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RESEARCH PAPER

Cross activity of orthologous WRKY transcription factors in wheat and *Arabidopsis*

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Abstract

WRKY proteins are transcription factors involved in many plant processes including plant responses to pathogens. Here, the cross activity of TaWRKY78 from the monocot wheat and AtWRKY20 from the dicot *Arabidopsis* on the cognate promoters of the orthologous *PR4*-type genes *wPR4e* and *AtHEL* of wheat and *Arabidopsis*, respectively, was investigated. *In vitro* analysis showed the ability of TaWRKY78 to bind a –17/+80 region of the *wPR4e* promoter, containing one *cis*-acting W-box. Moreover, transient expression analysis performed on both TaWRKY78 and AtWRKY20 showed their ability to recognize the cognate *cis*-acting elements present in the *wPR4e* and *AtHEL* promoters, respectively. Finally, this paper provides evidence that both transcription factors are able to cross-regulate the orthologous *PR4* genes with an efficiency slightly lower than that exerted on the cognate promoters. The observation that orthologous genes are subjected to similar transcriptional control by orthologous transcription factors demonstrates that the terminal stages of signal transduction pathways leading to defence are conserved and suggests a fundamental role of *PR4* genes in plant defence. Moreover, these results corroborate the hypothesis that gene orthology imply similar gene function and that diversification between monocot and dicot has most likely occurred after the specialization of WRKY function.

Key words: *Arabidopsis thaliana*, gene expression, gene orthology, *PR4* genes, transient expression analysis, transcription, regulation, *Triticum aestivum*.

Introduction

Several investigations suggest that pathogenesis related (PR) proteins participate in plant defence mechanisms as many of them are endowed with antimicrobial activity against plant pathogens. Different types of PR proteins have been recognized and classified into 17 families on the basis of structural–functional characteristics and serological relatedness (Van Loon *et al.*, 2006a). Most genes and related proteins are induced by different stress stimuli, such as infection by viruses, bacteria, and fungi and treatment with the defence-related phytohormones salicylic acid (SA), jasmonic acid (JA) or ethylene (ET). PR proteins and genes have recently been extensively reviewed (van Loon *et al.*,

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane carboxylic acid; CaMV, cauliflower mosaic virus; CD-WRKY, C-terminal WRKY domain; EMSA, electrophoretic mobility shift assay; ERFs, ethylene-responsive element binding factors; ET, ethylene; GST, glutathione-S-transferase; INA, 2,6-dichloroisonicotinic acid; IPTG, isopropyl β-D-1-thiogalactopyranoside; JA, jasmonic acid; LUC, luciferase; Me-JA, methyl-jasmonate; PDB, potato dextrose broth; PEG, polyethylene glycol; PR, pathogenesis related; qPCR, quantitative PCR; SA, salicylic acid; TCV, turnip crinkle virus; TFs, transcription factors; UTR, 5'-untranslated region.

2006*a*, and references therein; Sels *et al.*, 2008). Although intensive studies on PR proteins have been carried out, their precise role in defence responses remained elusive since enzymatic activities or functions are still unknown for some of them.

PR4 proteins show antifungal activity against several phytopathogenic fungi and were demonstrated to possess ribonucleasic activity correlated to the antifungal capacity (Caporale *et al.*, 2004; Bertini *et al.*, 2009). Genes encoding PR4 proteins were studied in rubber tree, potato, tobacco, tomato, *Arabidopsis* (*Arabidopsis thaliana*), barley, maize, and wheat, while mature proteins have been characterized only in tobacco, tomato, barley, and wheat (Stanford *et al.*, 1989; Broekaert *et al.*, 1990; Linthorst *et al.*, 1991; Svensson *et al.*, 1992; Caruso *et al.*, 1993, 1996, 1999, 2001; Potter *et al.*, 1993; Ponstein *et al.*, 1994; Bertini *et al.*, 2006).

PR4 protein studies are now shifted almost entirely to the analysis of the expression of the corresponding genes following biotic and abiotic stress conditions. A common feature of *PR4* genes is that they belong to a multigene family differentially regulated during development or upon fungal infection. Regulated gene expression is one of the most complex activities in cells. It involves many transcription factors (TFs) that contribute to basal transcription or mediate a response to developmental, environmental or metabolic cues. Moreover, regulatory proteins play a central role in the plant defence response and the elucidation of their action mechanisms should provide important insights into the molecular basis of resistance to pathogens (Rushton and Somssich, 1998). Depiction of either *cis*-acting regulatory elements or *trans*-acting factors underlying the activation of pathogen-induced gene expression represents a fundamental contribution to the understanding of the terminal stages of signal transduction pathways leading to defence responses. A detailed study of the *PR4* promoter and the analysis of *cis*-acting elements within its regulatory region could provide a molecular tool for the understanding of responsiveness to elicitors or to plant endogenous signals involved in defence.

In the past ten years, *cis*-acting elements and their cognate *trans*-acting factors have been described in some dicotyledonous plants but the analysis in monocots is still poor and needs further insight. Several promoters of *PR* genes have been characterized in tobacco (Niggeweg *et al.*, 2000; Yang *et al.*, 2000; Butterbrodt *et al.*, 2006). Deletion and linker-scanning analysis of the *Arabidopsis PR-1* gene promoter showed that mutations in the bZIP TF binding site abolished inducibility by SA and its synthetic homologue 2,6-dichloroisonicotinic acid (INA) (Lebel *et al.*, 1998). The sequence AGCCGCC, known as the GCC box, was also shown to be a *cis*-acting transcriptional element of *PR* genes and has been found in the promoters of several basic *PR* genes of dicots, conferring ET inducibility of tobacco *PR* genes encoding β-1,3-glucanase, PR-1 and PR5-d. This element is recognized by ET-responsive element binding factors (ERFs), one of the best-characterized classes of DNA-binding proteins among those implicated in *PR* gene expression (Singh *et al.*, 2002; Oñate-Sanchez *et al.*, 2007). Another family of TFs implicated in plant defence is represented by (R)WRKY, recognizing the sequence (T)(T)TGAC(C/T), known as the W-box (Robatzek *et al.*, 2001; Pandey and Somssich, 2009). These TFs appear to be involved in the regulation of several physiological processes that are important to plants, including pathogen defence, biosynthesis of secondary metabolites, and senescence (Eugelm *et al.*, 1999, 2000; Hinderhofer and Zentgraf, 2001; Robatzek and Somssich, 2001). WRKY factors were generally regarded as being plant specific, but their identification in the protist *Giardia lamblia* and the slime mould *Dictyostelium discoideum* suggests an earlier origin (Ulker and Somssich, 2004; Pan *et al.*, 2009).

Previously, the isolation of a wheat *PR4* gene (*wPR4e*), coding for Wheatwin5, and its structural/functional characterization was reported (Bertini *et al.*, 2006). The 5'-untranslated region of *wPR4e* was characterized *in silico* for the presence of regulatory *cis*-acting elements revealing the presence of several abiotic and biotic stress-responsive elements, including two W-boxes recognized by WRKY TFs (Bertini *et al.*, 2006). In *Arabidopsis*, the WRKY TF gene family (*AtWRKY*) can be subdivided into three different groups based on the number of WRKY domains and of certain features of the Zn finger-like motif that is characteristic for WRKY TFs. AtWRKY proteins with two WRKY domains belong to group I, whereas most proteins with one WRKY domain belong to groups II and III. The latter two groups differ from each other for the type of Zn finger domain. Generally, the WRKY domains of group I and group II members have the same type of finger motif C2H2, whereas in group III, the WRKY domain contains a C2HC motif. Sequence-specific binding of WRKY TFs of group I to their cognate DNA is mediated by the C-terminal WRKY domain, while the function of the N-terminal WRKY domain still remains unknown. The WRKY TF superfamily consists of 74 members in *Arabidopsis* (Eulgem and Somssich, 2007), and of 98 and 102 members in the rice (*Oryza sativa*) subspecies japonica and indica, respectively (Ross *et al.*, 2007). Recently, 45 members of WRKY TFs have been described in barley (*Hordeum vulgare*) and putative orthologues of the HvWRKY proteins in *Arabidopsis* and rice have been assigned (Mangelsen *et al.*, 2008). Presently, reports on wheat WRKY TFs are quite scanty. However, due to the hexaploid nature of *Triticum aestivum*, this plant species is expected to contain more WRKY genes than rice. Wu and collaborators reported the isolation and expression profiles of 15 wheat WRKY TFs that have been classified on the basis of the corresponding orthologous sequence of rice (Wu *et al.*, 2008). Currently, the GenBank database contains 43 different wheat WRKY sequences belonging to all three groups of this gene family.

The isolation and functional characterization of a WRKY TF from wheat, named *TaWRKY78*, is reported here. Using both *in vitro* and *in vivo* assays, it is demonstrated that TaWRKY78 is able to bind to and positively regulate the *wPR4e* promoter. Moreover, the *PR4*-type gene *AtHEL* from *Arabidopsis* (Potter *et al.*, 1993) that represents the

orthologue of *wPR4e* was studied and its 5'-untranslated region was characterized. The orthologue of *TaWRKY78* in *Arabidopsis*, namely *AtWRKY20*, was also isolated and its ability to positively regulate the *AtHEL* promoter in transient expression assays was demonstrated. Finally, the ability of both WRKY TFs to activate the orthologous cognate promoters of *wPR4e* and *AtHEL* was demonstrated, suggesting that this mechanism is essentially conserved across monocot and dicot species.

Materials and methods

Cultivation of wheat plants and treatments

Wheat (*Triticum aestivum* cv. San Pastore) was kindly supplied by the Istituto Nazionale per la Cerealicoltura (S. Angelo Lodigiano, Italy). Macroconidia from sporodochia of *Fusarium culmorum* (isolate ISPaVe485), supplied by the Istituto Sperimentale per la Patologia Vegetale (Roma, Italy), were collected from 10 d cultures grown at 21 °C and were suspended in sterile water. Wheat seeds were sterilized with sodium hypochlorite (3.0% w/v), washed several times in sterile water, and dried in a fume hood. Dried sterilized seeds were placed on sterile agar plates for 72 h at 21 °C and then inoculated with *F. culmorum* by adding 10 µl of a conidial suspension (3×10^5 spores ml^{-1} H_2O) to each coleoptile. Inoculated and control seedlings treated with sterile water (100 seeds for each sample) were allowed to grow at 21 °C in the dark for different periods of time. At each sampling time, germinating seeds were harvested, immediately frozen in liquid nitrogen, then stored at −80 °C prior to further analysis. The experiment was carried out three times independently under the same conditions.

