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RESEARCH PAPER

Gene expression profiling and silencing reveal that monolignol biosynthesis plays a critical role in penetration defence in wheat against powdery mildew invasion

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Abstract

Cell wall apposition (CWA) formation is one of the first lines of defence used by plants to halt invading fungi such as powdery mildew. Lignin is a complex polymer of hydroxylated and methoxylated phenylpropane units (monolignols) and lignification renders the cell wall more resistant to pathogen attack. The role of monolignol biosynthesis in CWA-mediated defence against powdery mildew penetration into cereals is demonstrated here using RNA interference (RNAi)-mediated gene silencing and enzyme-specific inhibitors. Thirteen cDNAs representing eight genes involved in monolignol biosynthesis were cloned from an expression sequence tag (EST) library derived from the epidermis of diploid wheat (*Triticum monococcum*) infected with *Blumeria graminis* f. sp. *tritici* (*Bgt*). Differential expression patterns were found for these genes in susceptible and resistant plants after infection. Transcripts of phenylalanine ammonia lyase (*PAL*), caffeic acid *O*-methyltransferase (*CAOMT*), ferulic acid hydroxylase (*FAH*), caffeoyl-CoA *O*-methyltransferase (*CCoAMT*), and cinnamyl alcohol dehydrogenase (*CAD*) were accumulated, particularly in the epidermis. RNAi-mediated transient gene silencing in the epidermis led to a higher penetration efficiency of *Bgt* than in the controls. Gene silencing also compromised penetration resistance to varying degrees with different genes against an inappropriate pathogen, *B. graminis* f. sp. *hordei* (*Bgh*). Co-silencing led to greater penetration of *Bgt or Bgh* than when the genes were silenced separately. Fluorescence emission spectra analyses revealed that gene silencing hampered host autofluorescence response at fungal contact sites. These results illustrate that monolignol biosynthesis is critically important for host defence against both appropriate and inappropriate pathogen invasion in wheat.

Key words: Cell autofluorescence, cell wall apposition, cereal, methylated monolignol.

Introduction

Lignin is, after cellulose, the second most abundant terrestrial biopolymer accounting for approximately 30% of the organic carbon in the biosphere (Boerjan *et al.*, 2003). It is crucial for the structural integrity of plant cell walls. Lignin deposition is one of the final stages of xylem cell differentiation and takes place mainly during secondary thickening of the cell wall (Chen *et al.*, 2006). It is a complex polymer of hydroxylated and methoxylated phenylpropane units linked via oxidative coupling, probably catalysed by both peroxidases and laccases (Boudet *et al.*, 1995). Lignin from angiosperms usually contains two major monolignols, 4-hydroxy, 3-methoxy substituted guaiacyl (G) units and 4-hydroxy, 3,5-dimethoxy substituted syringyl (S) units, joined by at least five different types of linkages (Davin and Lewis, 1992; Fig. 1).

Lignification has the potential to act in several ways in plant defence against pathogen infection. It can establish mechanical barriers to pathogen invasion, chemically modify

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Abbreviations: PAL, phenylalanine ammonia lyase; C3H, *p*-coumarate 3-hydroxylase; CAOMT, caffeic acid *O*-methyltransferase; F5H, ferulic acid 5-hydroxylase; 4CL, 4-hydroxycinnamoyl-CoA ligase; CCoAMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase.

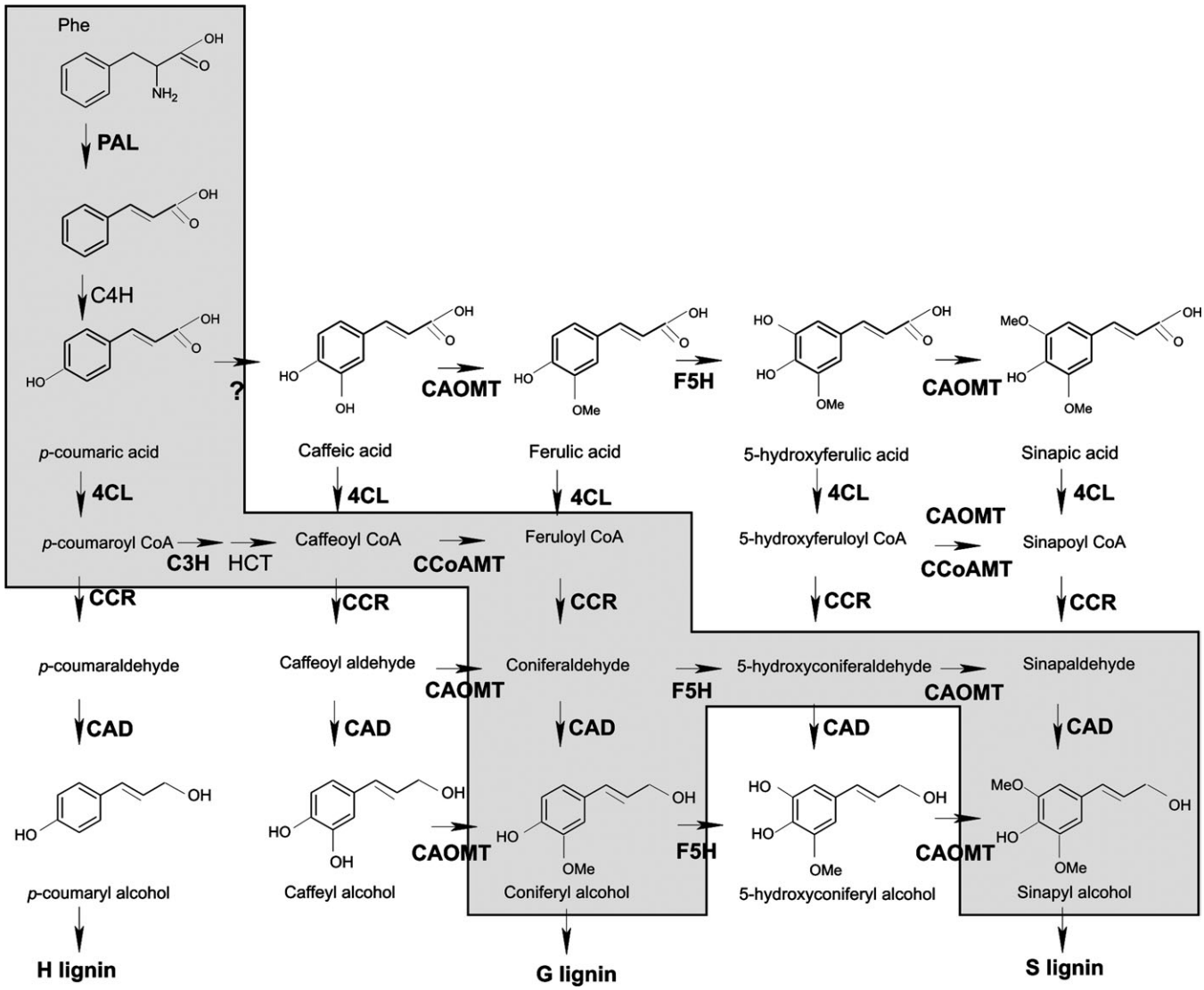

Fig. 1. The monolignol biosynthesis pathway. The grey route is the most favoured in angiosperms. All the enzymatic reactions presented in the pathway have been demonstrated at least *in vitro*. Because of the variety of isoenzymes and kinetic properties, alternative routes through the metabolic pathway may exist. For reactions with a question mark, the respective enzyme is unknown. PAL, phenylalanine ammonia lyase; C3H, *p*-coumarate 3-hydroxylase; CAOMT, caffeic acid *O*-methyltransferase; F5H, ferulic acid 5-hydroxylase; 4CL, 4-hydroxycinnamoyl-CoA ligase; CCoAMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; HCT, *p*-hydroxycinnamoyl-CoA reductase; C4H, cinnamate 4-hydoxylase.

