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Ectopic expression of *PtaRHE1*, encoding a poplar RING-H2 protein with E3 ligase activity, alters plant development and induces defence-related responses

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Abstract

RING (really interesting new gene)-H2 domain-containing proteins are widely represented in plants and play important roles in the regulation of many developmental processes as well as in plant–environment interactions. In the present report, experiments were performed to unravel the role of the poplar gene *PtaRHE1*, coding for a RING-H2 protein. *In vitro* ubiquitination assays indicate a functional E3 ligase activity for PtaRHE1 with the specific E2 ubiquitin-conjugating enzyme UbcH5a. The overexpression of *PtaRHE1* in tobacco resulted in a pleiotropic phenotype characterized by a curling of the leaves, the formation of necrotic lesions on leaf blades, growth retardation, and a delay in floral transition. The plant gene expression response to *PtaRHE1* overexpression provided evidence for the up-regulation of defence- and/or programmed cell death-related genes. Moreover, genes coding for WRKY transcription factors as well as for mitogen-activated protein kinases, such as wound-induced protein kinase (WIPK), were also found to be induced in the transgenic lines as compared with the wild type. In addition, histochemical β-glucuronidase staining showed that the *PtaRHE1* promoter is induced by plant pathogens and by elicitors such as salicylic acid and cellulase. Taken together, these results suggest that the E3 ligase PtaRHE1 plays a role in the ubiquitination-mediated regulation of defence response, possibly by acting upstream of WIPK and/or in the activation of WRKY factors.

Key words: Defence response, E3 ligase, *Nicotiana tabacum*, *Populus tremula*×*P. alba*, RING-H2.

Introduction

The poplar gene *PtaRHE1*, coding for a RING (really interesting new gene)-H2 domain-containing protein, has been identified through a cDNA-amplified fragment length polymorphism (AFLP) screening aimed at discovering genes whose expression is differential between the top and the base of 6-month-old *Populus tremula*×*P. alba* stems (van Raemdonck *et al.*, 2005). *In situ* RT-PCR localization of *PtaRHE1* in poplar stems undergoing secondary growth showed that this gene is mainly expressed within the cambial zone and, more particularly, in ray initials and derivatives (van Raemdonck *et al.*, 2005). RING domains

are characterized by four pairs of conserved cysteine (C) and histidine (H) residues coordinating two zinc ions in a cross-brace structure. Depending on the nature of metal ligands, but also on the spacing between pairs of metal ligands, RINGs have been classified into two main families, namely RING-HC (C_3HC_4) and RING-H2 ($C_3H_2C_3$), and into other minor RING variants (Saurin *et al.*, 1996; Jensen *et al.*, 1998; Stone *et al.*, 2005). As described by van Raemdonck *et al.* (2005) and in Supplementary Fig. S1 available at *JXB* online, the closest homologue (54% identity) to PtaRHE1 is the *Arabidopsis thaliana* ATL2

(*Arabidopsis Toxicos en Levadura*) (At3g16720) (Martínez-García *et al.*, 1996) whose family members are characterized by a transmembrane (TM) domain, a basic domain, a conserved domain, a RING-H2 domain, and a highly divergent region in the C-terminal part of the protein (Serrano *et al.*, 2006). As described by Serrano *et al.* (2006), the two T-DNA insertional mutants of ATL2 are not knock-out mutants and show no phenotype, and unfortunately cannot be used for complementation.

There is well-documented evidence showing that many plant RING domain-containing proteins act as E3 ubiquitin (Ub) ligases by promoting ubiquitination of specific target proteins. Ub attachment can be accomplished in different ways (including protein monoubiquitination, multiple mono-ubiquitination, and polyubiquitination) that determine the target's fate (Haglund and Dikic, 2005). The ubiquitination of protein targets requires the successive activity of the Ub-activating enzyme (E1), the Ub-conjugating enzyme (E2), and the Ub ligase (E3) which confers specificity to the degradation process (Schwechheimer *et al.*, 2009). Mono-ubiquitination is considered to function as a regulatory signal that can mediate the activity, subcellular localization, or conformation of a protein (Haglund and Dikic, 2005). Polyubiquitination produced by the linkage of Ub to K48 of another Ub moiety is known as a signal for proteosomal degradation of modified target proteins via the Ub–26S proteasome pathway (Bachmair *et al.*, 2001; Vierstra, 2003; Moon *et al.*, 2004). The E3 ligase activity of 64 recombinant RING-containing *A. thaliana* proteins has been investigated by *in vitro* ubiquitination assays (Stone *et al.*, 2005). Although >70% of these RING proteins were capable of mediating polyubiquitination *in vitro*, using AtUBC8 as E2 or other *A. thaliana* E2s from different subfamilies, 17 RING-H2 proteins were not, possibly as a consequence of misfolding in the expression host, or requirements for specific cofactors or E2 partners (Kraft *et al.*, 2005; Stone *et al.*, 2005).