To follow the time-course induction of gene expression by chemical inducers, surface-sterilized wheat seeds were submerged in freshly prepared sterile solutions of SA (5 mM) (Mallinckrodt Baker, Deventer, The Netherlands) or methyl-jasmonate (MeJA) (5 mM) (Serva, Brunschwig Chemie, Amsterdam, The Netherlands) for 4 h at room temperature. As a control, seeds were submerged in sterile water under the same conditions. Treated and control seeds (100 seeds for each sample) were placed on sterile agar plates and allowed to grow in the dark at 21 °C for different periods of time. At each sampling time, seedlings were harvested, immediately frozen in liquid nitrogen and then stored at −80 °C prior to further analysis.

For wounding experiment, three or four needle-prick wounds were applied to 3-d-old seedlings grown on sterile agar plates at 21 °C. Seedling samples from wounded and control plants were harvested at 1, 2, and 3 d after injury, immediately frozen in liquid nitrogen, and then stored at −80 °C until further analysis. Chemical induction and wounding experiments were carried out three times independently.

Cultivation of Arabidopsis *plants and treatments*

Seeds of *Arabidopsis* accession Col-0 were sown in quartz sand. After 2 weeks, seedlings were transferred to 60 ml pots containing sand/potting soil mixture that was autoclaved twice for 20 min with a 24 h interval (Pieterse *et al.*, 1998). Plants were cultivated in a growth chamber with an 8/16 h (200 µE m^{-2} s^{-1}) day/night cycle, temperatures of 24/20 °C, and 70% relative humidity for a further 3 weeks. Plants were watered on alternate days and received half-strength Hoagland solution (Hoagland and Arnon, 1938) containing 10 µM Sequestrene (CIBA-Geigy, Basel, Switzerland) once a week.

Chemical induction treatments of *Arabidopsis* were performed by dipping leaves of 5-week-old plants in aqueous solution containing 0.015% (v/v) Silwet L-77 (Van Meeuween Chemicals BV, Weesp, The Netherlands), supplemented with 1 mM SA, 100 µM MeJA or 1 mM 1-aminocyclopropane-carboxylic acid (ACC) (Sigma, Schnelldorf, Germany). Control plants were treated with 0.015% (v/v) Silwet only. Leaf rosettes were harvested at time 0, 3, 6, 24, and 48 h after treatment, immediately frozen in liquid nitrogen and then stored at −80 °C before further analysis.

For wounding experiments, four needle-prick wounds were applied to 5-week-old plants. Leaf rosettes from wounded and control plants were harvested at 0, 3, 6, 24, and 48 h after injury, immediately frozen in liquid nitrogen, and then stored at −80 °C before further analysis.

Fusarium oxysporum f.sp. *conglutinans* was grown on potato dextrose agar at room temperature for 2–3 weeks. Spores were taken up in sterile 0.2× potato dextrose broth (PDB), filtered through Miracloth (Calbiochem-Novabiochem, San Diego, CA), and counted with a Burker chamber (Merck ABS, Dietikon, Switzerland). Spore suspensions were diluted to 1×10^5 spores ml^{-1} in 0.2× PDB and 5 ml were used to inoculate the shoot collar. Control plants were treated with 0.2× PDB only.

Leaf rosettes were harvested at 0, 3, 6, 24, and 48 h after treatment and immediately frozen in liquid nitrogen. Each experiment was carried out three times independently.

cDNA production from wheat coleoptiles and Arabidopsis *leaves*

Total RNA was isolated from control and treated wheat coleoptiles according to Prescott and Martin (1987). One µg of total RNA was reverse-transcribed using oligo$(dT)_{20}$ primers (Invitrogen, Breda, The Netherlands), 10 mM dNTPs, and 200 U of SuperScript II (M-MLV) reverse transcriptase (Invitrogen, Breda, The Netherlands), according to the manufacturer's instruction. PCR with primers designed on the barley *actin* gene (*Hordeum vulgare*, clone cMWG0645, EMBL accession no. AJ234400) (Table 1) using total RNA as template was carried out in order to check for genomic DNA contamination.

Two µg of RNA from 5-week-old *A. thaliana* ecotype Col-0 leaves, prepared as described previously by Van Wees *et al.* (2000), was digested with DNase Turbo DNA-freeTM (Ambion, Huntingdon, United Kingdom) according to the manufacturer's instructions. To check for genomic DNA contamination, PCR with primers designed on *EIL2* (At5g21120) (Table 1) were carried out. DNA-free total RNA was converted to cDNA using oligo$(dT)_{20}$ primers, 10 mM dNTPs, and 200 U SuperScriptTM III Reverse Transcriptase (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions.

TaWRKY78 *expression analysis by quantitative PCR*

Quantitative PCR (qPCR) analysis was performed in optical 96-well plates with MX3000P (Stratagene, La Jolla, CA, USA), using SYBR Green to monitor dsDNA synthesis. Gene-specific primers for *TaWRKY78* amplification are reported in Table 1. qPCR reactions were performed in a volume of 20 µl, containing 0.5 µl cDNA, 1 µl of gene-specific primers (10 pmol μl^{-1}), 12.5 µl of SYBR Green Master Mix (Stratagene), and 0.375 µl of Rox, diluted 1:500. The following PCR program was used for all PCR reactions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s. Melting curves were recorded after cycle 45 by heating from 55 °C to 95 °C with a ramp speed of 1.9 °C min^{-1}. Transcript level normalization was carried out using primers of the constitutively expressed 18S rRNA gene (Table 1). Relative quantitative analysis was performed using the 4 d control as calibrator.

Three independent experiments were performed and gave similar results.

Isolation and expression of the TaWRKY78 C-terminal domain

The TaWRKY78 C-terminal domain coding sequence was amplified utilizing cDNA from 4-d-old wheat coleoptiles using primers based on the wheat clone wlmk1.pk0035.d9:fis (Acc. no.

Table 1. PCR primers

Gene name	Primer name	Use	Sequence 5' to 3' (restriction endonuclease site is in bold)	Restriction endonuclease site
TaWRKY78	TaWRKY78 F	qPCR	TGGATGATGGGTATCGTTGG	
	TaWRKY78 R	qPCR	CATACGTTGTTATCACTGATTTAGG	
	C-term F	RT-PCR	CGC**GGATCC**GATGATGGGTATCGTT	*Bam*HI
	C-term R	RT-PCR	CCG**GAATTC**TCAAGGGACTTCATGGTT	*Eco*RI
	TaWRKY78T F	RT-PCR	GC**GGATCC**ATGTCCCCGGCGCGGC	*Bam*HI
	TaWRKY78T R	RT-PCR	C**GAGCTC**TCATGGACCCATGACCAAGT	*Sac*
wPR4e	Δ4 F	PCR probe	GTGGCGAAGAGCGACTCCCATTCCAGT	
	Δ4 R	PCR probe	CATCTTTGTCAACGCAGC	
	ΔpPR4e F	PCR	CCC**AAGCTT**CCGTATGCCCCTAACAAATG	*Hin*dIII
	ΔpPR4e R	PCR	CG**CCTAGG**CTTTGTCAACGCAGCGGA	*Bam*HI
AtHEL	AtHEL F	PCR probe	ATCTGCTGCAGTCAGT	
	AtHEL R	PCR probe	ACTGTGGCAATGAGCTCA	
	pHEL F	PCR	CCC**AAGCTT**TTGAAGCGGACTTGATATGGG	*Hin*dIII
	pHEL R	PCR	AACTGCAGGATCGATAAGTCTTTGTTTTCTTGG	*Pst*I
	ΔpHEL F	PCR	CCC**AAGCTT**TTAGCCGCCTCTCCTCATTA	*Hin*dIII
	ΔpHEL R	PCR	AA**CTGCAG**AAGGTCACTGGTCAAAGTCATCTG	*Pst*I
	AtHELQ F	qPCR	CGGCAAGTGTTTAAGGGTGAAG	
	AtHELQ R	qPCR	TGCTACATCCAAATCCAAGCCT	
AtWRKY20	AtWRKY20 F	PCR probe	AAGGGATCTGGCGTCTACAACT	
	AtWRKY20 R	PCR probe	AGGCTGATGGTGTCTGTTTCAT	
	AtWRKY20T F	RT-PCR	GC**TCTAGA**ATGAACCCTCAAGCTAATGAC	*Xba*I
	AtWRKY20T R	RT-PCR	A**AGGCCT**TTACGGACCCGATTGTACT	*Stu*I
	AtWRKY20 dom F	RT-PCR	GCG**GATCC**GATGATGGTTATAGA	*Bam*HI
	AtWRKY20 dom R	RT-PCR	CC**CTCGAG**ATCATGATCGTGTTT	*Xho*I
rRNA18S	rRNA18S F	qPCR	GGTACGTGCTACTCGGATAACC	
	rRNA18S R	qPCR	TCTCCGGAATCGAACCCTA	
barley actin	Act F	RT-PCR	ATCGTGGGGCGCCCCAGGCACC	
	Act R	RT-PCR	CTCCTTAATGTCACGCACGATTTC	
EIL2	EIL2 F	RT-PCR	CAGATTCTATGGATATGTATAACAACAA	
	EIL2 R	RT-PCR	GTAAAGAGCAGCGAGCCATAAAG	
UBI10	UBI10 F	qPCR	AAAGAGATAACAGGAACGGAAACATAGT	
	UBI10 R	qPCR	GGCCTTGTATAATCCCTGATGAATAAG	

The table shows the primers used during PCR, RT-PCR and Q-RT-PCR experiments.