cell walls to be more resistant to cell wall-degrading enzymes, increase the resistance of walls to the diffusion of toxins from the pathogen to the host and of nutrients from the host to the pathogen, produce toxic precursors and free radicals, and lignify and entrap the pathogen (Nicholson and Hammerschmitt, 1992). Lignification renders the cell wall more resistant to mechanical pressure applied during penetration by fungal appressoria (Vance *et al.*, 1980) and more water resistant and thus less accessible to cell wall-degrading enzymes. In addition to the role of lignin as a preformed resistance factor, induced lignification has been proposed as an active resistance mechanism of plants to fungi (Nicholson and Hammerschmitt, 1992).

The phenylpropanoid pathway that provides the lignin-building monolignol units is strongly activated after infection by pathogens or treatment with elicitors (Pakusch and Matern, 1991; Jaeck *et al.*, 1992). In infected plants, deposition of phenylpropanoid compounds is a part of the cell wall reinforcement that restricts pathogen invasion (Nicholson and Hammerschmitt, 1992; Kauss *et al.*, 1993). Accumulation of soluble and cell wall–bound phenolics in plant tissues challenged by fungal pathogens has also been suggested as a defence strategy (von Röpenack *et al.*, 1998).

Attempts by fungi to penetrate plant tissues are often accompanied by the localized appositional growth of the plant cell walls at the point of attack called 'papilla'. Histochemical and solubility tests indicated that lignified material is a major component of these cell wall appositions (CWAs) and surrounding halo areas. Lignification was detected within 4 h after CWAs became visible, and

intensified with time after infection with a compatible pathogen in wheat, Japanese radish, and other species (Nicholson and Hammerschmitt, 1992). When compatible and incompatible pathogens were inoculated into a given species, greater concentrations of induced lignin were sometimes recovered from resistant than from susceptible tissues (Nicholson and Hammerschmitt, 1992).

CWAs are associated with H_2O_2 production at the site of fungal infection (Thordal-Christensen *et al.*, 1997; Wei *et al.*, 1998), which is involved in cross-linking of cell wall structural protein (Levine *et al.*, 1994) and essential for the formation of lignin polymer precursors (Christensen *et al.*, 2000). Recently, it was shown that Fe^{3+} accumulation at the fungal interaction site is required for H_2O_2 accumulation (Liu *et al.*, 2007). CWAs are thought to block penetration of pathogens if they are deposited rapidly and contain antifungal phenolic substances in sufficient quantities (Bushnell, 2002). Through the use of *mlo*-barley (resistant to barley powdery mildew penetration), it was demonstrated that, unlike normal cell walls, mature CWA areas could not be digested with cellulases; they retain their autofluorescence after cellulase treatment (von Röpenack *et al.*, 1998). In penetration-resistant wheat, removing lignin from the leaf by irradiating with UV light also removed autofluorescence and allowed cellulose digestion. Thus, cellulose fibres in the CWA regions are tightly bonded to phenolics/lignins, making them impervious to cellulase enzymes and, presumably, more difficult for fungi to penetrate (Bushnell, 2002). In highly resistant wheat cultivars, infected plant cells react hypersensitively and die within a few hours after fungal penetration, which prevents the formation of functional haustoria and, hence, further infection. The hypersensitive response (HR) is accompanied by intracellular accumulation of lignin or lignin-like material (Beardmore *et al.*, 1983).

Thus, lignin in CWAs and HR cells is an indicator of the contribution made by oligomeric or polymeric phenolic compounds to basal and non-host resistance (Huckelhoven, 2007). For instance, inhibitors of PAL, such as α-aminooxy-β-phenylpropaonic acid, 2-aminoindan-2-phosphonic acid or inhibitor of CAD, such as 2-hydroxyphenylalanine acetic acid (1,l-dimethyl ester) were reported to increase the susceptibility of barley to *Blumeria graminis* (Kruger *et al.*, 2002) and to decrease the resistance of *Arabidopsis* to *Peronospora parasitica* (Mauch-Mani and Slusarenko, 1996).

Induced lignification around the penetration sites or infection centres is generally accompanied by the increased activity of a number of enzymes. Inoculated wheat shows increased PAL and *O*-methyltransferase activities as localized lignin deposition occurs (Nicholson and Hammerschmitt, 1992). The accumulation of several defence-related transcripts has also been reported after barley is infected with powdery mildew, including *PAL* (Boyd *et al.*, 1995) and cinnamoyl-CoA reductase (*CCR*) (Caldo *et al.*, 2004). Genetic evidence for the role of monolignol in cell wall-associated defence is rare, however, either because of the indispensability of lignin for the plant form or as a result of the redundancy among enzymes involved in the monolignol pathway. The creation of a metabolic sink for tryptophan by the introduction of tryptophan decarboxylase by genetic means resulted in a decrease of soluble and cell wall-bound phenolics in potato, which, in turn, enhanced susceptibility to *Phytophthora infestans* (Yao *et al.*, 1995). Transgenic tobacco with suppressed expression of PAL showed reduced basal resistance to *Cercospora nicotianae* (Maher *et al.*, 1994). No global gene expression or genetic studies have been reported to date to reinforce correlative evidence of the role of monolignol biosynthesis in CWA-associated penetration defence against powdery mildew attack in cereals.

In a previous study (Bhuiyan *et al.*, 2007), it was demonstrated that the transcriptional activation of genes of methyl unit biosynthesis was linked to CWA formation in response to *Bgt* infection of diploid wheat (*einkorn* wheat*; Triticum monococcum;* hereafter wheat). From a series of gene expression studies it was also demonstrated that methyl units were transferred to lignin, polyamine, and ethylene pathways rather than to glycine betaine or nicotianamine biosynthesis following pathogen invasion (Bhuiyan *et al.*, 2007). The regulation of monolignol biosynthetic genes in response to powdery mildew infection is shown here and their functional role in penetration defence through the use of RNAi gene silencing is demonstrated. It was found that silencing genes in this pathway increased susceptibility to *Bgt* infection and compromised resistance to a non-host pathogen, *Bgh*, in wheat. In addition, pharmacological studies with inhibitors of PAL and CAOMT lent support to conclusions derived from molecular genetic analyses.

Materials and methods

Plant materials and powdery mildew infection

Powdery mildew-susceptible Tm441 (accession number TG13182) and CWA-based resistant Tm453 (accession number TG13192) lines of *Triticum monococcum* L. (AA genome) (Liu *et al.*, 2005; Bhuiyan *et al.*, 2007) were used throughout. Plant growth and maintenance of the powdery mildew isolates were performed as previously described by Bhuiyan *et al.* (2007).

