intensity of metabolites: $RRC = RM/SM$ and $RRI = (RP/RM)/(SP/SM)$. Significance (t-test): *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.  

Fig. 5. Virus-induced gene silencing of HvWIN1. (A) Schematic representation of BSMV-based virus-induced gene silencing (VIGS) vectors. The HvWIN1 cDNA fragment was cloned to pPSL038-1 vector (modified BSMV vector) downstream of the yb gene (BSMV/HvWIN1). The vector carrying either the phytoene desaturase (PDS) gene (BSMV/PDS) or without any plant gene (BSMV:00) served as positive control and negative control, respectively. The region in the HvWIN1 with gradient fill depicts the part of the gene used for silencing. (B) Phenotypes of the spikes from barley cultivar C19831 rub-inoculated with either BSMV/PDS or BSMV:00 constructs. Spikes were rub-inoculated with either BSMV:00 (negative control) or BSMV/PDS (positive control) constructs at flag leaf stage and photographed at different time intervals. dpi, days post rub-inoculation. (C) Relative fungal biomass calculated as relative gene copy number of tri6 at 6 dpi. (D) Relative transcript expression measured at 72 hpi. RM, mock-treated resistant samples; RP, pathogen-treated resistant samples; BSMV:00, barley plants rub-inoculated with negative control; BSMV/HvWIN1, barley plants rub-inoculated with BSMV vector containing HvWIN1. Significant differences in expression levels in RP+BSMV:00 compared with RP+BSMV/HvWIN1 using student’s t-test: *, $P<0.05$. A colour version of this figure is available at JXB online.
can be channelized to pathways such as the octadecanoid pathway for the biosynthesis of jasmonic acid and its derivatives, which further trigger several downstream defense-related target genes. However, in our study the FFAs such as palmitic acid and linoleic acid, along with glycerolipids such as 1-palmitoylglycerol 3-phosphate and PI (16:0/0:0) or 1-hexadecanoyl-sn-glycero-3-phospho-(1’-myo-inositol), produced high FC in abundance (Table 1), appeared to have been used as monomeric units to biosynthesize cutin (Domínguez et al., 2015). These results tempted us to explore the possible mechanism of cutin biosynthesis as one of the major defense strategies in response to pathogen attack. Therefore, we conducted a stepwise approach in qPCR analysis for some of the genes involved in the biosynthesis of FFAs and further down to cutin biosynthesis. Genes known to be involved in cuticle biosynthesis from Arabidopsis were selected for BLAST analysis to identify those showing maximum homologies in the barley genome database.

KAS2 is one of the important genes involved in FFA biosynthesis inside plastids (Beld et al., 2015). Through analysis of mutants, some members of the LACS family, cytochrome P450 oxidases and GPAT have been shown to be required for cutin biosynthesis (Pollard et al., 2008). LACS family members are required to activate FFAs to acyl-CoA for use by GPATs whereas members of the P450 family, such as CYP88 and CYP77A, were reported to be involved in ω-hydroxylation and midchain hydroxylation of FFAs (Pollard et al., 2008; Grausem et al., 2014). In barley, transcriptional silencing of CYP96B22 based on VIGS led to a decrease in penetration resistance of barley plants to Magnaporthe, host and nonhost isolates (Delventhal et al., 2014). GPAT catalyzes the transfer of an acyl group from acyl-CoA or acyl-ACP to the sn-1 position of the sn-glycerol-3 phosphate (G3P) (Murata and Tasaka, 1997). More than eight genes in Arabidopsis belonging to different GPAT families catalyzing either membrane or storage lipid biosynthesis have been reported. The GPAT6 falls under a different family that is involved in cutin or suberin biosynthesis in flowers and seed coats (Beisson et al., 2007, 2012; Li et al., 2007). GPAT6 is strongly expressed in flowers, and gpat6 mutants are substantially reduced in all C16 cutin monomers (DCA, 16-hydroxy- and 10,16-dihydroxypalmitates), while over-expression of GPAT6 increased these monomers (Beisson et al., 2012). After cutin monomers are synthesized, they are exported across the endoplasmic reticulum (ER) and the plasma membrane, through the polysaccharide cell wall to the nascent cuticular membrane using ATP-binding cassette (ATP) transporters. In Arabidopsis, the CER5/ABC transporter ABC transporter G family (ABC) member 12 (AT1G51500) encodes an ABC transporter that is involved in cuticular wax biosynthesis (Kunst and Samuels, 2009). Recently, an ABCG transporter that played an important role in cuticle deposition was reported in wild barley and rice. A spontaneous mutation, eibl.b, in wild barley led to reduced capacity to retain leaf water, a phenotype associated with reduced cutin deposition and a thin cuticle (Chen et al., 2011).