BT009430) containing a WRKY mRNA of group I (C-term and C-term R; Table 1). Amplification was performed over 30 cycles in a Progene Termal cycler using Biotaq DNA Polymerase (Bioline) with the following conditions: denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min, and elongation at 72 °C for 1 min. PCR products were separated by agarose gel electrophoresis and the appropriate band was recovered using the Wizard SV Gel and PCR clean-up system (Promega, Madison, WI) and ligated into pGEM-T® Easy (Promega). The identity of the inserted DNA fragment was confirmed by sequencing using an ABI PRISM 310 analyzer (Applied Biosystems, Courtaboeuf, France). The insert was subcloned into pGEX-4T1 plasmid between *Bam*HI and *Eco*RI sites. The recombinant vector, containing the coding sequence of the TaWRKY78 C-terminal domain linked to glutathione-*S*-transferase (GST), was named pGEX-CD-TaWRKY78. The expression construct was transformed into *Escherichia coli* strain BL21 for production of recombinant protein. Transformed cells were grown in 2× YT medium containing ampicillin (100 μg ml^{-1}) at 37 °C to an absorbance of 0.6 at 600 nm. Protein expression was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested 2 h after induction by centrifugation (8000 rpm, 4 °C, 10 min). Bacterial cell pellets containing recombinant fusion protein (GST:CD-TaWRKY78) were resuspended in 50 mM TRIS-HCl, pH 8.0, containing 1 mM EDTA (TE buffer), 100 μg ml^{-1} lysozyme, and a protease inhibitor cocktail. After incubation on ice for 15 min, the mixture was lysed by sonication and treated with DNase at 37 °C for 60 min. After centrifugation at 12 000 rpm, 4 °C for 30 min, the GST:CD-TaWRKY78 was purified from supernatant using Glutathione-Sepharose 4B resin (Amersham).

Southwestern analysis for GST:CD-TaWRKY78

Purified GST:CD-TaWRKY78 and GST alone (negative control) were subjected to SDS-PAGE and transferred to nitrocellulose membrane by electroblotting. The proteins were renatured by incubation of the membrane in 20 mM TRIS-HCl, pH 7.5 containing 150 mM NaCl, 2.5 mM DTT, 2.5% (v/v) Nonidet P-40, 10% (v/v) glycerol, and 3% BSA, overnight at 4 °C, under gentle shaking. The membranes were then washed twice with the binding buffer containing 10 mM TRIS-HCl, pH 8.0, 50 mM NaCl, 1 mM DTT, 5% (v/v) glycerol, and 0.125% (w/v) BSA. The hybridization with the biotinylated DNA probes was carried out adding 500 ng of the probe into the binding buffer and it was extended for 6 h at 4 °C, under gentle shaking. The 97 bp probe, spanning the −17/+80 region of the *wPR4e* promoter named *wPR4e*-Δ4, was amplified by PCR using the primers Δ4 for and Δ4 rev reported in Table 1 and the previously described pUC3.3X/S plasmid (Bertini *et al.*, 2006) as template. The reverse primer was biotinylated to the 3' end. The PCR reaction was performed as

described above with an annealing temperature of 56 °C. The *wPR4e*-Δ3 probe, spanning the +40/+69 region of the *wPR4e* promoter, was synthesized annealing specific biotinylated oligos.

After hybridization, the membranes were washed twice with binding buffer and once with PBS 1×. Streptavidin horseradish peroxidase-conjugated immunoglobulins were used to detect the interaction. Positive bands were detected using 4-chloro-1-naphthol (Sigma-Aldrich) as a chromogen.

Electrophoretic mobility shift assay (EMSA)

The *wPR4e*-Δ4 probe was prepared by PCR using 5'-[γ-^{32}P]-labelled forward primer. The labelling mixture contained 12.5 pmol of Δ4 forward primer, 4 µl of [γ-^{32}P]-ATP, and 20 U of kinase enzyme (Amersham Pharmacia Biotech, Uppsala, Sweden). The reaction mixture was incubated for 30 min at 37 °C and then 20 min at 65 °C. The primer end-labelled fragment was purified with Push Column Beta Shield Device (Stratagene) and used in the PCR reaction performed as above.

The DNA-protein binding reaction (25 µl) contained TRIS-HCl 5 mM pH 8.0, KCl 50 mM, $MgCl_2$ 6.25 mM, glycerol 5% (v/v), NP-40 0.8% (v/v), recombinant proteins (GST:CD-WRKY78 and GST), and 58 fmol (4 ng) of labelled *wPR4e*-Δ4. For specific competition, a 50-fold or 300-fold excess of not-labelled Δ4 was used. For non-specific competition, 50 ng and 100 ng of poly (dI-dC) were used. DNA-protein binding was performed at 4 °C for 30 min and resulting complexes were resolved on a 5% (w/v) polyacrylamide gel in 0.5× TBE at 4 °C.

AtHEL *and* AtWRKY20 *expression analysis by Northern blot*

For RNA extraction, at least five *Arabidopsis* plants for each treatment were harvested at the time points indicated. RNA isolation was performed as described previously by Van Wees *et al.* (2000). For RNA-blot analysis, 12.5 µg of RNA was denatured using glyoxal and dimethyl sulphoxide (Sambrook *et al.*, 1989), electrophoretically separated on a 1.5% (w/v) agarose gel, and blotted onto Hybond-N^+ membranes (Amersham) by capillary transfer. The electrophoresis and blotting buffer were 10 mM and 25 mM sodium phosphate (pH 7.0), respectively. RNA blots were hybridized with *AtHEL* (At3g04720) and *AtWRKY20* (At4g26640) specific probes that were labelled with [α-^{32}P]-CTP by random primer labelling. The *AtHEL* and *AtWRKY20* probes were generated through PCR on *A. thaliana* cDNA using gene-specific primers (Table 1).

To check for equal loading, the blots were stripped and hybridized with a probe for 18S ribosomal RNA. Blots were exposed for autoradiography and signals visualized using a Bio-Rad Molecular Imager FX (Bio-Rad, Veenendaal, The Netherlands).

DNA genomic extraction from Arabidopsis *leaves*

Arabidopsis leaves from 5-week-old plants were grinded in liquid nitrogen. Genomic DNA was extracted in a buffer containing TRIS 200 mM, NaCl 250 mM, EDTA 25 mM, and SDS 0.5% (w/v) and, after centrifugation for 5 min at 14 000 *g*, was precipitated in isopropanol. After centrifugation as above, the pellet was air-dried and dissolved in TRIS-EDTA 10 mM.

PR4 *promoters and WRKY TF isolation*

The −958/+80 *wPR4e* promoter region, named *ΔpPR4e*, was amplified from pUC3.3X/S (Bertini *et al.*, 2006) using the primers ΔpPR4e for and ΔpPR4e rev listed in Table 1. The 1015 bp promoter of *AtHEL* gene, named *pHEL* and its −589/−488 region, defined as *ΔpHEL*, were amplified from genomic DNA, using the primers reported in Table 1.

TaWRKY78 coding sequence (Acc. no. HM013818) was amplified using cDNA from wounded wheat coleoptiles harvested 1 d after injury utilizing primers based on the wheat clone wlmk1.pk0035.d9:fis (Table 1). The *AtWRKY20* coding sequence (TAIR code: At4g26640) was amplified using cDNA from 5-week-old *A. thaliana* ecotype Col-0 leaves using the primers reported in Table 1. Amplifications were performed as above with the following annealing temperatures: 60 °C *(ΔpPR4e*), 62 °C (*pHEL* and *TaWRKY78*), 65 °C (*ΔpHEL*), 61.5 °C (*AtWRKY20*), and elongation at 72 °C for 1 min. Amplicons were purified from agarose gel as above, cloned in pGEM®-T Easy and fully sequenced.

Isolation and expression of AtWRKY20 C-terminal domain

The AtWRKY20 C-terminal domain coding sequence was amplified using cDNA from 5-week-old *A. thaliana* ecotype Col-0 leaves using primers based on the known sequence of the TF (TAIR: At4g26640) (AtWRKY20 dom F and AtWRKY20 dom R, Table 1). Amplification was performed over 30 cycles in a Progene Termal cycler using Biotaq DNA Polymerase (Bioline) with the following conditions: denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, and elongation at 72 °C for 1 min. Sequence analysis of the PCR product and subcloning into pGEX-4T1 plasmid between *Bam*HI and *Xho*I sites were performed as previously described for GST:CD-TaWRKY78. The recombinant vector, containing the coding sequence of the AtWRKY20 C-terminal domain linked to glutathione-*S*-transferase (GST), was named pGEX-CD-AtWRKY20. The expression and the purification of GST:CD-AtWRKY20 was performed as previously reported for GST:CD-TaWRKY78.

Southwestern analysis for GST:CD-AtWRKY20

Purified GST:CD-AtWRKY20 and GST alone (negative control) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane by electroblotting. The Southwestern analysis was performed as previously described for GST:CD-TaWRKY78 using the promoter fragments *AtHEL*-Δ5 and *AtHEL*-Δ6 as probes. The 21 bp *AtHEL*-Δ5, spanning the −569/–549 region of the *AtHEL* promoter, and the 20 bp *AtHEL*-Δ6 probe, spanning the –589/–570 region of the *AtHEL* promoter, were synthesized annealing specific biotinylated oligos.

Transient expression assay using mesophyll protoplasts

In order to test the *in vivo* interaction between *PR4* promoters and WRKY TFs, *Arabidopsis* protoplasts were isolated from 5-week-old Col-0 plants and transformed with plasmid DNA using the polyethylene glycol (PEG)–calcium transfection protocol described by Yoo *et al.* (2007). PEG-calcium transfection of plasmid DNA was performed with protoplasts at a density of 1 000 000 ml^{-1}. Plasmids used for transient expression analyses are described in Table 3. Protoplasts were co-transformed with the effector plasmids *35S*::*TaWRKY78* or *35S*::*AtWRKY20* with each of the following reporter plasmids: *pPR4e*::*GUS*, *ΔpPR4e*::*GUS*, *pHEL*::*GUS*, *ΔpHEL*::*GUS*. In control samples, protoplasts were transformed with reporter plasmids alone. As an efficiency transformation control, all samples were co-transformed with a reference plasmid carrying the firefly luciferase (*LUC*) gene under the control of the *35S* promoter in a ratio of 2:1:2 (reporter:reference:effector) with a maximum of 5 µg of total plasmids in each experiment. Protoplasts were harvested 24 h after transformation and frozen in liquid nitrogen. GUS and LUC activities were measured as described by Pré *et al.* (2008). GUS activity was related to LUC activity to correct for transformation efficiency. Average of GUS–LUC ratios from five experiments were reported.