Plasmid construct

RNAi constructs were made by a combined ligation/recombination (LR) method using plasmid pIPKTA30N as the final GATEWAY destination vector (Douchkov *et al.*, 2005). For entry vector constructs, EST sequences of target genes (*TmPAL*, *TmCAOMT*, *TmCCoAMT*, and *TmCAD*) were amplified by PCR using EST specific primers. Purified DNA from the selected cDNA clones was used as the template for the PCR reaction. Amplified fragments were ligated into the TOPO cloning vector as described by the manufacturer, Invitrogen. The positive clones were used in the LR reaction. EST fragments in an entry vector were cloned into the RNAi destination vector pIPKTA30N as inverted repeats by a single LR reaction, for which Gateway

LR clonaseII enzyme mix (Invitrogen) was used. All constructs were verified by sequencing of both inverted repeats after PCR amplification.

Microprojectile bombardment and challenge inoculation

Leaf segments of 7-d-old wheat seedlings were used for microprojectile bombardment as described by Christensen *et al.* (2004). Plasmid pCAMBIA3301:GUS or plasmid pUbi:GFP served as internal controls of transformation efficiency. For experiments measuring the effect of pathogen attack, leaf segments were bombarded with a GFP or GUS together with an empty RNAi vector construct followed by challenge inoculation with either *Bgh* or *Bgt* 4 h after bombardment. For experiments measuring the effect of individual gene silencing on host or non-host–pathogen interactions, wheat leaf segments were co-bombarded with an RNAi construct plus pCAMBIA 35S:GUS or pUbi:GFP followed by challenge inoculation with *Bgt* or *Bgh* after 4 h of bombardment. Inoculation density was usually 180–220 conidia mm^{-2}. Interaction phenotypes of GUS-stained, transformed epidermal cells were determined 40 h after inoculation via light microscopy by counting the number of GUS-stained cells and the number of fungal haustoria in these cells. Fungal structures in transformed cells were stained with Coomassie Brilliant Blue. In the case of GFP, either epi-fluorescence or confocal microscopy (LSM510: Zeiss, Oberkochen, Germany) was used. Penetration efficiency was calculated as the number of penetrated cells divided by the number of attacked cells multiplied by 100 and used as a measure of the resistance of bombarded cells.

Northern blot analysis

Isolation of total RNA by phenol/chloroform extraction and Northern blotting were performed as described by Bhuiyan *et al.* (2007). Samples (20 μg) were separated on a 1.2% denaturing agarose gel, stained with 0.02% methylene blue, photographed to allow for comparison of RNA loading, transferred onto a GeneScreen Plus Hybridization Transfer membrane (NEN Life Science Products, Inc. Boston), UV cross-linked or baked at 80 °C, and hybridized at 65 °C in Quickhyb solution (Stratagene) with [^{32}P] dCTP radiolabelled probes consisting of the entire coding region of the genes under investigation. All Northern blots were repeated independently at least twice.

Semi-quantitative reverse transcriptase polymerase chain reaction

Semi-quantitative RT-PCR was performed as described by Bhuiyan *et al.* (2007) with some modifications. Total RNA was isolated from the *Bgt*-infected epidermal and mesophyll cells of the susceptible line. cDNA syntheses were performed using superscript reverse transcriptase (Invitrogen Life Technologies, Burlington, ON, Canada). mRNA was isolated from total RNA by the PolyATract mRNA isolation system (Promega Corporation, Madison, WI, USA). To ensure that equal amounts of mRNA were used, a positive control encoding the *TmGAPD* gene was normalized using primers GAPDF and GAPDR. PCR amplifications were performed using *Taq* polymerase (Amersham Biotech, Piscataway, NJ, USA) under the following conditions: 94 °C for 20 s, 52–62 °C for 30 s, and 72 °C for 60 s for 30 cycles in a thermal Eppendorf mastercycler (Hamburg, Germany). RT-PCR of all clones was repeated independently at least twice.

Treatment with inhibitors

Harvested leaves had their cut ends immediately immersed in distilled water or inhibitor solution and were left to take up their respective solutions for 6 h. Leaves were then removed from solutions and placed, adaxial sides up, on 0.5% agar for inoculation. One to two hours after inoculation, the cut ends were again submerged in distilled water after being trimmed to remove air embolisms. Leaves were then returned to their respective solutions and incubated for another 36 h at room temperature.

Microscopy study

The storage of leaf segments, the staining of fungal structures and microscopy were carried out as described by Bhuiyan *et al.* (2007). To detect autofluorescent compounds, the epidermis was cleared in 95% ethanol, equilibrated in a solution of lactic acid, glycerol, and water (1:1:1 by vol.), mounted, and examined by an epi-fluorescence or confocal microscope LSM510 (Zeiss, Oberkochen, Germany). To visualize callose, the method of Humphry *et al.* (2006) was followed. Emission spectra of CWA and halo areas of transformed cells were recorded from 410 nm to 590 nm at an excitation wavelength of 405 nm.

Results

Expression of genes involved in monolignol biosynthesis are induced in response to Bgt *attack*

A cDNA library derived from wheat epidermis infected with *Bgt* (Liu *et al.*, 2005; Bhuiyan *et al.*, 2007) was searched in order to identify monolignol biosynthesis specific ESTs to investigate the role of monolignol biosynthesis in CWA-mediated defence against powdery mildew invasion. A complete set of eight monolignol biosynthetic genes was found from among 13 cDNAs. The nucleotide and protein sequences of all cDNAs were analysed by comparison with the database using BLAST and the closest orthologues were identified (Table 1).

The temporal expression pattern of the eight monolignol genes, in response to powdery mildew infection of a pair of susceptible and resistant wheat lines, was assessed over a 144 h post inoculation (hpi) period by Northern blotting (Fig. 2). The gel blot data show that the steady-state level of *TmPAL, TmCCR, TmCCoAMT, TmF5H, TmCAOMT*, and *TmCAD* transcripts were accumulated in response to infection but at different time points in the susceptible line. *TmPAL* and *TmCCR* showed the earliest expression at 3

Table 1. *List of expressed sequence tags of monolignol genes isolated from* Blumeria graminis *f. sp. tritici infected wheat (*Triticum monococcum*) epidermis cDNA library*

Accession no.	Gene name	Copy	Closest orthologue (accession no.; identity; reference)	*E* value
EU099348	*TmPAL*	2	*Triticum aestivum* (AY005474; 98%)	4e-123
EU099347	*TmC3H*	1	*Oryza sativa* (XM_482757; 74%)	3e-93
EU099349	*TmF5H*	2	*O. sativa* (NM_197316; 83%)	4e-50
EU099350	*Tm4CL*	2	*O. sativa* (X52623; 80% ; Zhao *et al.*, 1990)	2e-46
EU099351	*TmCCoAMT*	1	*O. sativa* (AY644637; 89%)	4e-91
EU099352	*TmCCR*	1	*Lolium perenne* (AY061888; 80%; McInnes *et al.*, 2002)	1e-22
EU099353	*TmCAD*	3	*O. sativa* (DQ234272; 84%; Zhang *et al.*, 2006)	3e-157
DQ862834	*TmCAOMT*	1	*T. aestivum* (AY226581; 94%)	1e-70