The data reported in the literature indicate that RING proteins are associated with plant growth and development as well as with plant–environment interactions (Schwechheimer *et al.*, 2009). For instance, in *Arabidopsis*, COP1 is involved in the repression of photomorphogenesis (von Arnim and Deng, 1994; Subramanian *et al.*, 2004), BIG BROTHER in organ size by restricting the duration of cell proliferative growth (Disch *et al.*, 2006), RHF1a and RHF2a in the formation of male and female gametophytes (Liu *et al.*, 2008*a*), HUB1 and 2 in the control of cellular development during leaf and root development (Fleury *et al.*, 2007) and in flowering time control (Cao *et al.*, 2008), SHA1 in shoot apical meristem maintenance (Sonoda *et al.*, 2007), XBAT32 in lateral root development (Nodzon *et al.*, 2004), and RIE1 in seed development (Xu and Li, 2003), and in rice, EL5 was associated with root development (Koiwai *et al.*, 2007).

Other RING finger proteins are involved in the regulation of hormone signalling pathways in *A. thaliana*, such as AIP2 (Zhang *et al.*, 2005), KEG (Stone *et al.*, 2006), and SDIR1 (Zhang *et al.*, 2007) in abscisic acid (ABA)

signalling, SINAT5 in auxin response (Xie *et al.*, 2002), and BRH1 in brassinosteroid signalling (Molnár *et al.*, 2002). In addition, RING proteins have been shown to regulate the response to biotic and abiotic stresses as well as to be involved in plant defence (Craig *et al.*, 2009). For instance, RIN2 and RIN3 are involved in the *RPM1-* and *RPS2-*dependent hypersensitive response (HR) (Kawasaki *et al.*, 2005), BAH1/NLA in the regulation of salicylic acid (SA) accumulation (Yaeno and Iba, 2008), RING1 in the triggering of the programmed cell death (PCD) pathway (Lin *et al.*, 2008), XERICO in the regulation of drought tolerance through alteration of the ABA signalling pathway (Ko *et al.*, 2006), and HOS1 in regulating cold responses (Lee *et al.*, 2001).

Here, it is shown that the recombinant PtaRHE1 protein is a functional E3 ligase as demonstrated by its autoubiquitination. To characterize further the role of PtaRHE1, its overexpression was investigated. Instead of the poplar model, heterologous expression in the tobacco model plant was chosen since tobacco produces typical angiosperm wood and homozygous lines can be rapidly obtained. *Arabidopsis* was not selected in this study since although it produces secondary xylem, it lacks ray parenchyma cells (Chaffey *et al.*, 2002) where *PtaRHE1* was found to be precisely expressed (van Raemdonck *et al.*, 2005). Over-expressing *PtaRHE1* resulted in dramatic alterations of leaf phenotype as well as in up-regulation of defence genes and genes encoding WRKY transcription factors. Challenging transgenic tobacco plants with different stresses showed that the *PtaRHE1* promoter is responsive to several plant pathogens and to cellulase (Cel), as well as to ABA and SA. All together, these data suggest that *PtaRHE1* might be part of the overall signal cascades involved in plant defence and development.

Materials and methods

Plant material and growth conditions

Non-transgenic and transgenic tobacco plants (*Nicotiana tabacum* cv. Havana) were grown aseptically on Murashige and Skoog (MS) medium (Micro and 1/2 concentration Macro elements including vitamins; Duchefa) supplemented with 200 mg l^{-1} kanamycin (Duchefa) when needed. Cultures were incubated at 23±2 °C under a 16 h light photoperiod (70 µmol m^{-2} s^{-1}, cool-white fluorescent lamp; Osram). Sown seeds, or acclimatized plants, were cultivated on soil in a growth chamber under a 16 h light photoperiod at 24 °C.

Plant treatment

For biotic stress treatment, 19-day-old *pPtaRHE1::GUS* plantlets grown on phytagel (0.2%, w/v) solidified MS medium were inoculated in 20 ml of liquid MS medium containing *Rhodococcus fascians* (strain D188), *Pseudomonas syringae* pv *tabaci*, or *Agrobacterium tumefaciens* (strain C58) (500 µl of overnight bacterial culture in 2 ml of liquid YEB medium).