Using Arabidopsis model studies the barley genes were predicted and used for expression analysis using qPCR. Our results suggested upregulation of KAS2, CYP86A2, CYP89A2 and LACS2 (Fig. 2), which have important roles in cuticle biosynthesis. Transcriptional regulation of cuticle biosynthesis has mainly been explored in model plants like Arabidopsis, however, in crops such as barley and wheat, information is lacking. Regulation of cuticle biosynthesis is intricate, involves a complex network of genes, hormones and TFs, and is influenced by various factors like environment, plant development and pathogen attack (Yeats and Rose, 2013). WIN1 was the first TF that was identified in...
having a role in cutin biosynthesis. Overexpression of \( \text{WIN1}/\text{SHN1} \) led to an altered cutin composition, while silencing led to reduced cutin deposition and affected water permeability in Arabidopsis (Kannangara et al., 2007). Silencing of \( \text{SHN3} \) in tomato resulted in morphological alterations of the fruit epidermis and significant reduction in cuticular lipids. It was demonstrated that \( \text{SHN3} \) activity is mediated by control of genes associated with cutin metabolism and epidermal cell patterning (Shi et al., 2013). In barley, on the basis of positional cloning, a \( Nud \) gene on chromosome arm 7HL that has homology to the Arabidopsis \( \text{WIN1}/\text{SHN1} \) TF, was proposed to control biosynthesis of the hull (hulled caryopses have caryopses with adhering hulls at maturity, whereas the hulless or naked caryopses are free-threshing variant phenotypes) (Taketa et al., 2008).

Literature-based studies coupled with our bioinformatics analysis on the protein–DNA interactions network using STITCH 4 software highlighted \( \text{WIN1} \) TF (Supplementary Fig. S3) as potentially having a significant role in FHB resistance. \( \text{HvWIN1} \) was cloned from five different barley genotypes (Supplementary Table S5), including CI9831 and H106-371, to find if there is any functional polymorphism. While the \( \text{HvWIN1} \) sequence was the same at coding sequence level, the susceptible genotype had an extra intron (Supplementary Fig. S4A, B). Many genes are known to differ in intron number and arrangement without affecting function. Analysis of transcript abundance of \( \text{HvWIN1} \) suggested enhanced gene expression for resistant genotype CI9831 upon pathogen inoculation (Fig. 4). Since similar levels of expression were detected in RM and SM samples, the presence of an extra intron does not affect constitutively the basal levels of \( \text{HvWIN1} \) expression. Since the absence of an intron was found in just one resistant genotype (CI9831), further studies including sequencing of \( \text{WIN1} \) from other resistant genotypes and their validation are needed to conclude the role of introns in gene regulation and disease resistance. The variation in gene expression could be due to a number of other reasons and is beyond the scope of this study.