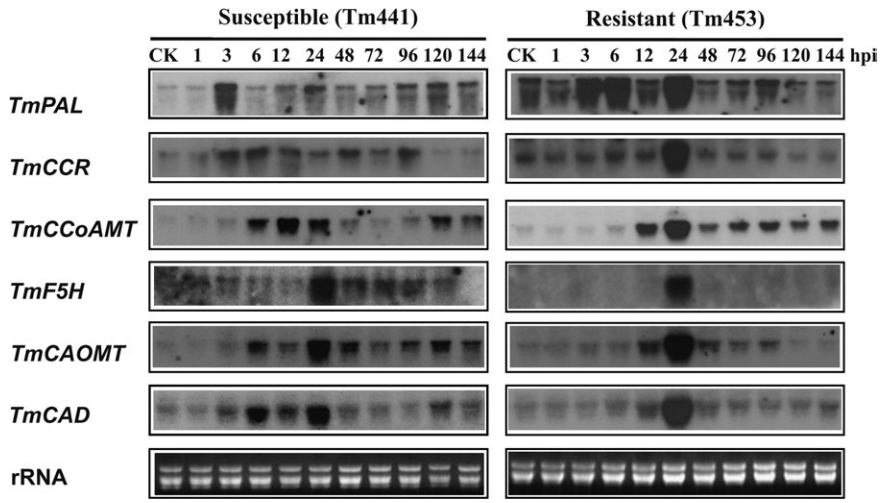

Fig. 2. Northern blot analysis of monolignol biosynthesis genes in response to *Blumeria graminis* f.sp. *tritici* infection. Total RNA was isolated at 0–144 hpi from 10-d-old primary leaves inoculated with *B. graminis* f.sp. *tritici* conidia. Uninoculated leaves were used as controls (CK). The transcript levels of the genes were analysed with ^{32}P-labelled cDNA probes for *TmPAL*, *TmCAOMT*, *TmF5H*, *TmCCoAMT*, *TmCCR*, and *TmCAD*. Total RNA was loaded at 20 μg per lane and equal loading was monitored by methylene blue staining of ribosomal RNA.

hpi, that was also observed with a peroxidase gene, *TmPRX1*, in a previous study (Liu *et al.*, 2005). Transcripts of *TmCAOMT* and *TmCAD* were strongly accumulated at 6 hpi, followed by a slight decrease at 12 hpi and then a sharp increase at 24 hpi. A similar expression pattern was also observed with *TmSAMS1*, a key gene of the methyl cycle in our previous study (Bhuiyan *et al.*, 2007). The expression peaks at 6 hpi and 24 hpi coincide with attempted penetration time points of primary (4–6 hpi) and appressorial germ tubes (16–24 hpi), respectively (Collinge *et al.*, 2002). Transcripts of *TmCCoAMT* were accumulated by 6 hpi, were sharply higher at 12 hpi, decreased through to 96 h before increasing again at 120 hpi and 144 hpi. Expression of *TmF5H* was induced by 24 hpi, and remained induced through to 120 hpi. Transcripts of *Tm4CL* (*4-hydroxycinnamoyl-CoA ligase*) and *TmC3H* (p-*coumarate 3-hydroxylase*) were not accumulated by *Bgt* infection in the susceptible line (data not shown).

During the incompatible interaction of the fungus and the resistant (CWA-mediated) line (Liu *et al.*, 2005; Bhuiyan *et al.*, 2007), transcripts of *TmPAL, TmCCR, TmCCoAMT, TmF5H, TmCAOMT*, and *TmCAD* strongly accumulated at 24 hpi (Fig. 2). A similar pattern of expression was observed in the case of methyl cycle (Bhuiyan *et al.*, 2007) and peroxidase genes (Liu *et al.*, 2005). Unlike the compatible interaction, there was no observable expression peak of these genes at 3 hpi or 6 hpi in the resistant line except moderately in the case of *TmPAL*. Strong expression at the early time points is also reported with *TmMetSyn*, *TmSAMS1* of methyl cycle genes (Bhuiyan *et al.*, 2007), and with a peroxidase gene, *TmPRX1* (Liu *et al.*, 2005). The accumulation of *TmCCoAMT, TmCAOMT*, and *TmCAD* transcripts started to increase at 12 hpi (Fig. 2). Transcripts of *Tm4CL* and *TmC3H* were not accumulated in either line (data not shown) suggesting that a transcriptional regulation of these genes is not directly involved in a response to *Bgt* attack.

Epidermis shows up-regulation of monolignol biosynthesis genes in response to Bgt *infection*

The epidermis provides the major line of defence against infection by the surface-growing powdery mildew fungus.

To investigate further whether the expression patterns of the monolignol biosynthesis genes differ between epidermis and other cell types, samples from susceptible line epidermis and mesophyll tissues were collected at 0 hpi and 24 hpi and amplified by RT-PCR using gene-specific primers. One marker gene, *Cab* (Chlorophyll *a*/*b* binding protein) was tested as an indicator of mesophyll contamination in epidermal tissues. One methyl cycle gene (*TmSAMS1*) and one peroxidase gene (*TmPRX1*) were also tested for comparison with monolignol genes. Distinct expression patterns among monolignol genes were found in the semi-quantitative RT-PCR analysis (Fig. 3).

The expression of *TmPAL* was slightly induced in the epidermis; expression of *TmCAOMT* and *TmF5H* was induced in both epidermis and mesophyll tissues; expression of *Tm4CL* was not induced in either epidermis or mesophyll but, rather, was reduced in mesophyll tissue; the transcript of *TmCCoAMT* was accumulated both in the epidermis and the mesophyll; transcripts of *TmCCR* and *TmCAD* were both accumulated in the epidermis, slightly in the former case, much more so in the latter; the level of mRNA for *TmC3H* was unchanged before and after inoculation in both tissues, a result that correlates well with observations from Northern blotting. These data demonstrated that monolignol genes have distinct tissue-specific expression.

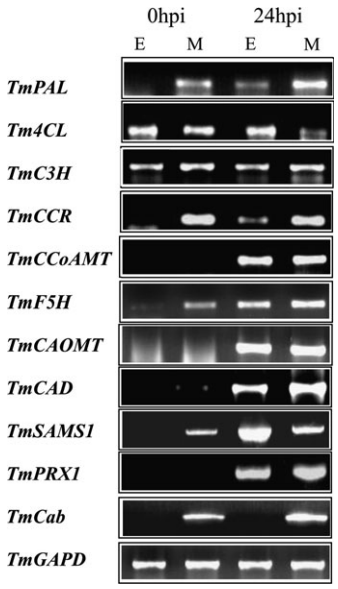

Fig. 3. RT-PCR analysis of tissue-specific expression of monolignol biosynthesis in response to *Blumeria graminis* f.sp. *tritici* infection. *TmSAMS1* (Bhuiyan *et al.*, 2007) and *TmPRX1* (Liu *et al.*, 2005) genes were tested for comparison with the expression pattern of monolignol genes. Total RNA was isolated from epidermal (E) and mesophyll (M) tissues at 0 hpi and 24 hpi and reverse transcribed to cDNA. The expression of a chlorophyll *a*/*b* binding protein (*TmCab*) and a glyceraldehydes 3-phosphate dehydrogenase gene (*TmGAPD*) was used as an indicator of mesophyll contamination in epidermal tissues and a control of mRNA normalization, respectively.

Induction of *TmPAL*, *TmCAOMT*, *TmCCoAMT*, *TmF5H*, *TmCCR*, and *TmCAD* gene expression after pathogen attack was predominantly in the epidermis. This suggests that an elevated level of these transcripts is required for the activation of monolignol biosynthesis in the epidermis as a component of the host–defence response. As demonstrated in previous studies with the methyl cycle (Bhuiyan *et al.*, 2007) and peroxidase genes (Liu *et al.*, 2005), expression of some monolignol genes was also up-regulated in mesophyll tissues suggesting that these genes also play a role in host defence in mesophyll tissues.