To test the influence of TaWRKY78 on *HEL* endogenous expression, *Arabidopsis* protoplasts were isolated from 5-week-old Col-0 plants and transformed with plasmid DNA using the polyethylene glycol (PEG)–calcium transfection protocol as above. Protoplasts were transformed with 1 µg of the plasmid *35S*::*TaWRKY78*. As a control, protoplasts were transformed with

pUC::*GUS* (empty plasmid). Protoplasts were harvested 24 h after transformation.

RNA isolation from protoplasts and qPCR

Total RNA was isolated from protoplasts transformed with *35S*::*TaWRKY78* plasmid with Trizol (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions and cDNA preparation was carried out as above. qPCR analysis was performed in optical 96-well plates with a MyIQ™ Single Color Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands), using SYBR Green to monitor dsDNA synthesis.

HEL gene-specific primers (Table 1) were designed previously by Seo *et al.* (2008). Primers for *TaWRKY78* amplification are reported in Table 1. The transcript level normalization was carried out using primers of the constitutively expressed gene *UBI10* (At4g05320) (Table 1). qPCR reactions were performed in a volume of 15 µl, containing 1 µl cDNA, 0.5 µl of each of the two gene-specific primers (10 pmol μl^{-1}), and 3.5 µl of 2× IQ SYBR Green Supermix reagent (Bio-Rad, Veenendaal, The Netherlands). The following PCR program was used for all PCR reactions: 95 °C for 3 min; 40 cycles of 95 °C for 30 s, 59.5 °C for 30 s, and 72 °C for 30 s. CT (threshold cycle) values were calculated using Optical System Software, version 1.0 for MyIQ™ (Bio-Rad, Veenendaal, The Netherlands). Transcript levels were calculated by using the $2^{\Delta\Delta CT}$ formula. Melting curves were recorded after cycle 40 by heating from 55 °C to 95 °C with a ramp speed of 1.9 °C min^{-1}.

Results

Isolation and characterization of a novel wheat WRKY *TF gene*

Previously (Bertini *et al.*, 2006), the 5′-untranslated region (UTR) of the *wPR4e* promoter was analysed using PLACE or PlantCARE databases and several known regulatory motifs interacting with different TFs families involved in numerous regulatory processes were found. Given the presence of two W-boxes (TTGAC) at positions –1092/–1096 and +72/+76, recognized by WRKY TFs (Ilker and Somssich, 2004), this work aimed to test their ability to be recognized *in vitro* by TFs belonging to the WRKY family.

On the basis of the first reported complete wheat WRKY sequence present in a data bank (clone wlmk1.pk0035.d9:fis), primers were designed that allowed a WRKY TF to be isolated from mRNA extracted from wounded wheat coleoptiles. Comparison with WRKY sequences present in the data bank and, in particular, with that of clone wlmk1.pk0035.d9:fis, revealed the isolation of a novel WRKY TF that has been named *TaWRKY78* after the orthologous sequence of rice (Ross *et al.*, 2007). Multiple protein sequence alignments between homologous WRKY TFs from wheat, *Arabidopsis*, and rice have been carried out (see Supplementary Fig. S1 at *JX*B online). Sequence identity is very high (90%) when sequences from monocots are compared, whereas t it is lower (50%) when sequences from dicots are considered. In particular, a high level of identity was only found for *TaWRKY17*, which therefore appears to be the only possible *TaWRKY78* isoform isolated so far. A phylogenetic tree was built up using the same WRKY TFs sequences demonstrating that wheat WRKY TFs are more closely related to the *Oryza* TFs than to those of *Arabidopsis* (see Supplementary Fig. S2 at *JX*B online).

To investigate the expression pattern of *TaWRKY78* following chemical treatment as well as wounding or pathogen infection, wheat seeds were treated with aqueous solutions of SA or MeJA. In addition, 3-d-old coleoptiles were either wounded with three or four needle-pricks or infected with a *Fusarium culmorum* spore suspension. In order to compare the expression pattern of *TaWRKY78* in coleoptiles of the same age, seedlings generated from SA or MeJA-treated seeds were harvested after 4, 5, and 6 d; by contrast, infected or wounded coleoptiles were harvested 1, 2, and 3 d after treatments.

TaWRKY78 expression was analysed by qPCR using specific primers (Fig. 1). 18S rRNA was used as an internal standard for RNA normalization. The expression of *TaWRKY78* was weakly affected by MeJA and almost unaffected by SA treatment, wounding or *F. culmorum* infection. Similarly, expression of the cognate wPR4e gene was previously demonstrated to be induced by MeJA, wounding and *F. culmorum* infection (Bertini *et al.*, 2006).

In vitro *interaction between wheat GST:CD-TaWRKY78 and the* wPR4e *promoter*

In order to evaluate the *in vitro* interaction between the TaWRKY78 and the *wPR4e* promoter, a truncated version of the TF carrying only the C-terminal WRKY domain (CD-TaWRKY78) was synthesized using specific primers. The recombinant protein was produced as a fusion partner with gluthatione-*S*-transferase (GST) (GST:CD-TaWRKY78).

A promoter fragment of 97 bp spanning the –17/+80 region of the *wPR4e* promoter was produced by PCR using specific primers. This DNA fragment, named *wPR4e*-Δ4, contains several *cis*-acting elements besides one W-box (TTGAC) located at +72/+76 relative to the putative transcription start site (Fig. 2A). This promoter fragment

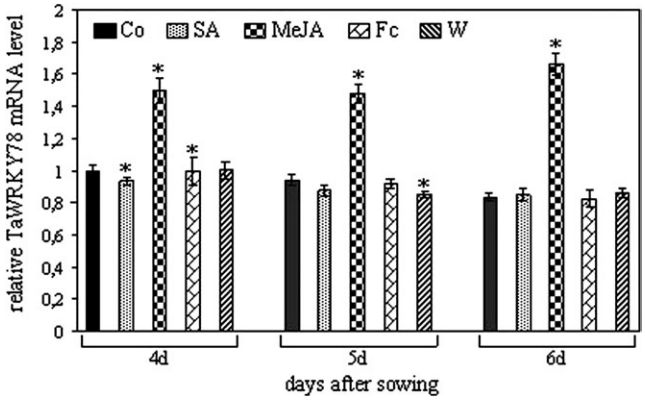

Fig. 1. qPCR analysis of *TaWRKY78* transcript levels in wheat coleoptiles after SA (5 mM) and MeJA (5 mM) treatments, *F.* culmorum (Fc) infection, and wounding. Time-course expression was analysed in both control and treated coleoptiles harvested 4, 5, and 6 d after sowing. Bars represent means ±SD. Asterisks indicate statistically significant differences compare to control plants (Student's *t* test, $P < 0.05$).

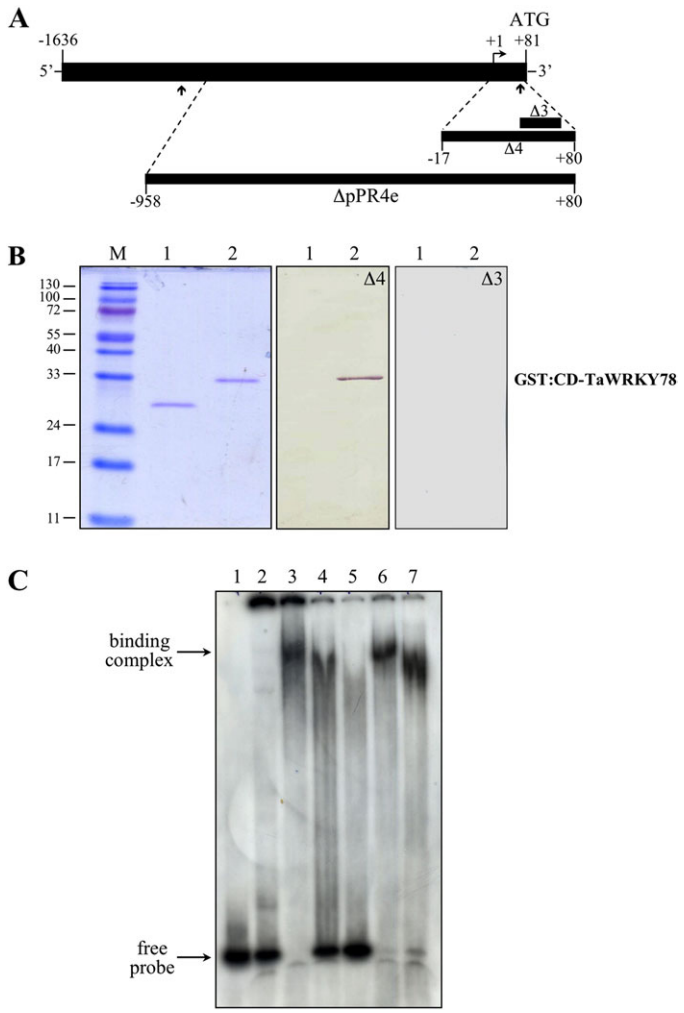

Fig. 2. (A) Schematic overview of the *wPR4e* promoter. W boxes (↑) and promoter fragment positions are indicated. +1, Transcriptional start site; +81, ATG position. (B) SDS-PAGE (left panel) of GST control protein (lane 1) and GST:CD-TaWRKY78 recombinant fusion protein (lane 2). Southwestern (central and right panels) using biotinylated *wPR4e*-Δ4 and *wPR4e*-Δ3 DNA fragments as a probe, respectively. M, Molecular weight markers. (C) EMSA assay using *wPR4e*-Δ4 fragment as a probe. Negative controls, ^{32}P-probe without protein (lane 1) and ^{32}P-probe with GST protein (lane 2). Binding assay (lane 3), ^{32}P-probe with GST:CD-WRKY78. Competitive binding assay (lanes 4 and 5), addition of increasing molar excess (50× and 300×, respectively) of non-radioactive probe. Competitive binding assay with non-specific probe (lanes 6 and 7), addition of increasing amount of poli dI-dC (50 ng and 100 ng, respectively).

was used in Southwestern experiments aimed at verifying its ability to be recognized by GST:CD-TaWRKY78 through its W-box element. Another promoter fragment without any predicted *cis*-acting element was used as a negative control. This DNA fragment, named *wPR4e*-Δ3 (Fig. 2A), spanned the +40/+69 region of the *wPR4e* promoter and was produced by amplification with specific biotinylated oligos. Purified GST:CD-TaWRKY78 was subjected to SDS-PAGE analysis (Fig. 2B, left panel, lane 2) followed by electroblotting on nitrocellulose membranes. The membranes were individually probed with the biotinylated *wPR4e*-Δ4 (Fig 2B, central panel) and *wPR4e*-Δ3 (Fig. 2B, right panel) promoter fragments and the resulting binding was shown by using peroxidase-conjugated streptavidin antibody. Purified GST was used as a control (Fig. 2B, all panels, lane 1). As can be observed, the fusion protein is able to bind the *wPR4e*-Δ4 promoter fragment through its CD-TaWRKY78 (Fig. 2B, central panel, lane 2) domain, while no binding was observed using *wPR4e*-Δ3 (Fig 2B, right panel, lane 2).