For abiotic stress treatment, 12-day-old *pPtaRHE1::GUS* plantlets grown in solid MS medium were transferred to fresh liquid MS medium containing ABA (150 µM), H$_2$O$_2$ (10 mM), SA (50 µM), NaCl (300 mM), Cel (100 µg ml^{-1}), spermidine (0.5 mM), or

spermine (0.5 mM). For each treatment, seedlings were harvested after 8 h for β-glucuronidase (GUS) staining.

Vector construction for PtaRHE1 *overexpression and plant transformation*

For overexpression, the coding sequence of *PtaRHE1* (AY780430), cloned in pCR®4-TOPO® (Invitrogen, Merebelke, Belgium), was amplified with the primer attb1RHE1 5′-AAAAAGCAGGCT-TAATGGACCCAGACTCG-3′ to flank the *att*B1 recombination site at the 5′ end of the coding sequence and the primer 5′-AGAAAGCTGGGTCTTAACACCGAGTTTGC-3′ to flank the *att*B2 recombination site to the 3′ end of the sequence with the stop codon. A second PCR using primers amplifying the entire *att*B1 and *att*B2 sequences was performed, according to the supplier's instructions (Invitrogen). The PCR fragment was cloned in the Gateway™ vector pDONR221 yielding the entry clone RING BP2, which was then recombined with the Gateway™-compatible T-DNA destination vector pK7WG2 containing a cauliflower mosaic virus (CaMV) 35S promoter (Karimi *et al.*, 2002), in reactions mediated by the Gateway™ BP and LR Clonase™ Enzyme Mix (Invitrogen).

The resulting RLR1 construct was mobilized to *Agrobacterium* strain C58C1^Rif containing the plasmid pGV2260. *N. tabacum* was transformed by the leaf disc protocol according to Deblaere *et al.* (1987), using thidiazuron (1 mg l⁻¹) instead of benzylaminopurine. The number of T-DNA inserts was assessed by segregation of T$_0$ offspring on selective medium (MS supplemented with 200 μg ml⁻¹ kanamycin). Eight T$_1$ seedlings of each one-copy line were grown in the greenhouse and their seeds were sown on selective medium to identify homozygous lines (T$_2$).

Semi-quantitative RT-PCR and real-time quantitative RT-PCR (RT-qPCR) analyses

Total RNA from leaves of 6-week-old plants was prepared using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) then treated with DNase I (DNA-*free*™ from Ambion, Austin, TX, USA). RNA quality and quantity were assessed with a Bioanalyzer 2100 (Agilent). Single-stranded cDNA was synthesized using the Reverse Transcription System (Promega, Madison, WI, USA). For semi-quantitative PCR, reactions were performed using Promega's MasterMix. Expression levels of *PtaRHE1* and *EF1α* were assessed by means of RT-PCR using the primers as described in Supplementary Table S1 at *JXB* online.

RT-qPCR analysis was performed as described by Vandeputte *et al.* (2007), in an ABI 7900 system (Applied Biosystems). Transcriptional changes were calculated based on the comparative ΔΔC$_T$ method as described by Livak and Schmittgen (2001) and are reported as ratios between expression in transgenic lines overexpressing *PtaRHE1* (RLR1-1-1 and RLR1-5-7) and wild-type (WT) plants. The C$_T$ value of each gene was normalized to the C$_T$ value of the reference gene *EF1α*. The expression of each gene was investigated in three biological replicates. Primer pairs used for RT-PCR analysis were designed according to the cDNA sequences present in public databases (Supplementary Table S1 at *JX*B online). Criteria for designing primers (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) were a primer size between 18 and 25, an optimal T_m at 60 °C, and a product size ranging from 200 bp to 250 bp.

PtaRHE1 promoter cloning and analysis

Genomic DNA was extracted from *P. tremula*×*P. alba* (clone INRA 717-1B4) aerial parts using a DNeasy Plant Mini Kit (Qiagen). *pPtaRHE1* was recovered using a GenomeWalker™ Kit (Clontech), according to the manufacturer's instructions. Several rounds of genome walking were performed with the following gene-specific primers: GSP1 5′-GTTACTTACTCATCTAACCGGGTCAAG-3′, GSP2 5′-ACAGTACCTCTTCTTTCCCTACTTAGC-3′, GSP1 5′-