Functional role of monolignol biosynthesis genes in response to Bgt *attack*

To characterize the functional role of monolignol genes, RNAi-mediated gene-silencing assays in susceptible wheat epidermal cells were conducted by biolistic transformation and subsequent microscopic analysis of the interaction of *Bgt* with transformed cells. Independent and combinatorial gene silencing with RNAi constructs of *TmPAL*, *TmCAOMT*, *TmCCoAMT*, and *TmCAD* were undertaken, with co-bombardment of GUS- or GFP–expressed constructs as positive controls for transformations. RNAi-based suppression enhanced the penetration efficiency of *Bgt* compared with the controls; the average penetration efficiency was increasing from 40% to about 60% by single gene silencing (Fig. 4). The highest penetration efficiency (64%) was obtained in the case of *TmCAD* silencing. Co-silencing of *TmPAL+TmCAOMT*, *TmPAL+TmCAD*, and *TmCAOMT+TmCAD* was performed in epidermal cells and, in all cases, penetration efficiency was enhanced over that of independent silencing. The marginally highest rate of penetration (78%) was observed with *TmPAL+TmCAD* co-silenced cells (Fig. 4).

Silencing monolignol genes hamper autofluorescence of papilla regions in response to Bgt *infection*

The fluorescence emission spectra of CWAs and the surrounding halo areas beneath the appressorial germ tube (AGT) interaction sites of transformed cells were also analysed to evaluate the autofluorescence response of papilla regions after gene silencing. Autofluorescence intensity was significantly reduced in the papilla region of silenced cells (Fig. 5A, B). In general, the fluorescence intensity of the CWAs is higher than that of the halo area. Fluorescence was decreased in CWAs and halo areas of all independent gene-silenced cells compared with the controls. The source of the peaks appearing in the papilla autofluorescence spectra could not be identified, but it was clear that some peaks were shifted in silenced cells. Co-silencing of *TmPAL+TmCAOMT*, *TmPAL+TmCAD*, and *TmCAOMT+TmCAD* significantly reduced the fluorescence intensity over that of independent silencing (Fig. 5A, B). Among these three combinations, *TmCAOMT+TmCAD* showed the lowest autofluorescence in both CWA and halo areas.

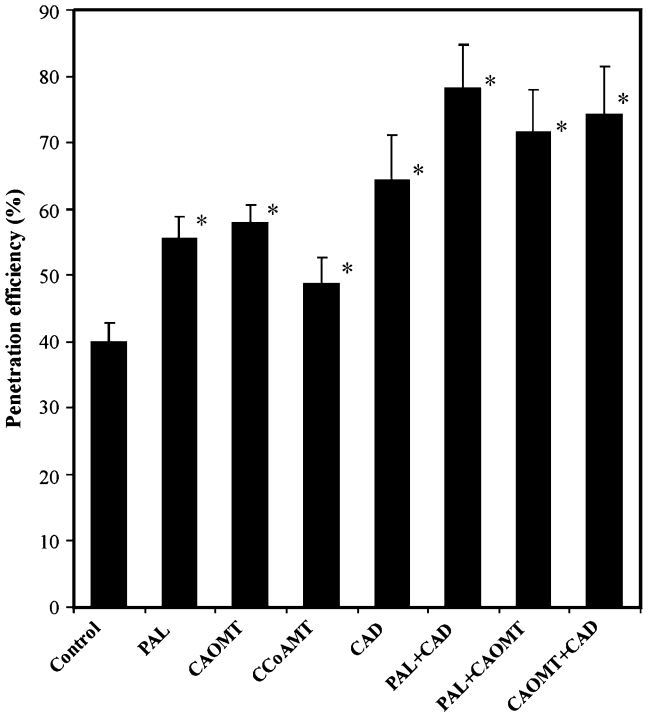

Fig. 4. RNAi-mediated monolignol gene silencing induces super-susceptibility to wheat. Average penetration efficiency of *Bgt* in a wheat susceptible line due to gene silencing. After 4 h of bombardment, leaves were inoculated with a high density of *Bgt* conidiophores and successful entry into epidermal cells was evaluated with microscopy as described in the Materials and methods. Data shown represent mean ±standard deviation from at least three experiments in which, as a minimum, 100 GUS-stained cells were evaluated. Asterisks besides columns indicate $P < 0.05$ (Student's *t* test) compared to the negative control (GUS only).

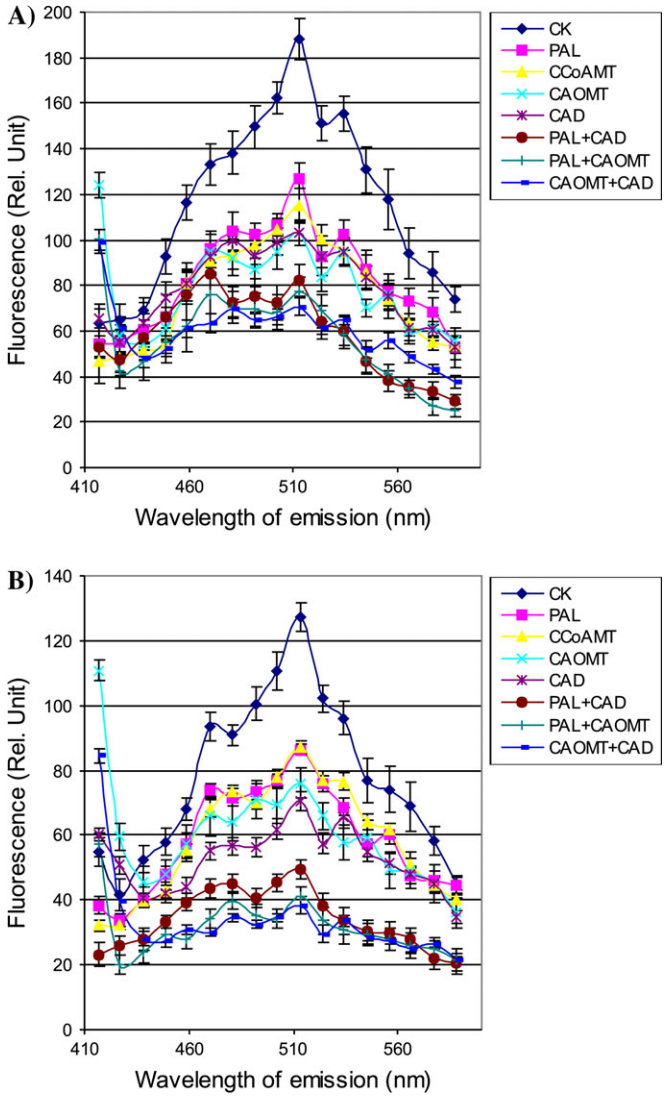

Fig. 5. Fluorescence emission spectra of CWA and halo areas of monolignol gene-silenced cells. (A) Emission spectra of CWAs, (B) emission spectra of halo areas. A binary vector harbouring GUS was co-bombarded with RNAi vectors. HA, halo area; CWA: cell wall apposition. Fluorescence emission spectra and autofluorescence were measured by confocal laser scanning microscopy. Spectra shown are means of replicate measurements of at least ten papillae per treatment. Data shown represent the mean ±standard deviation of ten papillae per treatment. Scale bar corresponds to 10 μm.