Association of RR metabolites with genes is not enough to claim the role of that gene in FHB resistance, and they need to be functionally validated. To prove the role of \( \text{HvWIN1} \) in FHB disease resistance and transcriptional regulation of downstream target genes, we carried out transient gene silencing of \( \text{HvWIN1} \) in FHB disease resistance and transcriptional regulation of downstream target genes, we carried out transient gene silencing of \( \text{HvWIN1} \) based on VIGS (Fig. 5A), which is an easy and rapid knockdown technique to study gene function in plant development, biotic and abiotic stress resistance (Senthil-Kumar et al., 2008; Cakir et al., 2010; Ramegowda et al., 2014) and has been successfully utilized in several crops like barley, wheat and potato (Scofield et al., 2005; Lee et al., 2012; Delventhal et al., 2014; Yogendra et al., 2015a). In our study, silencing of \( \text{HvWIN1} \) in CI9831 resulted in a susceptible phenotype as evidenced by increased fungal biomass measured by increased copy number of \( \text{tr} \) (Fig. 5C). Also, the transcript abundance of \( \text{HvWIN1} \) was significantly reduced due to knockdown of the gene (Fig. 5D). These results present compelling evidence of the involvement of \( \text{HvWIN1} \) in FHB disease resistance. Since the amount of FFAs like linoleate (FC = 39.00–3.09) and palmitic acid (FC = 6.56–1.3) was decreased dramatically, it was inferred that \( \text{HvWIN1} \) has a role in the regulation of fatty acid biosynthetic genes. However, transcript expression analysis of \( \text{KAS2} \) did not reflect the same upon silencing of \( \text{HvWIN1} \). This could be due to the involvement of other fatty acid biosynthetic genes such as \( \text{acyl-CoA carboxylase} \), or other genes. A TF can bind to several downstream \( R \) genes and regulate biosynthesis of several RR metabolites. However, the high upregulation of \( \text{CYP86A2}, \text{CYP89A2} \) and \( \text{LACS2} \) upon pathogen invasion in resistant genotype CI9831 was significantly reduced upon \( \text{HvWIN1} \) silencing (Fig. 6), proving regulation of these genes by this TF. In one of the studies in Arabidopsis, \( \text{LACS2} \) was proved to be directly targeted by \( \text{WIN1} \) (Kannangara et al., 2007). Our results suggest that these genes may be under the transcriptional control of \( \text{WIN1} \). However, promoter analysis of these genes, except for \( \text{KAS2} \) to which promoter sequence was not available, failed to lead to any significant insight, as these promoters did not have the GCC box that is necessary for \( \text{WIN1} \) to bind to these promoters. This was consistent with other studies in Arabidopsis where, surprisingly, none of the promoters under \( \text{WIN1} \) control contained identifiable GCC boxes (Kannangara et al., 2007). It seems that \( \text{HvWIN1} \) either controls FFA biosynthesis and cuticle biosynthesis genes using some unknown mechanism or in coordination with some other TFs that may regulate these genes (Alves et al., 2014). A similar kind of conclusion has been made in another study in Arabidopsis (Kannangara et al., 2007). In Arabidopsis and Torenia fournieri, MIXTA-like \( \text{MYB} \) TFs \( \text{MYB106} \) and \( \text{MYB16} \) that regulated epidermal cell morphology, were also shown to regulate cuticle development coordinately with \( \text{HvWIN1}/\text{SHN1} \) (Oshima et al., 2013).

In conclusion, our metabolomics, gene expression and functional validation data revealed the important role of \( \text{HvWIN1} \) in regulating genes involved in cuticle biosynthesis. Based on our results, we have proposed a model (Fig. 7) explaining the regulation of genes involved in FFA
biosynthesis by HvWIN1 upon F. graminearum infection, leading to reinforcement of the cuticle. Since the germ tube of F. graminearum enters spikelets between the lemma and palea, gaining access through stomata on the inner side of the lemma and palea, or by direct penetration through the cuticle, the deposition of these FFAs to reinforce the cuticle can pose a physical barrier leading to disease resistance. Taken together, HvWIN1 could be used in breeding programs or used to replace it in FHB-susceptible cultivars, if this gene is nonfunctional, based on genome editing to improve resistance in barley against FHB (Shan et al., 2014).

Supplementary data

Supplementary data are available at JXB online.

Fig. S1.Canonical discriminant analysis of significant (P<0.05) metabolites in spikelets of barley resistant (C19831) and susceptible (H106-371) genotypes upon F. graminearum or mock inoculation.

Fig. S2. In silico fragmentation of resistance-related (RR) metabolites.

Fig. S3. Protein–DNA interactions network for WIN1 TF based on integration of experimental and manually curated evidence with text-mining information and interaction predictions using STITCH 4 software (http://stitch.embl.de).

Fig. S4. (A) Alignment of nucleotide sequence of HvWIN1 from C19831 (resistant) with susceptible barley genotypes (H106-371, AC Metcalfe, CDC Copeland, Zhedar-2). (B) Alignment of amino acid sequence of coding regions of HvWIN1 from C19831 (resistant) and susceptible barley (H106-371, AC Metcalfe, CDC Copeland, Zhedar-2) genotypes.

Table S1. Primers used for the expression analysis of various H. vulgare genes involved in cuticle biosynthesis.

Table S2. BLAST analysis of HvWIN1 fragment used for VIGS experiment.

Table S3. Resistance-related (RR) metabolites (P>0.05) detected in the spikelets of barley genotypes inoculated with water or spores of F. graminearum.

Table S4. Details of the genes predicted in barley and used in expression analysis in the present work.

Table S5. List of barley genotypes from which HvWIN1 was sequenced and submitted to the NCBI database with GenBank accession numbers.

Table S6. Promoter analysis of potential HvWIN1 target genes under study.

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References

Aharoni A, Dixit S, Jetter R, Thoenes E, van Arkel G, Pereira A. 2004. The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in Arabidopsis. The Plant Cell 16, 2463–2480.

Aives MS, Dadaalto SP, Goncalves AB, de Souza GB, Barros VA, Fietto LG. 2014. Transcription factor functional protein-protein interactions in plant defense responses. Proteomes 2, 85–106.