TCTCGAGTCTGGGTCCATTTCTTGAAT-3′, GSP1 5′-AGAG-GAGGAGGACGAGGTAGGTTCTTG-3′, and GSP2 5′-TGAT-CAAACCAATTCACCTTCCTCTCA-3′. The promoter sequence was amplified with primers F 5′-CAAGTTGCAACCGGATTATG-3′ and R 5′-TTCAATTGGTGGATCTCTCG-3′, cloned in the pCR®4-TOPO® vector (Invitrogen), and sequenced. Prediction of potential *cis*-elements was performed using the PLACE database (Higo *et al.*, 1999; http://www.dna.affrc.go.jp/htdocs/PLACE). Gateway® (Invitrogen) *att*B1 and *att*B2 sequence extensions were added for cloning into the Gateway®-compatible binary T-DNA destination vector pKGWFS7 (Karimi *et al.*, 2002) allowing the fusion of the *PtaRHE1* promoter with both *GFP* (green fluorescent protein) and *GUS* reporter genes in reactions mediated by the Gateway® BP and LR clonase® Enzyme mix (Invitrogen). Transgenic tobacco plants were produced as described above. Five homozygous lines were identified as having a similar pattern of expression during seedling development. One of these lines, RLR6-11-6, was selected for detailed expression analysis. Histochemical GUS staining was performed as described by Hemerly *et al.* (1993). Seedlings were examined under a light binocular (Olympus SZX-ILLK200), and roots and cross-sections under a light microscope (Olympus BX 60). Images were acquired with a Colorview II Soft Imaging System (Olympus).

Production of PtaRHE1 and PtaRHE1-Ct recombinant proteins

To produce the full-length protein, *PtaRHE1* was amplified by PCR (1 min at 94 °C, 1 min at 60 °C, 1 min 30 s at 68 °C for 30 cycles, followed by 10 min at 68 °C) with primers F 5′-AAAAAGCAGGCTTAATGCAGAAAGAAAAA-3′, and R 5′-AGAAAGCTGGGTATAGATAAAAGGCATA-3′. To produce PtaRHE1 where the N-terminal TM and the basic domains were deleted (PtaRHE1-Ct), the same coding sequence was amplified by PCR (1 min at 94 °C, 1 min at 50 °C, 1 min 30 s at 68 °C for 30 cycles, followed by 10 min at 68 °C) with the primers F 5′-AAAAAGCAGGCTACCACATGCCAGATTCT-3′ and R 5′-AGAAAGCTGGGTATAGATAAAAGGCATA-3′. PCRs were performed using the Platinum *Pfx* polymerase (Invitrogen). The pBAD-DEST49 expression system (Invitrogen) was used to produce recombinant proteins with horseradish peroxidase (HRP)–thioredoxin as an N-terminal fusion partner (14 kDa) of the cloned gene product, a V5 epitope, and a hexahistidine (6 His) tag (4 kDa) as C-terminal fusion partners, resulting in fusion proteins with an expected mass of 50.9 kDa for PtaRHE1 and 42.6 kDa for PtaRHE1-Ct.

In vitro autoubiquitination assay

PtaRHE1 and PtaRHE1-Ct were expressed in the *Escherichia coli* strain TOP10 (Invitrogen) for recombinant protein production and purification. Protein production was induced with 0.02% arabinose for 3 h at 37 °C. After pellet lysis, the PtaRHE1-His and PtaRHE1-Ct-His proteins were bound to Ni-NTA beads (Invitrogen), washed, but not eluted. The proteins were allowed to refold in a buffer containing zinc ions (20 nM HEPES pH 7.4, 150 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.05% Triton X-100, 0.02 mM ZnCl$_2$, 1.5 mM MgCl$_2$). For the *in vitro* autoubiquitination assay, ATP-containing buffer [50 mM HEPES pH 7.4, 2 mM ATP, 5 mM MgCl$_2$, 2 mM dithiothreitol (DTT), 0.2 mM ZnCl$_2$], haemagglutinin (HA)-tagged Ub (human recombinant), E1 Ub-activating enzyme (rabbit recombinant), and several human recombinant E2 Ub-conjugating enzymes, including UbcH5a, UbcH5b and UbcH5c, purchased from BostonBiochem, were used as reagents. Ubiquitination reactions including negative controls for E3, E2 and Ub were incubated at 30 °C for 1 h. The reactions were stopped by adding SDS loading buffer and incubation at 65 °C for 10 min. The samples were subjected to 8% SDS–PAGE and blotted on Immobilon®-P polyvinylidene difluoride (PVDF) membranes (Sigma). The PtaRHE1-His and PtaRHE1-Ct-His proteins were detected by penta/tetra His antibody (Qiagen) and

Ub or the ubiquitinated proteins by anti-HA antibody (Roche) against HA-Ub.

Accession number

Sequence data for the *PtaRHE1* promoter region can be found in the GenBank/EMBL data libraries under the accession number GQ174438.