Silencing monolignol genes allows penetration of non-host pathogen, Bgh*, in wheat*

Success in making wheat leaves significantly more susceptible to *Bgt* through the down-regulation of monolignol genes by the application of RNAi-mediated gene silencing, led to investigating whether this approach would lead to penetration by inappropriate or non-host pathogens. The independent gene silencing of *TmPAL*, *TmCAOMT*, *TmCCoAMT*, and *TmCAD* compromised penetration resistance to *Bgh* in wheat to varying degrees (Fig. 6A). No penetration was observed in the controls (Fig. 6A, B, upper panel). Figure 6B (lower panel) shows an example of the successful penetration of *Bgh* in a *TmCAOMT*-silenced cell of a wheat leaf where GUS was used as a marker gene. A GFP-expressing *TmCAOMT*-silenced cell was also susceptible to *Bgh* penetration (data not shown). The efficiency of *Bgh* penetration showed differences from those in the case of *Bgt* and individual gene silencing. The most efficient penetration was observed with *TmCAOMT*-silenced cells (11%). Co-silencing increased the penetration ability of *Bgh* with maximum penetration efficiency (15%) obtained when *TmCAOMT* and *TmCAD* were silenced together. Significantly higher efficiencies of penetration were also found with *TmPAL+TmCAD* (9%) and *TmPAL+TmCAOMT* (14%) silenced cells than when these genes were silenced separately (Fig. 6A).

Inhibiting PAL or CAOMT increases penetration efficiency to Bgt *and compromises penetration resistance of* Bgh *in wheat*

PAL is the first enzyme in the phenylpropanoid and monolignol biosynthesis pathways. Previous studies by

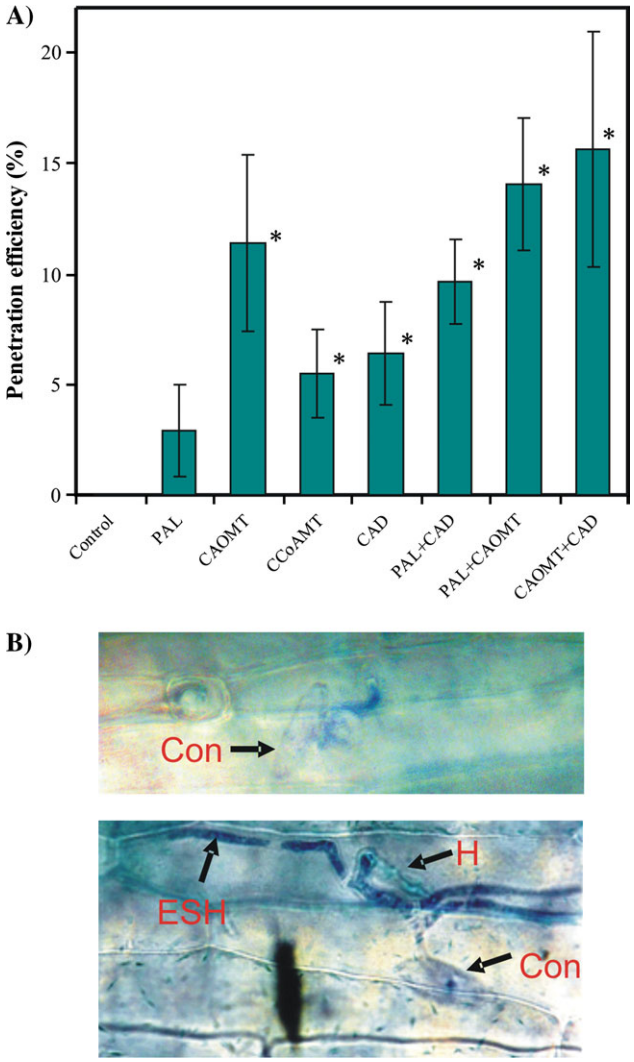

Fig. 6. RNAi-mediated gene silencing compromises penetration resistance of the non-host fungus, *Blumeria graminis* f.sp. *hordei* (*Bgh*). (A) Penetration efficiency of *Bgh* in the susceptible wheat line was evaluated by at least three independent experiments for each construct. Bars represent standard errors. (B) GUS expressing transformed cell that was inaccessible to *Bgh* (upper panel) and a *TmCAOMT*-silenced cell that was penetrated by *Bgh* (lower panel). The fungus formed a haustorium (H) and elongated secondary hyphae (ESH) on the leaf surface. Conidia were indicated by Con. After 4 h of bombardment, leaves were inoculated with a high density of *Bgh* conidiophores and successful entry into the epidermis cells were evaluated with microscopy as described in the Materials and methods. Data shown represent mean ±standard deviation from at least three experiments in which, as a minimum, 100 GUS-stained cells were evaluated. Asterisks besides columns indicate $P < 0.05$ (Student's *t* test) compared to the negative control (GUS only).

others showed that the PAL-specific inhibitors, α-aminooxy-β-phenylpropaonic acid and 2-aminoindan-2-phosphonic acid, at a concentration of 1 mM, led to a reduction in papilla autofluorescence and, at the same time, increased the penetration rate of powdery mildew fungi into barley, oat, and wheat (Carver *et al.*, 1992; Zeyen *et al.*, 2002). Another PAL-specific inhibitor, 4-hydroxybenzoic hydrazide (HBH) (Sigma), was used here and its effect on the penetration efficiency of powdery mildew into wheat leaves was followed. The penetration efficiency of *Bgt* increased at all concentrations of inhibitor up to 1 mM, the highest being from 44% in the control to 60.3% at 0.5 mM inhibitor concentration (Fig. 7A). At 2 mM, the efficiency of penetration decreased, suggesting that this concentration of inhibitor was toxic to the plant.

In the case of CAOMT, pyrocatechol (*O*-dihydroxybenzene; DHB) was used as an inhibitor, which has been shown to be a substrate for CAOMT and thus competes with the normal substrate for the cofactor *S*-adenosyl methionine (SAM) (Poulton and Butt, 1975). Upon *O*-methylation, a methyl group from SAM is transferred to DHB resulting in the formation of *S*-adenosyl-L-homocysteine, which is known to be a potent inhibitor of CAOMT activity (Poulton and Butt, 1975). DHB was applied at concentrations between 0.01 mM and 5 mM, the most efficient being 2.0 mM, which increased the penetration efficiency of *Bgt* from 44% to 58% (Fig. 7B). DHB applied at 5 mM or lower did not cause any damage to or necrotic lesions on wheat leaves.

Different concentrations of HBH and DHB were applied to wheat leaves followed by infection with *Bgh* to evaluate the effect of both inhibitors on the penetration efficiency of an inappropriate pathogen. It was found that both inhibitors compromised the penetration resistance of wheat to this non-host pathogen. In the case of the PAL inhibitor, low frequencies of penetration were observed at concentrations between 0.1 mM and 1 mM, with a peak effect of 1.8% at 0.5 mM (Fig. 7C). High penetration was observed with *Bgh* when the CAOMT inhibitor was applied (Fig. 7D). All concentrations from 0.1 mM to 5 mM were effective in compromising penetration resistance, the most efficient concentration being 2.0 mM, which allowed penetration of 7.8% of *Bgh* conidia (Fig. 7D).