Bai G, Shaner G. 2004. Management and resistance in wheat and barley to Fusarium head blight 1. Annual Review of Phytopathology 42, 135–161.

Beisson F, Li Y, Bonaventure G, Pollard M, Ohroge JB. 2007. The acyltransferase GPAT5 is required for the synthesis of suberin in seed coat and root of Arabidopsis. The Plant Cell Online 19, 351–368.

Beisson F, Li-Beisson Y, Pollard M. 2012. Solving the puzzles of cutin and suberin polymer biosynthesis. Current Opinion in Plant Biology 15, 329–337.

Beld J, Lee DJ, Burkard MD. 2015. Fatty acid biosynthesis revisited: structure elucidation and metabolic engineering. Molecular BioSystems 11, 38–59.

Bollina V, Kumaraswamy GK, Kushalappa AC, Choo TM, Dion Y, Rioux S, Faubert D, Hamzehzarghani H. 2010. Mass spectrometry-based metabolomics application to identify quantitative resistance-related metabolites in barley against Fusarium head blight. Molecular Plant Pathology 11, 769–782.

Borisjuk N, Hrmova M, Lopato S. 2014. Transcriptional regulation of cuticle biosynthesis. Biotechnology Advances 32, 526–540.

Broun P, Poindexter P, Osborne E, Jiang CZ, Riechmann JL. 2004. WIN1, a transcriptional activator of epidermal wax accumulation in Arabidopsis. Proceedings of the National Academy of Sciences, USA 101, 4706–4711.

Bushnell W. 2001. What is known about infection pathways in Fusarium head blight? National Fusarium Head Blight Forum Proceedings, 2001.105.

Cakir C, Gillespie ME, Scofield SR. 2010. Rapid determination of gene function by virus-induced gene silencing in wheat and barley. Crop Science 50, S77–S84.

Cakir C, Scofield S. 2008. Evaluating the ability of the barley stripe mosaic virus-induced gene silencing system to simultaneously silence two wheat genes. Cereal Research Communications 36, 217–222.

Chamarti SK, Kumar K, Gunnaiah R, Kushalappa AC, Dion Y, Choo TM. 2014. Identification of fusarium head blight resistance related metabolites specific to doubled-haploid lines in barley. European Journal of Plant Pathology 138, 67–78.

Chen G, Komatsuda T, Ma JF, et al. 2011. An ATP-binding cassette subfamily G full transporter is essential for the retention of leaf water in both wild barley and rice. Proceedings of the National Academy of Sciences, USA 108, 12354–12359.

Choo TM, Vegier B, Shen QQ, Martin RA, Ho KM, Savard M. 2004. barley traits associated with resistance to fusarium head blight and deoxynivalenol accumulation. Phytopathology 94, 1145–1150.

Delventhal R, Falter C, Strugala R, Zellerhoff N, Schaffrath U. 2014. Ectoparasitic growth of Magnaporthe on barley triggers expression of the putative barley wax biosynthesis gene CYP96B2 which is involved in penetration resistance. BMC Plant Biology 14, 26.

Domínguez E, Heredia-Guerrero JA, Heredia A. 2015. Plant cuticle genes: unanswered questions. Trends in Plant Science 20, 551–558.

Grausem B, Widemann E, Verdier G, Nosbächs D, Aubert Y, Beisson F, Schreiber L, Franke R, Pinot F. 2014. CYP77A19 and CYP77A20 characterized from Solanum tuberosum oxidize fatty acids in vitro and partially restore the wild phenotype in an Arabidopsis thaliana cutin mutant. Plant, Cell & Environment 37, 2102–2115.

Gunnaiah R, Kushalappa AC, Duggavathi R, Fox S, Somers DJ. 2012. Integrated metabolite- proteomic approach to decipher the mechanisms by which wheat QTL (Fhb1) contributes to resistance against Fusarium graminearum. PloS One 7, e40695.

Hamzehzarghani H, Kushalappa A, Dion Y, Rioux S, Comeau A, Yaylayan V, Marshall W, Mather DE. 2005. Metabolic profiling and factor analysis to discriminate quantitative resistance in wheat cultivars against fusarium head blight. Physiological and Molecular Plant Pathology 66, 119–133.

Jansen C, Von Wettstein D, Schäfer W, Kogel K-H, Felk A, Maier FJ. 2005. Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted Fusarium graminearum. Proceedings of the National Academy of Sciences, USA 102, 16892–16897.
Kachroo A, Kachroo P. 2009. Fatty acid-derived signals in plant defense. Annual Review of Phytopathology 47, 153–176.