Results

In vitro ubiquitination assays indicate that PtaRHE1 is a functional E3 ligase

In order to investigate the enzymatic activity of PtaRHE1, recombinant PtaRHE1 proteins were produced in *E. coli* and purified for *in vitro* autoubiquitination assays. Since PtaRHE1 possesses a putative N-terminal TM domain that may interfere with its production in *E.coli* (as reported for EL5 by Takai *et al.*, 2002), two recombinant forms of PtaRHE1 were produced. The first form was the full-length protein (PtaRHE1) and the second form was a truncated protein (PtaRHE1-Ct) where the TM and the basic domains were deleted (Supplementary Fig. S2 at *JXB* online). The His-tagged PtaRHE1 and PtaRHE1-Ct proteins were purified and subjected to autoubiquitination in the presence of ATP, HA-tagged Ub (human recombinant), E1 (rabbit recombinant), and several human recombinant E2s, in-cluding UbcH5a, UbcH5b, and UbcH5c. After completion, these reactions were separated by SDS–PAGE and trans-ferred to a PVDF membrane for immunodetection of the PtaRHE1 and PtaRHE1-Ct proteins and HA-Ub. The overlaps of either PtaRHE1 or PtaRHE1-Ct and HA-Ub signals on the membranes were verified to determine whether these proteins were coupled to Ub or not. As shown in Fig. 1A, following immunostaining with the anti-HA antibody, a band with a mol. wt of 60.3 kDa, corresponding to a shift of PtaRHE1 by 9.4 kDa (corre-sponding to the molecular weight of one Ub moiety), was observed only when UbcH5a was used as E2. A comparable pattern, albeit weaker, was obtained using the anti-His antibody against PtaRHE1 (Fig. 1B). These data clearly show that PtaRHE1 possesses an E3 ligase activity. Similarly to EL5 (Takai *et al.*, 2002), PtaRHE1 uses specific E2 enzymes, in the present case UbcH5a, while UbcH5b and UbcH5c were not able to mediate the ubiquitination reaction. The truncated PtaRHE1-Ct also showed a prefer-ence for UbcH5a. However, in this case, only monoubiqui-tination patterns could be detected in the overlapping HA and His signals (Fig. 1C, D, respectively), suggesting that PtaRHE1-Ct might have a reduced activity and therefore that the deleted TM domain or areas near to it play a role in the PtaRHE1 ubiquitination activities. Hetero- or homodimerization has been shown to be essential for the function of many E3 ligases (Nikolay *et al.*, 2004;
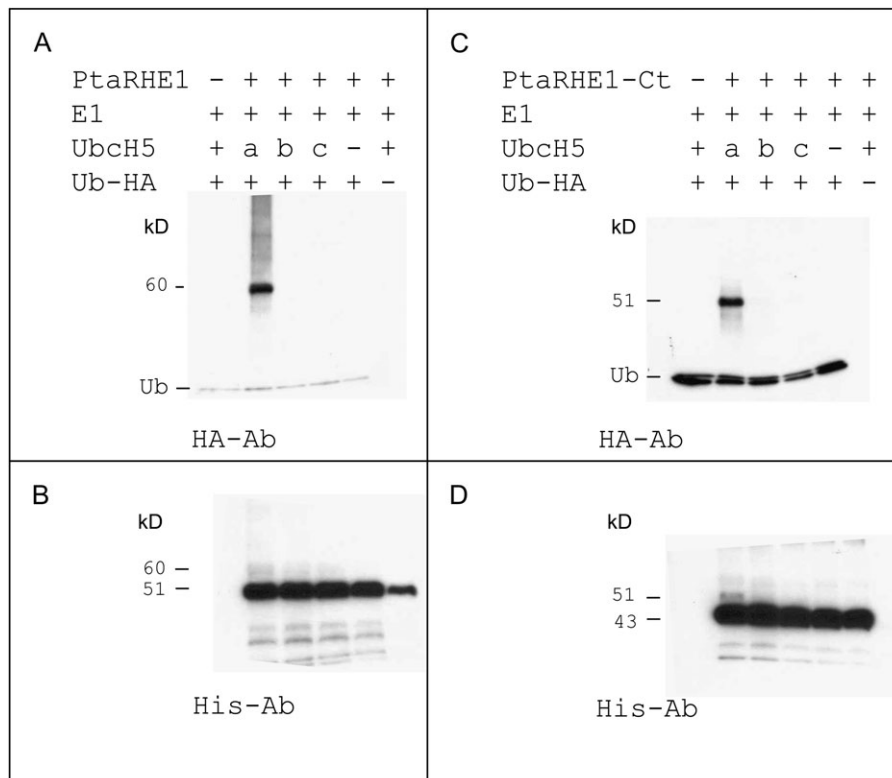

Fig. 1. E3 Ub ligase activity of PtaRHE1 and PtaRHE1-Ct proteins. (A) and (B) E3 ligase activity of HRP–thioreodoxin–PtaRHE1-6His fusion protein. (C) and (D) E3 ligase activity of HRP–thioreodoxin–PtaRHE1-Ct-6His fusion protein. Anti-HA antibody was used to detect Ub and ubiquitinated proteins (A, C) and anti-His antibody was used to detect His-tagged PtaRHE1 and PtaRHE1-Ct (B, D). Three different E2s, UbcH5a–c, were tested in this assay.