The autofluorescence and callose depositions were also measured at the AGT contact sites with samples of leaves after *Bgh* infection and with or without CAOMT inhibitor treatment. Autofluorescence of CWAs was reduced in inhibitor-treated samples when compared with the controls (Fig. 7E). The emission spectra of CWA and halo areas were also measured by applying CAOMT inhibitor. Fluorescence was decreased compared with the control; however, the intensity level was higher than that in *TmCAOMT* gene silencing (data not shown). A similar degree of callose deposition was found at the fungal interaction sites in both inhibitor-treated and -untreated samples (Fig. 7F).

Discussion

The functional role of monolignol biosynthesis genes in response to powdery mildew invasion in epidermal tissues of diploid wheat was investigated here through the use of

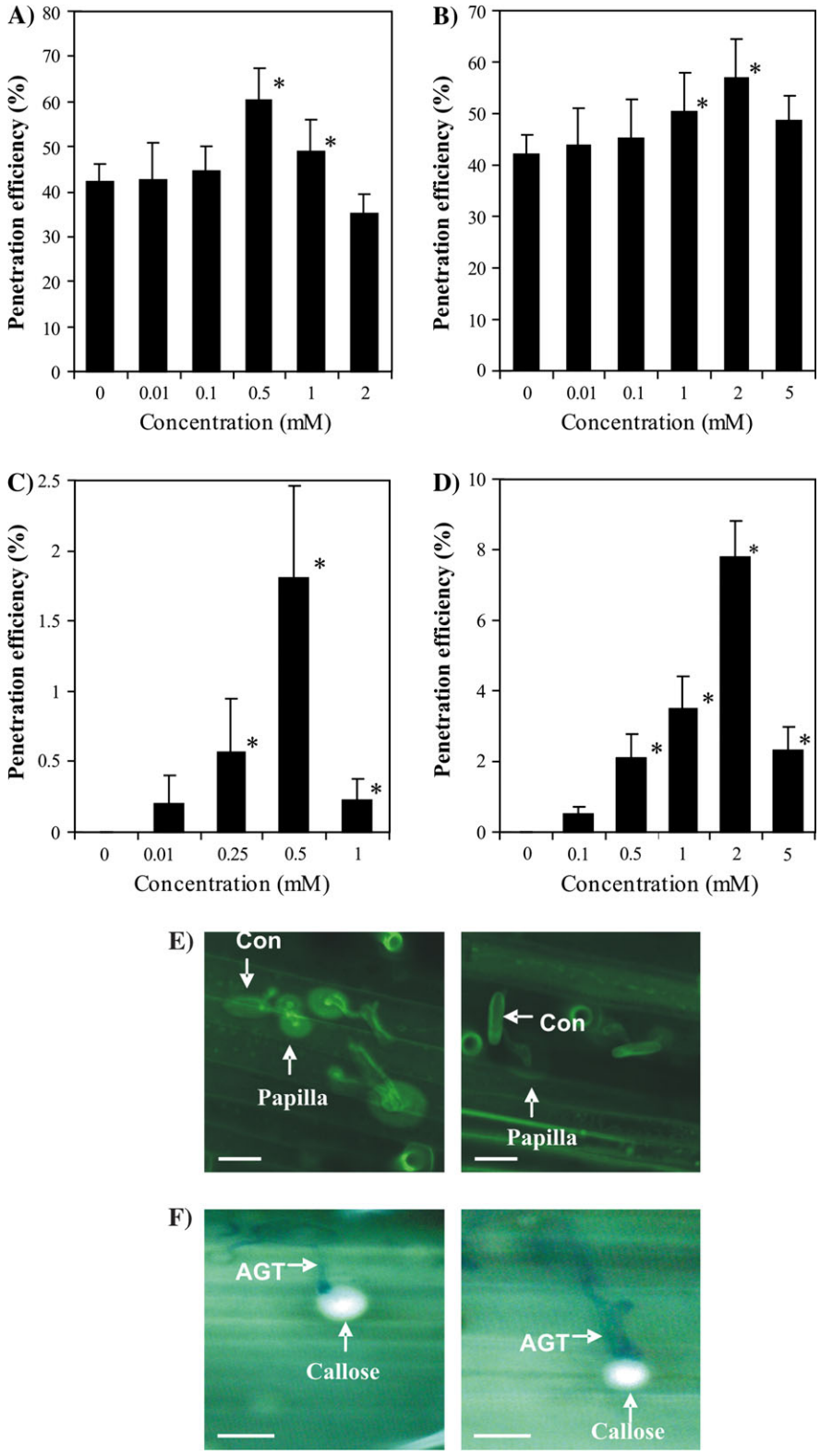

Fig. 7. Effects of PAL and CAOMT inhibitors on the penetration ability of *Bgt* (A, B) and *Bgh* (C, D). (A, C) PAL inhibitor; (B, D) CAOMT inhibitor; (E) autofluorescence and (F) callose deposition in response to fungal interaction in CAOMT inhibitor-treated (right panel) and untreated (left panel) sample leaves. Con, conidia; AGT, appressorial germ tube. Scale bar corresponds to 10 μm. Wheat leaves were exposed to various concentrations of PAL and CAOMT inhibitors for 6 h prior to inoculation with *Bgh*. After inoculation, leaves were again placed on inhibitor solutions for 36 h. For observation of autofluorescence and callose deposition, leaves were collected at 20 hpi. Penetration efficiency was scored as described in the Materials and methods. Data shown represent mean ±standard deviation from three independent experiments in which 100 interaction sites were evaluated. Asterisks besides the columns indicate $P < 0.05$ (Student's *t* test) compared with the negative control (0 mM inhibitor).

gene silencing assays and by enzyme-specific inhibitors. Both RNAi-mediated gene silencing and inhibitors increased susceptibility to an appropriate powdery mildew and compromised penetration resistance to an inappropriate powdery mildew fungus as well. This is the first report to demonstrate the importance of monolignol genes for penetration defence in plants against pathogen invasion through the use of RNAi-mediated gene silencing.

Expression of monolignol biosynthesis genes was induced in both susceptible and resistant wheat lines after *Bgt* attack. Reports by others on the induction of phenylpropanoid genes by pathogen infections in a number of plant species are consistent with our gene expression data (Dixon *et al.*, 2002; Koutaniemi *et al.*, 2007). In the resistant line, transcripts of all monolignol genes were accumulated at 24 hpi. A similar expression pattern was reported for methyl unit biosynthetic genes in the same resistant line in a previous study (Bhuiyan *et al.*, 2007). Usually, AGT attempted to penetrate into the host cells during 16–24 hpi (Collinge *et al.*, 2002). Although the expression of the monolignol genes studied here was at 24 hpi in a resistant line, the expression probably started to up-regulate a little earlier than 24 hpi, like other defence genes demonstrated in the barley–powdery mildew interactions (Caldo *et al.*, 2004). Monolignol units are oxidized by peroxidase to produce lignin polymer; expression of peroxidase genes were shown by some of us in a previous study to be induced in both the epidermis and mesophyll tissues in wheat after *Bgt* attack (Liu *et al.*, 2005). These results, together with our data, suggest that both monolignol and peroxidase genes are coordinately regulated upon pathogen infection.