The *wPR4e*-Δ4 fragment was also used in EMSA in order to corroborate the above results. As shown in Fig. 2C, GST:CD-WRKY78 is able to recognize the unique W-box present in the promoter fragment (Fig. 2C, lane 3). An excess of unlabelled *wPR4e*-Δ4 fragment (Fig. 2C, lanes 4, 5) could effectively compete for binding with the labelled probe, whereas a non-competitor DNA was not able to hinder binding to the W-box (Fig. 2C, lanes 6, 7). Purified GST was used as a control (Fig. 2C, lane 2). It can thus be concluded that GST:CD-WRKY78 is able specifically to recognize and bind the W-box present in the *wPR4e* promoter *in vitro*.

In vivo *interaction between TaWRKY78 and the* wPR4e *promoter*

A previous study showed that a 1716 bp 5'-flanking region of the *wPR4e* gene fused to the GUS-encoding *uidA* reporter gene is able to drive expression of the reporter gene in transgenic tobacco plants (Bertini *et al.*, 2006). In order to study the regulation of the *wPR4e* promoter by TaWRKY78, an *in vivo* transient expression assay using polyethylene glycol (PEG)-mediated direct gene transfer into *Arabidopsis* leaf protoplasts was set up (Yoo *et al.*, 2007). To this aim *Arabidopsis* protoplasts were transfected with a plasmid carrying the *GUS* reporter gene under the control of the *wPR4e* promoter (*pPR4e*::*GUS* plasmid) containing two W-boxes, as described above. A plasmid containing the same *GUS* reporter gene under the control of a 1038 bp truncated version of the *wPR4e* promoter, referred to as *ΔpPR4e* (Fig. 2A), spanning the –958/+80 region and containing a single W-box localized at position +72/+76 (Δ*pPR4e::GUS* plasmid), was also used. Either of the above constructs was co-transfected in *Arabidopsis* leaf protoplasts together with a plasmid carrying the *TaWRKY78* coding sequence transcribed from the cauliflower mosaic virus (CaMV) *35S* promoter (*35S*::*TaWRKY78*). A plasmid carrying the *LUC* reporter gene under the control of the CaMV *35S* promoter (*35S::LUC*) was also used in PEG-mediated transfections to test transformation efficiency. Twenty-four hours later, total proteins were extracted from *Arabidopsis* protoplasts and assayed for LUC and GUS activities. GUS activity was normalized relative to LUC activity and the normalized value was considered to represent the strength of the transactivating function of the TF. As shown in Fig. 3, both the full-length *wPR4e* promoter and its W-box containing truncated version *ΔpPR4e* were able to drive the expression of the *GUS* reporter gene, even if at basal levels,

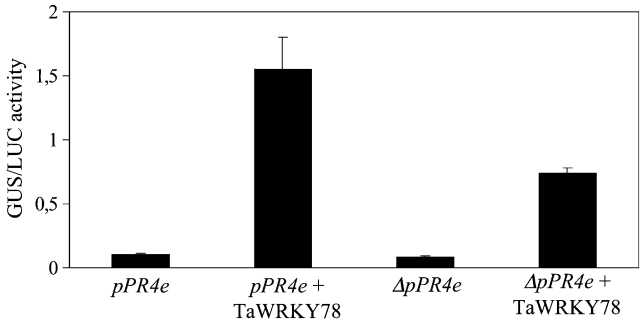

Fig. 3. Transcriptional activity of *pPR4e* and its truncated version (Δ*pPR4e*) with or without TaWRKY78 TF. Transient *GUS* expression driven by: *pPR4e*, *pPR4e* plus TaWRKY78, Δ*pPR4e*, and Δ*pPR4e* plus TaWRKY78. GUS expression increased 15-fold and 9-fold when TaWRKY78 was provided *in trans* to *pPR4e* and Δ*pPR4e*, respectively. Bars represent the average of GUS–LUC activity ratios from five transformations. Error bars represent SDs.

but their activities increased (15-fold and 9-fold induction, respectively) when the transactivating TF bound to the cognate *cis*-acting elements. This suggests that both W-boxes present in the *wPR4e* promoter are functionally active. Moreover, these results indicate that transient transformation of *Arabidopsis* protoplasts is an appropriate method for *in vivo* analysis of *cis*-acting elements.

Characterization of the Arabidopsis PR4-*type gene* AtHEL

In order to investigate whether *PR4* genes from different classes within the Anthophyta phylum are similarly regulated, *wPR4e* from the monocot wheat was compared with *AtHEL*, the orthologous *PR4* gene from the dicot *A. thaliana*. The latter gene belongs to class I of the PR4 family, since it encodes a preproprotein having a structure typical of hevein-related polypeptides. The deduced amino acid sequence contains a signal peptide for targeting to the secretory pathway, an N-terminal Cys-rich domain, a spacer region, a C-terminal domain related to the PR4s of class I, and a carboxy-terminal peptide of 13 amino acids involved in vacuolar sorting (Neuhaus *et al.*, 1991). Previous studies showed that *AtHEL* mRNA is inducible after both turnip crinkle virus (TCV) infection and ET treatment (Potter *et al.*, 1993). To investigate the expression pattern of *AtHEL* under different conditions, *AtHEL* transcript levels in response to leaf treatment with SA, MeJA or ACC and after fungal infection or wounding were analysed. Figure 4 shows the time-course of the induction of the *AtHEL* gene in 5-week-old *A. thaliana* ecotype Col-0 leaves by Northern blot analysis. Figure 4A demonstrates that the *AtHEL* gene is strongly induced upon treatment with MeJA or the ET precursor ACC within 24 h. In addition, wounding (Fig. 4B) and inoculation with the pathogen *Fusarium oxysporum* f.sp. *conglutinans* (Fig. 4C) strongly activated *AtHEL* gene expression. Exogenous application of SA did not affect the level of *AtHEL* mRNA accumulation, suggesting that the JA/ET signalling pathway predominantly regulates *AtHEL*

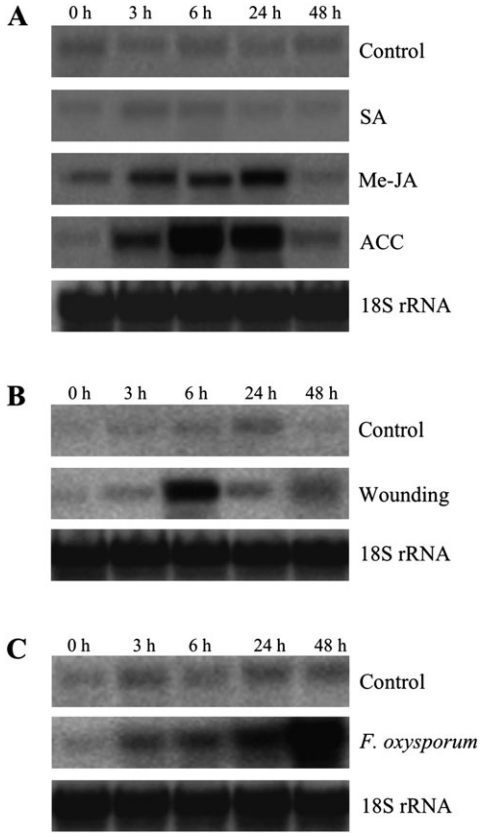

Fig. 4. Northern blot analysis of *AtHEL* gene expression in 5-week-old *Arabidopsis* Col-0 plants in response to SA (1 mM), MeJA (1 μM) and ACC (1 mM) treatments (A), wounding (B) and *F. oxysporum* infection (C). Leaf tissue was harvested at 0, 3, 6, 24, and 48 h after treatments for RNA analysis. Equal loading of RNA samples was checked using a probe for 18S rRNA.

gene expression. This latter evidence is further strengthened by the early induction observed after wounding. Moreover, as JA/ET-signalling is also associated with the plant response to pathogen infection (Pieterse *et al.*, 2009), the time-course expression pattern of 5-week-old *A. thaliana* ecotype Col-0 following *F. oxysporum* f. sp. *conglutinans* infection was verified. As shown in Fig. 4C, fungal infection activates the expression of the *AtHEL* gene, indicating that it may play a role in plant defence mechanisms triggered by both biotic and abiotic stresses, according to previous finding (Thomma *et al.*, 1998).