Subramanian *et al.*, 2004) and it could be that, due to the deletion, a dimerization has become impossible. Another possibility is that interactions with the other reaction components, such as E2, can not take place efficiently due to the deletion.

Overexpression of PtaRHE1 in transgenic tobacco triggers leaf curling, leaf blade necrosis as well as growth retardation and flowering delay

To investigate the role of *PtaRHE1*, transgenic tobacco lines overexpressing the full-length *PtaRHE1* coding sequence under the control of the CaMV 35S promoter were generated. Four independent T_2 transgenic lines, designated RLR1-1-1, RLR1-5-7, RLR1-7-6, and RLR1-9-3, were selected. Overexpression of *PtaRHE1* in these lines was confirmed by RT-PCR analysis, whereas no amplicon could be detected in the WT plant (Fig. 2). Among these lines, RLR1-1-1 and RLR1-5-7 were selected for further characterization. After 1 month of growth, transgenic lines showed a curling of the leaves and necrotic spots on leaf blades that were both absent in WT plants (Fig. 3A, B). The severity of the phenotype varied from plant to plant, and ranged from leaf curling of all leaves (RLR1-1-1) to leaf curling limited to the youngest leaves (RLR1-5-7). In addition, leaf development was strongly restricted in RLR1-1-1 as compared with RLR1-5-7 and the WT (Fig. 3B). Three-month-old transgenic lines showed an altered development as compared with the WT plants (Fig. 3C). Transgenic plants were shorter, mainly because they formed shorter internodes, and showed a delay in floral transition (Fig. 3C). After 4 months, *PtaRHE1* overexpressors reached the same size as the WT plants and floral transition occurred (Fig. 3D). The phenotype of the 4-month-old line RLR1-1-1 was characterized by a pronounced leaf curling (Fig. 3E) and by the appearance of chlorosis and necrosis on the leaf blades (Fig. 3F), whereas the WT leaves were flat (Fig. 3G) and did not show any necrosis (Fig. 3H). No apparent anatomical differences were noticed in transversal stem cross-sections, the main vein in leaves, and stomata density and distribution between transgenic lines and the WT (data not shown). However, transverse sections in leaves showed that RLR1-1-1 leaves (Fig. 3I) were less thick than WT leaves (Fig. 3J), possibly because intercellular spaces within the spongy parenchyma in the *PtaRHE1* overexpressors were smaller and less frequent than in the WT plants.

Several classes of genes encoding inducible defence-related proteins are up-regulated in transgenic plants overexpressing PtaRHE1

Since the phenotype of the *PtaRHE1* overexpressors, i.e. the formation of necrotic lesions, was reminiscent of possible cellular processes linked to cell defence and/or cell death, the expression of a number of genes reported to be induced during several associated mechanisms in plant cells was investigated. As shown in Table 1, the selected genes are classified into several categories including genes induced during defence and the HR, genes associated with cell death linked to proteasome malfunction, genes induced during apoptosis, or genes linked to age-mediated leaf senescence and oxidative stress. Specific primers, listed in Supplementary Table S1 at *JXB* online, were designed for these genes either using the tobacco sequences in the database or using the closest homologous sequences of *N. tabacum* by performing a BLAST N search in the non-redundant NCBI database. Their expression was analysed in leaves of 1-month-old T_2 plants of RLR1-1-1, RLR1-5-7, and the WT, when the phenotype was clearly visible. A quantitative analysis of the expression of the selected genes was performed by RT-qPCR using the cDNA made from three individual plants of each line. The overexpression of *PtaRHE1* in each individual plant was first checked by RT-PCR (Supplementary Fig. S3 at *JXB* online).