Our RT-PCR data clearly demonstrate that expression of key monolignol biosynthetic genes was in the epidermis, suggesting their involvement in the CWA-mediated host defence response. A few genes were also activated in mesophyll tissues. This is not surprising given the role of mesophyll cells in epidermal lignification. For example, others have reported that H_2O_2, which is required for cross-linking during lignin polymerization, accumulated in the mesophyll tissues before increasing in the epidermis in barley leaves in response to barley powdery mildew infection (Vanacker *et al.*, 2000). A rise of the PAL enzyme, together with mRNA transcription, was reported in barley epidermis upon powdery mildew infection (Zierold *et al.*, 2005), as in the present study. Rapid induction of PAL and 4CL mRNAs was reported in potato leaves after *Phytophthora infestans* infection (Fritzmeier *et al.*, 1987). In our study, *4CL* was neither induced in the susceptible nor the resistant lines, suggesting that our isoform of 4CL may not be involved in powdery mildew infection. In wheat, wounding or elicitation specifically led to the induction of a CAD isoform with a substrate preference for sinapyl alcohol (Mitchell *et al.*, 1999). CCoAMT was proposed to be involved in the formation of esterified ferulic acid in the cell wall as a pathogen defence response (Pakusch and Matern, 1991), although the enzyme is now believed to play a key role in the biosynthesis of lignin during vascular development (Ye *et al.*, 1994).

An EST library generated from specific conditions is a valuable tool for analysing the involvement of metabolic genes at a genomic level. The EST clones used for this study were collected from a library that was derived from the epidermis of a susceptible wheat line infected with *Bgt*. Therefore, it was expected that all clones encoding monolignol genes studied here would be closely related to pathogen-inducible host gene isoforms. Indeed, our gene expression and silencing results clearly showed that, except for *4CL* and *C3H*, all clones were linked to penetration defence in the host plant. Our gene silencing significantly increased the penetration rate of *Bgt* and compromised penetration resistance to *Bgh* in susceptible wheat plant. The penetration efficiencies of host-specific and non-host pathogens were not the same for each gene silenced, however. These suggest that the contribution of monolignols or their derivatives in the defence mechanisms of host and non-host pathogen interactions is rather complex.

Individual gene silencing or co-silencing significantly increased the penetration efficiency of both host and non-host pathogens compared with that by an inhibitor in susceptible plants. Autofluorescence intensity in the CWA and halo areas was reduced more so by gene silencing than by inhibitor application. Although quantitative data on lignin accumulation in the CWA and halo areas are not available, emission spectra of papilla regions clearly indicated that lignin or 'lignin-like material' accumulation was hampered by gene silencing as well as by inhibitors. Recently, it was shown that independent down-regulation of monolignol genes decreased lignin content in alfalfa (Chen *et al.*, 2006). In this study, co-silencing gave rise to a higher penetration efficiency and a lower fluorescence at the papilla than did individual gene silencing, which may indicate that the former reduces the total flux of lignin content to a greater extent than does the latter. Also it can not be excluded that our gene silencing might have decreased the soluble phenolic acids, monolignols or their derivatives in CWAs, which, in turn, might have affected the efficiency of the papilla to prevent fungal invasion. In a previous study, it was reported that soluble and cell-wall bound conjugates of ferulic acid (feruloyltyramine and feruloyl-3′-methoxytyramine) accumulates in onion epidermis at the sites of attempted penetration of *Botrytis allii* and these conjugates were found to be linked to the cell wall by ether bonds (McLusky *et al.*, 1999).

Interrupting monolignol biosynthesis by using PAL and CAD inhibitors provided evidence for a contribution of lignin apposition to powdery mildew cereal defence (Carver *et al.*, 1992; Kruger *et al.*, 2002). In this study, it is also shown that inhibiting PAL and CAOMT increased susceptibility to *Bgt*. Moreover, PAL and CAOMT inhibition is sufficient to allow penetration of a non-host fungus, albeit at a low frequency. CAOMT inhibition was more efficient than PAL, however. Autofluorescence was decreased in inhibitor-treated leaves in this study and also as others have shown previously (Carver *et al.*, 1992). In addition, it is shown here that interrupting monolignol biosynthesis did not hamper callose deposition at fungal interaction sites. This suggests

that callose deposition is regulated independently of monolignol biosynthesis in papillae.

In the present study, while monolignol gene silencing increased the penetration efficiency of *Bgt* up to 78%, it should be noted that the penetration efficiency of *Bgh* was only 15% in susceptible wheat line. Therefore, additional defence responses may be contributing to non-host resistance in wheat. This result suggests that monolignol biosynthesis plays a role in penetration resistance in both host and non-host pathogen invasions in cereals. Non-host resistance describes the immunity of an entire plant species against non-adapted pathogen species (Lipka *et al.*, 2005).

Not much is known about the mechanisms underlying non-host resistance in wheat. An actin cytoskeleton-based mechanism that involves vesicle movement and exocytosis and focuses a battery of defence activities at the infection site appears to be involved in non-host resistance in barley (Milkis *et al.*, 2007) and *Arabidopsis* (Yun *et al*, 2003). In *Arabidopsis*, it was demonstrated that pre- and post-invasion defences both contribute to non-host resistance (Lipka *et al.*, 2005). It is not known whether both pre and post-invasion defence mechanisms are involved in non-host resistance in cereals or monocots in general.

In this study, although gene silencing and inhibitor treatment compromised penetration resistance to *Bgh*, growth of the fungus was only followed up to 36–40 h after pathogen inoculation. It is not clear whether *Bgh* could continue growing and complete its life cycle on infected wheat. Based on these results it can be hypothesized that monolignol biosynthesis is a pre-invasive defence against non-host resistance in wheat. Further study is now underway to clarify whether monolignol biosynthesis also plays a role in the post-invasive defence mechanism in wheat.

Among all the genes studied here, silencing of *TmCAOMT* was the most effective in allowing penetration of both pathogens, especially in the case of the non-host pathogen. The effectiveness of the CAOMT inhibitor in allowing penetration of *Bgh* in wheat reinforced this result. Thus, it seems clear that methylated monolignol compounds are important for effective CWA formation. This finding is in agreement with our previous work where it was demonstrated that more methyl unit generation is crucial to mitigate multiple stresses (Bhuiyan *et al.*, 2007). CAOMT, which is responsible for the synthesis of sinapyl alcohol, the precursor of syringyl lignin units, methylates both the C3 and the C5 position of the phenolic ring (Boerjan *et al.*, 2003). CCoAMT methylates the C3 position of the phenolic ring and is necessary for the formation of the guaiacyl rather than the syringyl lignin units (Chen *et al.*, 2006). In this study, silencing of *TmCAOMT* increased the penetration rate for both the appropriate and inappropriate pathogens compared with *TmCCoAMT*. Most probably, silencing of *TmCAOMT* affected syringyl lignin unit formation, which, in turn, affected lignin accumulation in CWAs more effectively than the silencing of *TmCCoAMT* does. Therefore, it can be hypothesized that the syringyl lignin unit is more important than the guaiacyl unit in CWA formation. Recently, by chemical analysis, it was demonstrated that syringyl lignin is accumulated during the hypersensitive resistance response in wheat when leaves were treated with fungal elicitor or stem rust fungus (Menden *et al.*, 2007). This result, together with our finding, suggests that syringyl lignin may be the active form of lignification in both CWA and HR to protect cells against fungal invasion.

In conclusion, the RNAi-mediated gene silencing with inhibitor data in this study clearly demonstrate that monolignol biosynthesis plays a critical role in epidermal defence against both appropriate and inappropriate powdery mildew infection in wheat. An important question is whether this pathway has a similar role in dicot plants, an investigation that is now underway in our laboratory.

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