Characterization of the AtHEL *promoter*

To identify putative *cis*-acting regulatory elements within the *AtHEL* promoter, both PLACE and PlantCARE databases were used according to the 'pattern matching' approach (Lescot *et al.*, 2002). Inspection of 1015 bp upstream of the start codon revealed the presence of several sequences that match *cis*-regulatory elements described in other plant genes. Both TATA and CAAT-boxes were present, located at –28/–23 and –101/–98 bp upstream of the putative transcription start site (+1), respectively. Putative *cis*-acting elements present within the *AtHEL* promoter and known to be involved in

stress induction are detailed in Table 2 together with the corresponding consensus sequences, associated TFs, and responsiveness. Within the upstream region of the *AtHEL* promoter, ten E-box elements (CANNTG) recognized by the bHLH TF family were located in both orientations (Stalberg *et al.*, 1996). Both MYB core and MYC consensus *cis*-acting elements recognized by Myb and Myc TFs, respectively, were also identified in the *AtHEL* promoter. The latter TFs have been shown to be important water stress- and abscisic acid (ABA)-responsive *trans*-acting factors (Abe *et al.*, 1997). Among other *cis*-acting elements that can be detected in the *AtHEL* promoter there are DOF core and TAAAG motifs both recognized by Dof proteins known to be induced by SA and oxidative stress (Yanagisawa, 2004), and one GCC core recognized by EREBPs, a group of ET-responsive TFs. Finally, ten sequences that mach the consensus W-box sequence ((T)TGAC) found in several *PR* gene promoters (Eulgem *et al.*, 2000; 'lker and Somssich, 2004), are located at positions –427/–424, –494/–491, –502/–498, –507/–504, –569/–566, –680/–676, –837/–833, –914/–910, –923/–919, and –929/–925 relative to the putative transcription start site.

Isolation and characterization of the TaWRKY78 orthologue of A. thaliana

In *Arabidopsis* there are 72 expressed WRKY genes that are subdivided into three groups based on structural features (http://www.arabidopsis.org/browse/genefamily/WRKY.jsp). Based on the *TaWRKY78* sequence, the *Arabidopsis* orthologue *AtWRKY20* was identified. Since very often the expression of a gene reflects that of the TFs involved in its regulation, the question whether *AtWRKY20* is regulated by the same biotic and abiotic stimuli controlling *AtHEL* expression was addressed. To this end, a 500 bp long amplicon, was generated from *AtWRKY20* by PCR and used as a probe in Northern blot analysis. As shown in Fig. 5A, MeJA and ACC strongly induced *AtWRKY20,* whereas SA had little or no effect. Also wounding (Fig. 5B) and *F. oxysporum* infection (Fig. 5C) induced *AtWRKY20* expression, indicating that the expression patterns of *AtWRKY20* and *AtHEL* are very similar.

In vitro *interaction between wheat GST:CD-AtWRKY20 and the* AtHEL *promoter*

The C-terminal WRKY domain of *AtWRKY20* (CD-AtWRKY20) was produced as fusion protein with gluthatione-*S*-transferase (GST) (GST:CD-AtWRKY20), using specific primers.

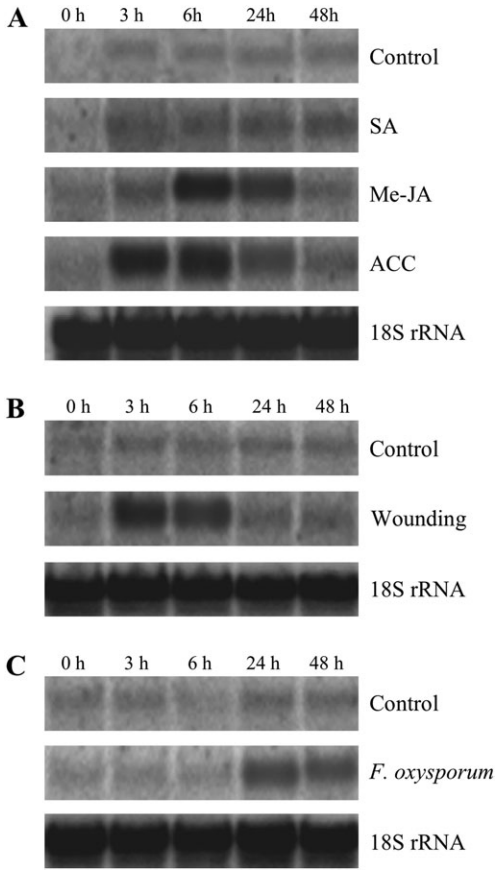

Fig. 5. Northern blot analysis of *AtWRKY20* gene expression in 5-week-old *Arabidopsis* Col-0 plants in response to SA (1 mM), MeJA (1 μM), and ACC (1 mM) treatments (A), wounding (B), and *F. oxysporum* infection (C). Leaf tissue was harvested at 0, 3, 6, 24, and 48 h after treatments for RNA analysis. Equal loading of RNA samples was checked using a probe for 18S rRNA.

Table 2. *In silico* pHEL promoter analysis

cis-element	Consensus sequence	Strand	Responsiveness	Associated TFs	Copy number	Database (PLACE)
DOF core	AAAG	(+)/(–)	Oxidative stress	Dof	11	S000265
DPBF core	ACACNNG	(–)	ABA	bZIP	2	S000292
E-box	CANNTG	(+)/(–)	ABA	bHLH	10	S000144
GCC core	GCCGCC	(+)	Ethylene	EREBPs	1	S000430
GT-1 consensus	GRWAAW	(+)/(–)	SA, Light	GT-1	13	S000198
MYB core	CNGTTR	(+)/(–)	Dehydration	Myb	2	S000176
MYC consensus	CANNTG	(+)/(–)	ABA, Dehydration	Myc	10	S000407
TAAAG motif	TAAAG	(+)	Oxidative stress, SA	Dof	2	S000387
W-box	(T)TGAC	(+)/(–)	SA, Wounding, Pathogen	WRKY	10	S000390
						S000447

Potential *cis*-acting regulatory elements identified in At*HEL* flanking regions by *in silico* 'pattern matching' search against PLACE database. Several features of the selected elements are also highlighted.

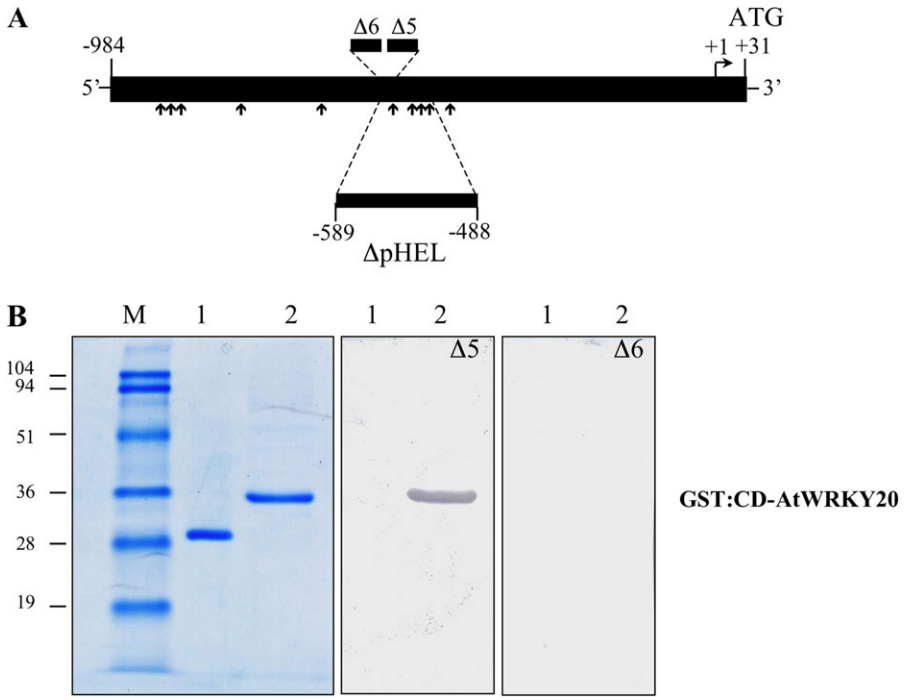

Fig. 6. (A) Schematic overview of the *AtHEL* promoter. W boxes (↑) and promoter fragment positions are indicated. +1, Transcriptional start site; +31, ATG position. (B) SDS-PAGE (left panel) of GST control protein (lane 1) and GST:CD-AtWRKY20 recombinant fusion protein (lane 2). Southwestern (central and right panels) using biotinylated *AtHEL*-Δ5 and *AtHEL*-Δ6 DNA fragments as a probe, respectively. M, molecular weight markers.

A promoter fragment of 21 bp (*AtHEL*-Δ5, Fig. 6A) containing one W-box and spanning the –549/–569 region of the *AtHEL* promoter was used in Southwestern experiments aimed at verifying its ability to be recognized by GST:CD-AtWRKY20 through its W-box element. Another fragment (*AtHEL*-Δ6, Fig. 6A) without any predicted *cis*-acting element and spanning the –570/–589 region of the *AtHEL* promoter was used as a negative control. Purified GST:CD-AtWRKY20 was subjected to SDS-PAGE analysis (Fig. 6B, left panel, lane 2) followed by electroblotting on nitrocellulose membranes. The membranes were individually probed with the biotinylated *AtHEL*-Δ5 (Fig. 6B, central panel) and *AtHEL*-Δ6 (Fig. 6B, right panel) promoter fragments and the resulting binding was evidenced by using peroxidase-conjugated streptavidin antibody. Purified GST was used as a control (Fig. 6B, all panels, lane 1). As can be observed, the fusion protein is able to bind the *AtHEL*-Δ5 promoter fragment through its CD-AtWRKY20 domain (Fig. 6B, central panel, lane 2), while no binding was observed using the *AtHEL*-Δ6 promoter fragment (Fig. 6B, right panel, lane 2).

In vivo *interaction between AtWRKY20 and the* AtHEL *promoter*

In order to study the regulation of the *AtHEL* promoter following *in vivo* interaction with AtWRKY20, the 1015 bp 5' flanking region of *AtHEL* gene, containing ten W-boxes, was fused to the GUS-encoding *uidA* reporter gene (*pHEL*::*GUS* plasmid) and used for transient expression assays based on PEG-mediated direct gene transfer into *Arabidopsis* leaf protoplasts (Yoo *et al.*, 2007). *Arabidopsis* protoplasts were co-transfected with *pHEL*::*GUS* plus a plasmid carrying the *AtWRK20* coding sequence transcribed from the CaMV *35S* promoter (*35S*::*AtWRKY20*). A plasmid carrying the *LUC* reporter gene was also used as an efficiency control. The same co-transformation experiment was also carried out using a 102 bp deletion of the *AtHEL* promoter, spanning the –589/–488 region defined as Δ*pHEL* (Fig. 6A) and containing four W-box localized at position –494/–491, –502/–498, –507/–504, and –569/–566 (Δ*pHEL*::*GUS* plasmid). Twenty-four hours after transfection, total proteins were extracted and assayed for LUC and GUS activities as above described. As shown in Fig. 7, both the *AtHEL* promoter and its deletion Δ*pHEL* were able to drive the expression of the *GUS* reporter gene, although at basal levels, but their activities increase 10-fold and 12-fold, respectively, when the transactivating TF binds to the cognate *cis*-acting elements. These results show that AtWRKY20 is able to positively regulate the *AtHEL* promoter through interaction with W-boxes.