As shown in Table 1, the two transgenic lines robustly expressed *PtaRHE1*, and RLR1-1-1, showing the strongest phenotype (Fig. 3), had four times more transcripts than RLR1-5-7. In RLR1-1-1, several genes coding for defence-related proteins were significantly induced. The transcript of *PR-1a* dramatically accumulated, with a relative increase of 250 000-fold as compared with the WT. Other defence-related genes were also up-regulated, including *PR-1b*, *TIZZ*, *PR5*, *SAR8.2.A*, *PR3*, *HSR201*, *HMGR2*, *WRKY12*, *EDS1*, *PR4*, and *WIPK*. Transcript levels of the tested genes related to proteasome cell death were not significantly different in the WT and in the transgenic lines. Two genes, *PR2* and *HIN1*, known to be induced during both the HR and proteasome cell death (Kim *et al.*, 2003), were also induced in RLR1-1-1. No significant changes in the transcript levels of the genes associated with apoptosis, senescence, or oxidative stress were detected in this transgenic line as compared with the WT. In the second transgenic line, RLR1-5-7, only some of the genes that are affected in line RLR1-1-1 had a significant change in expression, including *PR-1a*, *PR-1b*, *PR2*, *PR3*, *PR5*, *SAR8.2A*, and *TIZZ*. The gene expression analysis suggests that at least some phenotypic features observed in the lines overexpressing *PtaRHE1* seem to be associated with a defence-like response of the plants. To support this conclusion, *in planta* analysis of *PtaRHE1* gene expression in response to various stresses and during plant development was examined.

PtaRHE1 promoter-driven GUS activity is induced by various biotic and abiotic treatments and is developmentally regulated

A 1207 bp long sequence upstream of the ATG codon of *PtaRHE1* was isolated from *P. tremula*×*P. alba* by genome walking (Supplementary Fig. S4 at *JXB* online). The 74 bp 5′-untranslated region (UTR) initially recovered by rapid amplification of cDNA ends (RACE) (van Raemdonck *et al.*, 2005) was extended to 102 bp by the homology with the expressed sequence tag (EST) Q044A08 from the contig POPLAR.9452.C1 (98% sequence similarity). Using the PLACE database, the *PtaRHE1* promoter (*pPtaRHE1*)
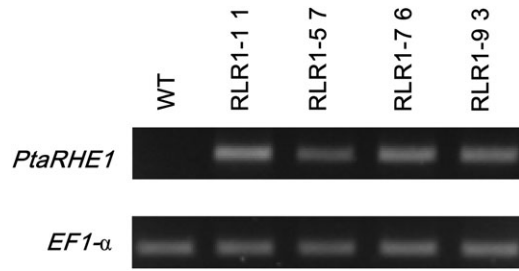
Fig. 2. Identification of transgenic lines overexpressing *PtaRHE1*. RT-PCR analysis of *PtaRHE1* expression in T_2 transgenic tobacco lines and the WT. *EF1α* was used as loading control.

was searched for the presence of *cis*-regulatory sequences. As shown in Supplementary Fig. S3 at *JXB* online, several putative CAAT and TATA boxes were found. A number of potential *cis*-acting elements have also been identified including two ABA-responsive elements (ABREs), eight W-boxes, 10 ARR1AT elements, 11 GT-1-binding motifs, one BS1 site, 10 POLLENLELAT52 elements, three ACG-TERD1 sequences, two ACGTABOX elements, six ROOT-MOTIFTAPOX1 motifs, one RAV1AAT sequence, seven MYB recognition sites, nine GTGA motifs, eight NOD-CON2GM sequences, 12 DOF recognition sites, three MYC recognition sequences, and one HDZIP2ATATHB2
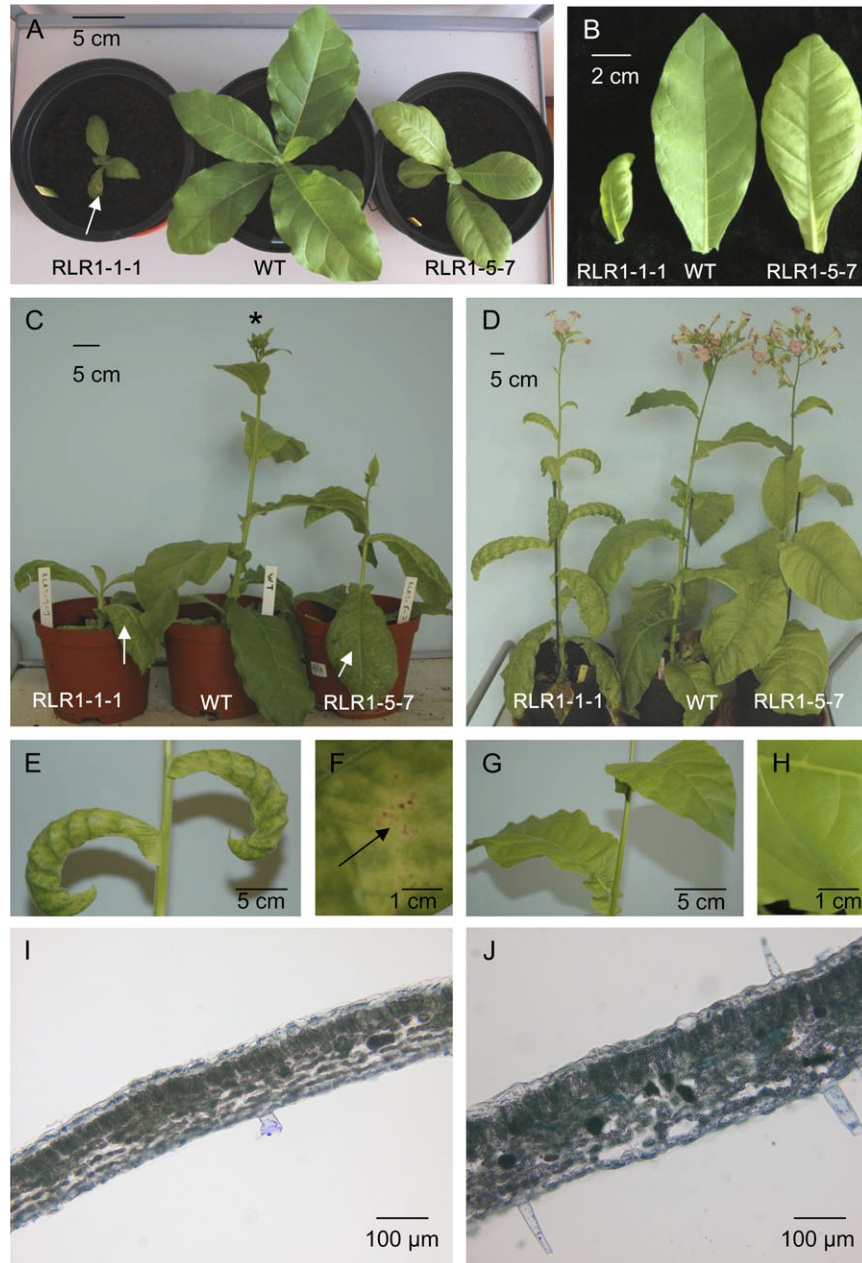