Kang Z, Buchenauer H. 2000. Ultrastructural and immunocytochemical investigation of pathogen development and host responses in resistant and susceptible wheat spikes infected by Fusarium culmorum. Physiological and Molecular Plant Pathology 57, 255–268.

Kannangara R, Branigan C, Liu Y, Penfield T, Rao V, Mouille G, Hoffe H, Pauly M, Riechemann, Broun P. 2007. The transcription factor WIN1/SHN1 regulates cutin biosynthesis in Arabidopsis thaliana. The Plant Cell 19, 1278–1294.

Kuhn M, Szkлярzny P, Dletscher-Frankild S, Blicher TH, von Mering C, Jensen LJ, Bork P. 2014. STITCH 4: integration of protein-chemical interactions with user data. Nucleic Acids Research 42, D401–D407.

Kumar A, Karre S, Dhokane D, Kage U, Hukkeri S, Kushalappa AC. 2015. Real-time quantitative PCR based method for the quantification of fungal biomass to discriminate quantitative resistance in barley and wheat genotypes to fusarium head blight. J. Cereal Sci 64, 16–22.

Kumaraswamy GK, Bollina V, Kushalappa AC, Choo TM, Dion Y, Rioux S, Mamer O, Faubert D. 2011a. Metabolomics technology to phenotype resistance in barley against Giberella zeae. European Journal of Plant Pathology 130, 29–43.

Kumaraswamy KG, Kushalappa AC, Choo TM, Dion Y, Rioux S. 2011b. Mass spectrometry based metabolomics to identify potential biomarkers for resistance in barley against fusarium head blight (Fusarium graminearum). Journal of Chemical Ecology 37, 846–856.

Kunst L, Samuels L. 2009. Plant cuticles shine: advances in wax biosynthesis and export. Current Opinion in Plant Biology 12, 721–727.

Kushalappa AC, Gunnaiah R. 2013. Metabo-lo-proteomics to discover plant biotic stress resistance genes. Trends in Plant Science 18, 522–531.

Kushalappa AC, Yogendra KN, Karre S. 2016. Plant innate immune response: quantitative and qualitative resistance. Critical Reviews in Plant Science 35, 38–55.

Lee WS, Hammond-Kosack KE, Kanyuka K. 2012. Barley stripe mosaic virus-mediated tools for investigating gene function in cereal plants and their pathogens: virus-induced gene silencing, host-mediated gene silencing, and virus-mediated overexpression of heterologous protein. Plant Physiology 160, 582–590.

Li Y, Beisson F, Koo AJ, Molina I, Pollard M, Ohroge J. 2007. Identification of acyltransferases required for cutin biosynthesis and production of cutin with suberin-like monomers. Proceedings of the National Academy of Sciences, USA 104, 18339–18344.

Lirak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25, 402–408.

Ma M, Yan Y, Huang L, Chen M, Zhao H. 2012. Virus-induced gene-silencing in wheat in skies and grains and its application in functional analysis of HMW-GS-encoding genes. BMC Plant Biology 12, 141.

McMullen M, Bergstrom G, De Wolf E, Dill-Macky R, Hershman D, Scofield SR, Huang L, Brandt AS, Gill BS. 2012. A land-plant-specific glycerol-3-phosphate acyltransferase with sn-2 preference and phosphatase activity producing 2-monoacylglycerol. Proceedings of the National Academy of Sciences, USA 107, 12040–12045.

Yeats TH, Rose JK. 2013. The formation and function of plant cuticles. Plant Physiology 163, 5–20.

Yogendra KN, Kumar A, Sarkar K, et al. 2015a. Transcription factor SlWPKY1 regulates phenylpropanoid metabolites conferred late blight resistance in potato. Journal of Experimental Botany 66, 7377–7389.

Yogendra KN, Kushalappa AC, Sarmiento F, Rodriguez E, Mosquera T. 2015b. Metabolomics deciphers quantitative resistance mechanisms in diploid potato clones against late blight. Functional Plant Biology 42, 284–298.

Zadoks JC, Chang TT, Konzak CF. 1979. A decimal code for the growth stages of cereals. Weed Research 14, 415–421.

Zhu H, Gilchrist L, Hayes P, Kleinhofs A, Kudrna D, Liu Z, Prom L, Steffenson B, Toojinda T, Vivar H. 1999. Does function follow form? Principal QTLs for Fusarium head blight (FHB) resistance are coincident with QTLs for inflorescence traits and plant height in a doubled-haploid population of barley. Theoretical and Applied Genetics 99, 1221–1232.