In vivo *interaction between TaWRKY78 and AtWRKY20 with orthologous* PR4 *promoters*

In order to verify whether the WRKY TFs isolated from wheat (TaWRKY78) and *Arabidopsis* (AtWRKY20) are able to recognize and regulate the expression of the orthologous *PR4* promoters *pHEL* and *pPR4e*, respectively, transient expression analysis was carried out. The constitutive level of

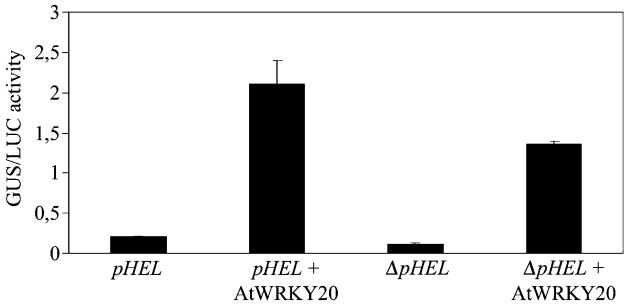

Fig. 7. Transcriptional activity of *pHEL* and its truncated version (Δ*pHEL*) with or without AtWRKY20 transcription factor. Transient GUS expression driven by: *pHEL*, *pHEL* plus AtWRKY20, Δ*pHEL* and Δ*pHEL* plus AtWRKY20. GUS expression increased 10-fold and 12-fold when AtWRKY20 was provided *in trans* to *pHEL* and Δ*pHEL*, respectively. Bars represents the average of GUS–LUC activity ratios from five transformations. Error bars represent SDs.

expression of the endogenous *AtHEL* gene was measured by qPCR analysis of *A. thaliana* protoplasts. Protoplasts isolated from *A. thaliana* were transfected with *35S*::*TaWRKY78* plasmid DNA in order to verify the ability of the wheat TF to regulate the *Arabidopsis AtHEL* promoter. Transfection with an empty vector (*pUC-GUS*) was used as a control. Following transformation, RNA was reverse-transcribed using oligo(dT) as reverse primer and the resulting cDNA was amplified using *AtHEL*-specific primers that allowed qPCR analysis. As shown in Fig. 8A, the *AtHEL* mRNA was constitutively expressed even though at low levels. The presence of TaWRKY78 was able to promote transcription of the endogenous *AtHEL* gene, increasing its expression level by 18-fold (Fig. 8A). The same cDNA was amplified using *TaWRKY78* specific primers. As expected, the transgene was not expressed in control samples transformed with the empty vector, whereas it was expressed at high levels in *Arabidopsis* protoplasts transfected with the plasmid carrying *TaWRKY78* (Fig. 8B). These results clearly show that the endogenous *AtHEL* promoter can be regulated *in vivo* by the wheat TF TaWRKY78.

Similar results were obtained when *Arabidopsis* leaf protoplasts were transfected with plasmids carrying the *AtHEL* promoter upstream of the GUS reporter gene (*pHEL*::*GUS*) and *35S*::*TaWRKY78*. Plasmids carrying the *LUC* reporter gene were also used as efficiency control. The above-described deletion of the *AtHEL* promoter (Δ*pHEL*) was also used to verify the ability of TaWRKY78 to regulate the 102 bp promoter fragment. As shown in Fig. 8C, the presence of the wheat TF yields an induction of about 6-fold of the *pHEL* promoter and has an even stronger effect (about 9-fold) on the Δ*pHEL*.

To study the regulation of the *wPR4e* promoter by AtWRKY20, *Arabidopsis* leaf protoplasts were co-transfected using *pPR4e*::*GUS* and *35S*::*AtWRKY20*. Plasmids carrying the *LUC* reporter gene were co-transferred as efficiency control. The Δ*pPR4e* deletion was also used to verify its responsiveness to the *Arabidopsis* TF. The results are presented in Fig. 8D. AtWRKY20 was able to recognize the orthologous cognate *wPR4e* promoter and to increase its expression level by 11-fold. Moreover, it was also able to bind the single W-box present in Δ*pPR4e* and to induce its expression by about 8-fold. Together these results indicate that each of the orthologous WRKY TFs from the monocot wheat and the dicot *Arabidopsis* can cross-activate cognate *PR4* promoters from other species, suggesting WRKY functioning is essentially conserved in these distant plant species.

Discussion

The identification of five new *PR4* genes from *T. aestivum* cv. S. Pastore, named *wPR4e*, *wPR4f-a*, *wPR4f-b*, *wPR4f-c*, and *wPR4g*, was recently reported (Bertini *et al.*, 2006). *In silico* analysis of the 5' untranslated region of *wPR4e* allowed the putative promoter region to be characterized in terms of regulative elements. Several abiotic and biotic stress-responsive elements were localized both on the coding and the antiparallel strand. Among them two W-boxes, the docking sites of WRKY TFs, are located upstream and downstream the transcription start site.

WRKY TFs are considered to play important regulatory functions in defence against biotic and abiotic stresses (Iker and Somssich, 2004; Eugelm and Somssich, 2007; Pandey and Somssich, 2009). Before the identification of two EST homologues to WRKY proteins, one from *Giardia lamblia*, a primitive protozoan, and another from *Dictyostelium discoideum*, a slime mould, the WRKY superfamily was considered to be restricted to the plant kingdom. The latter two organisms evolutionally precede the divergence of plants from animals and fungi, indicating an ancient origin of WRKYs (Eulgem *et al* 2000; Iker and Somssich, 2004; Pan *et al.*, 2009).

Functional characterization of the wheat TaWRKY78 TF

In this study the isolation of a wheat WRKY TF of group I, named TaWRKY78 on the basis of the orthologous sequence of rice, and its functional characterization are presented. The C-terminal domain of TaWRKY78, containing the functional WRKY motif, was able to bind *in vitro* the Δ4 promoter fragment of the *wPR4e* gene, containing several *cis*-acting elements along with one W-box (TTGAC) located at +72/+76 relative to the putative transcription start site. The GST:CD-WRKY78 fusion protein showed a strong W-box-dependent binding activity with the minimal promoter region using both Southwestern and EMSA assays, since a promoter fragment deleted of this element was not bound (Fig. 2, right panel). In order to confirm the ability of TaWRKY78 to recognize the *wPR4e* promoter, *in vivo* transient expression analyses were carried out using PEG-mediated direct gene transfer into *Arabidopsis* leaf protoplasts. Either the *wPR4e* promoter, containing two W-boxes, or its deletion Δ*pPR4e*, containing only one W-box, were able to drive the expression of the GUS gene reporter in the absence of TaWRKY78. However, GUS

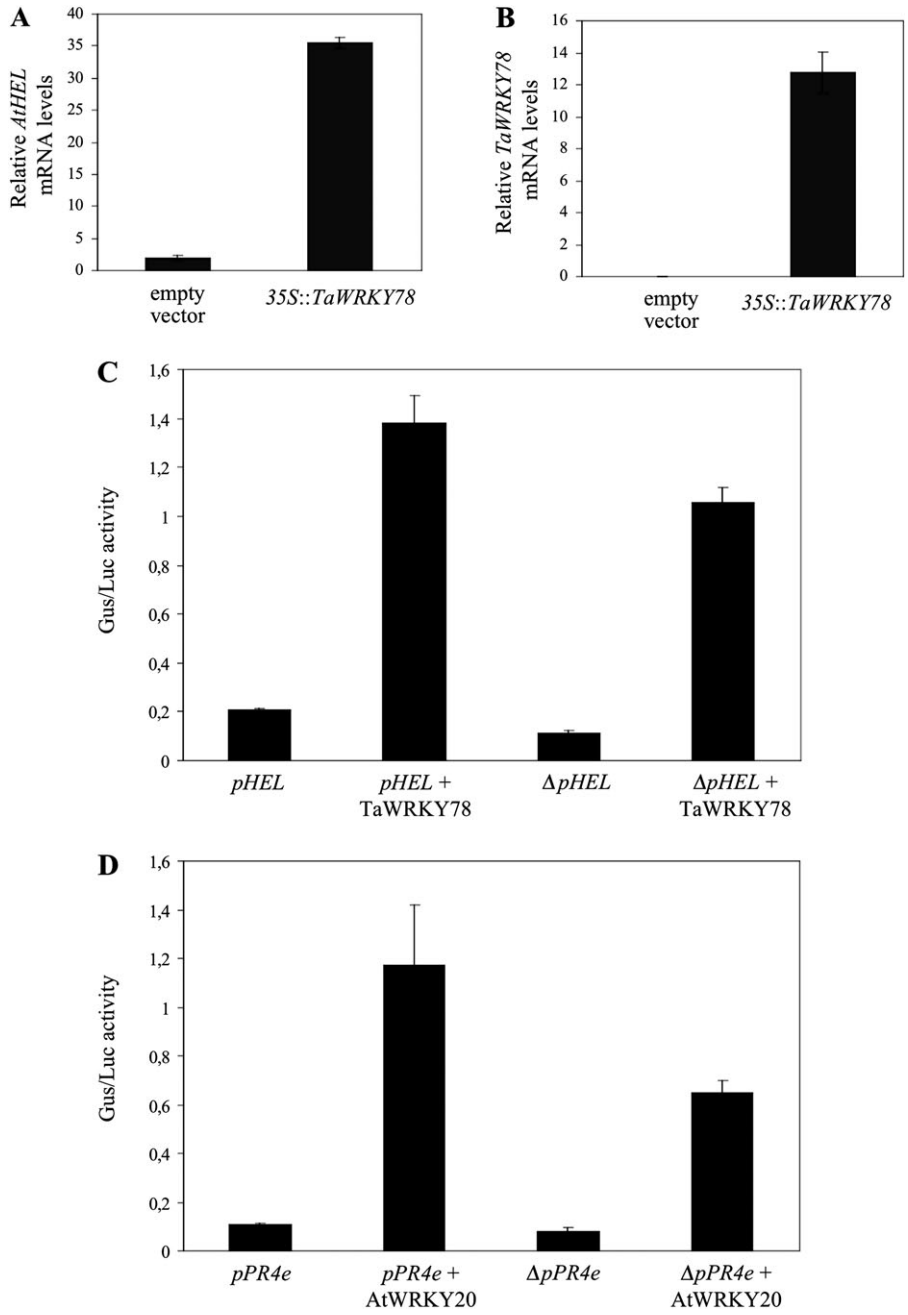

Fig. 8. qPCR analysis of *AtHEL* (A) and *TaWRKY78* (B) transcript level in *Arabidopsis* leaves protoplasts transformed with empty vector (A, B) and *35S*::*TaWRKY78* plasmid (A, B). *AtHEL* endogenous expression increases 18-fold when TaWRKY78 was provided *in trans* (A). High-level expression of *TaWRKY78* in protoplasts transformed with *35S*::*TaWRKY78* (B) indicates high transformation efficiency. Transcriptional activity of *pHEL* and Δ*pHEL* (C) and *pPR4e* and Δ*pPR4e* (D) with or without TaWRKY78 and AtWRKY20 transcription factor, respectively. Transient GUS expression driven by *pHEL, pHEL* plus TaWRKY78, Δ*pHEL*, and Δ*pHEL* plus TaWRKY78 (C); *pPR4e, pPR4e* plus AtWRKY20, Δ*pPR4e*, and Δ*pPR4e* plus AtWRKY20 (D). GUS expression increased 6-fold and 9-fold when TaWRKY78 was provided *in trans* to *pHEL* and Δ*pHEL*, respectively, and 11-fold and 8-fold when AtWRKY20 was provided *in trans* to *pPR4e* and Δ*pPR4e*, respectively. Bars represents the average of GUS-LUC activity ratios from five transformations. Error bars represent SDs.

activity increased by 15-fold and 9-fold, respectively, in the presence of TaWRKY78. These results clearly show that the wheat TF isolated here plays a positive role in regulating the *pPR4e* promoter and that both W-boxes are functionally active.

The expression pattern of *wPR4e* in wheat coleoptiles and roots following pathogen infection, treatment with SAR chemical inducers, and wounding had already been reported (Bertini *et al.*, 2006). *wPR4e* was found to be induced following MeJA treatment, *F. culmorum* infection and wounding with different strengths and efficiency. In this study, the responsiveness of *TaWRKY78* to similar treatments in young seedlings was investigated. The expression of *TaWRKY78* was weakly affected by MeJA (1.7-fold)

Table 3. Plasmid vectors

Plasmid	Relevant characteristics	Reference
pUC::GUS	*Hin*dIII/*Eco*RI fragment from pBI101.2 containing GUS reporter gene and Nost cloned in pUC8	Bertini *et al.* (2006)
pUC-35S::GUS	*Hin*dIII/*Xba*I CaMV 35S promoter from pBI121 cloned in pUC::GUS	This work
pUC-35S::*LUC*	*Bam*HI/*Sac*I *LUC* reporter gene from pGEM-*LUC* (Promega) cloned in pUC-35S::GUS instead of GUS	This work
pUC-pPR4e::GUS	*Bam*HI /*Sma*I pPR4e cloned in pUC::GUS upstream GUS	Bertini *et al.* (2006)
pUC-ΔpPR4e::GUS	*Hin*dIII/*Bam*HI ΔpPR4e cloned in pUC::GUS upstream GUS	This work
pUC-pHEL::GUS	*Hin*dIII/*Pst*I pHEL cloned in pUC::GUS upstream GUS	This work
pUC-ΔpHEL::GUS	*Hin*dIII/*Pst*I ΔpHEL cloned in pUC::GUS upstream GUS	This work
pUC35S::Ta*WRKY78*	*Bam*HI/*Sa* I Ta*WRKY78* cloned in pUC-35S::GUS instead of GUS	This work
pUC35S::At*WRKY20*	*Xba*I/*Stu*I At*WRKY20* cloned in pUC-35S::GUS instead of GUS	This work

The table showsthe characteristics of the plasmid vectors used during transient expression assays.

and almost unaffected by SA treatment, wounding or *F. culmorum* infection. It is well known that the final expression of a gene is the result of concerted action between several TFs, each of which responsive to different stimuli. In this case it is conceivable that, besides TaWRKY78, other TFs contribute to the full expression of the *wPR4e* gene in wheat seedlings.

Characterization of the Arabidopsis *orthologous PR4 gene* AtHEL

Several authors reported the importance of TFs in regulating plant defence genes (Desveaux *et al.*, 2005; Jalali *et al.*, 2006) and particularly the role of WRKY TFs in defence mechanisms (Iker and Somssich, 2004; Eugelm and Somssich, 2007; Pandey and Somssich, 2009). In order to understand better the transcriptional regulation of genes located downstream of signal transduction pathways leading to disease tolerance and the role of WRKY TF, a comparative analysis between members of the *PR4* family of defence genes, namely *wPR4e* and its orthologous gene from *Arabidopsis AtHEL*, was performed. Previous studies showed that *AtHEL* mRNA is inducible after TCV infection as well as ET and SA treatments (Potter *et al.*, 1993). Several authors confirmed the ET-mediated inducibility of the gene that is considered an ET/JA pathway marker (Seo *et al.*, 2008), while SA-mediated *AtHEL* induction was achieved using higher concentration of chemical inducer (5 mM) than normally used for *Arabidopsis*. To deepen the expression studies of the *AtHEL* gene, its transcription pattern in response to MeJA, SA, and ACC treatments as well as to pathogen infection or wounding was analysed. Northern analysis showed that *AtHEL* is strongly induced upon MeJA and ACC treatments as well as after *F. oxysporum* infection or wounding, whereas it is unaffected by SA treatments at the tested concentration (1 mM). It is well recognized that SA, JA, and ET accumulate in response to pathogen infection or herbivore damage (mimicked by wounding), leading to the activation of distinct sets of *PR* genes. In general, biotrophic pathogens activate preferentially the SA-mediated transduction pathway, whereas necrotrophic pathogens and herbivore insects induce the JA/ET-mediated signalling (Ton *et al.*, 2002). On the basis of the results reported in this paper it can be concluded that *AtHEL* expression follows a signal transduction pathway mediated by jasmonates or ET analogously to its wheat orthologue *wPR4e*. Further insight into the regulation of *AtHEL* expression comes from the characterization of its putative promoter region in terms of regulative elements. *In silico* analysis revealed the presence of several JA- and pathogen-responsive elements in the *AtHEL* promoter, adding significance to its induction pattern. Taken together these data strongly support an active role for *AtHEL* in the defence responses recruited through JA-dependent signal transduction pathways. Among several *cis*-acting regulatory elements known to be involved in plant defence, ten W-box elements, recognized by WRKY proteins, were identified, which were also found in the promoters of *Hevea brasiliensis Hev1.1* and *Hev2.1* (Pujade-Renaud *et al.*, 2005) as well as in the *wPR4e* promoter (Bertini *et al.*, 2006).

Functional characterization of the Arabidopsis *orthologous AtWRKY20 TF*

In order to address the question whether the regulation of WRKY-mediated PR4 gene expression in two very different plant species is similar, the *Arabidopsis* orthologue of wheat *TaWRKY78*, namely *AtWRKY20*, was isolated. Characterization of *AtWRKY20* revealed that it follows an expression pattern similar to the one reported above for *AtHEL*, i.e. strong induction after MeJA and ACC treatments as well as after *F. oxysporum* infection or wounding. This result strongly suggests that, besides other TFs, AtWRKY20 plays a dominant role in the expression of *AtHEL* gene. With the aim of deepening the interaction study between the *AtHEL* promoter and its regulator AtWRKY20, transient expression analyses were carried out using either the *AtHEL* promoter, containing ten W-boxes, or its truncated version Δ*pHEL*, containing four W-boxes. It is interesting to note that the 102 bp promoter fragment Δ*pHEL* is able to drive the expression of GUS reporter gene, though with lower efficiency than the whole *AtHEL* promoter. Moreover, AtWRKY20 is able to induce either *pHEL* or its truncated version Δ*pHEL* with comparable efficiency (Fig. 7). It can be speculated that the four W-boxes present in the *AtHEL*

promoter deletion might play a pivotal role in *AtHEL* gene regulation, whereas those localized distantly from the putative transcription start site might be almost inactive.

Cross activity of orthologous WRKY transcription factors in wheat and Arabidopsis

Once the capacity of TaWRKY78 and AtWRKY20 to regulate positively the corresponding *PR4* genes was established, the questions whether the wheat TF was able to recognize and regulate the *Arabidopsis AtHEL* promoter and whether the *Arabidopsis* TF was able to recognize and regulate the orthologue *wPR4e* were addressed. *In vivo* expression studies provided evidence that both TFs are able to cross regulate the orthologous *PR4* genes with an efficiency slightly lower than that exerted on the cognate promoters. The observation that orthologous genes are subjected to similar transcriptional control by orthologous TFs demonstrates that the terminal stages of signal transduction pathways leading to defence are highly conserved and suggests a fundamental role of PR4 proteins in plant defence. Moreover, these results corroborate the hypothesis of Mangelsen *et al.* (2008) that gene orthology implies similar gene function. Finally, in this study, evidence is provided that diversification between monocot and dicot has most likely occurred after the specialization of WRKY function.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Amino acid sequence alignment between TaWRKY78, AtWRKY20, OsWRKY78, and their paralogues: clone wlmk1.pk0035.d9:fis (Acc. no. BT009430), TaWRKY17 (Acc. no. EU665429.1), AtWRKY2 (Acc. no. AF418308.1), AtWRKY4 (Acc. no. AF425835.1), AtWRKY33 (Acc. no. NM_129404), and OsWRKY9 (Acc. no. AY341850.1).

Supplementary Fig. S2. Phylogenetic tree of the WRKY TFs aligned in Supplementary Fig. S1.

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