Fig. 3. Phenotype of transgenic lines overexpressing *PtaRHE1*. (A) and (B) One-month-old plants and third leaves, respectively. (C) Three-month-old plants. (D) Four-month-old plants. (E) and (F) Details of 4-month-old RLR1-1-1 leaves and leaf blade, respectively. (G) and (H) Details of 4-month-old WT leaves and leaf blade, respectively. (I) and (J) Hand-made cross-section in third leaves of 4-month-old RLR1-1-1 and WT plants, respectively. The arrows indicate necrotic spots. *, 3-month-old WT plant undergoing floral transition.

Table 1. Relative expression (fold) of a selection of genes associated with various types of cell processes linked to defence and/or cell death in two lines of PtaRHE1 overexpressors compared with the WT

The quantitative expression level of each gene was measured by RT-qPCR and each value is the relative accumulation of each gene transcript compared with that of *EF1α*. The data presented are the mean value of RNA preparations in three different individual plants of each line.

	$2^{e-\Delta\Delta CT}$ RLR1-1-1			$2^{e-\Delta\Delta CT}$ RLR1-5-7		
	Mean	**SE**	*t*-test (*P* value)	**Mean**	**SE**	*t*-test (*P* value)
PtaRHE1	828 735.18	300 840.44	≤0.01	209 593.44	57 530.96	≤0.01
1. Defence/elicitor-inducible genes						
PR-1a	256 701.17	176 302.06	≤0.01	8458.53	6585.74	≤0.01
PR-1b	120 967.23	85 436.22	≤0.01	1604.42	689.56	≤0.01
TIZZ	61 481.94	33 817.36	≤0.01	532.02	296.37	≤0.01
PR5	444.98	377.28	≤0.01	9.03	1.85	≤0.01
SAR8.2a	102.64	34.82	≤0.01	5.81	2.72	≤0.01
PR3	52.36	24.10	≤0.01	2.80	0.53	≤0.01
HSR201	22.92	7.78	≤0.01	0.57	0.10	NS[a]
HMGR2	20.16	10.37	≤0.01	0.68	0.02	≤0.01[a]
WRKY12	7.45	5.02	≤0.01	0.97	0.36	NS[a]
EDS1	9.05	3.52	≤0.01	1.25	0.29	NS[a]
PR4	5.84	1.47	≤0.01	0.98	0.29	NS[a]
WIPK	2.43	0.57	≤0.01	0.99	0.03	NS[a]
LSD1	1.93	0.35	≤0.01[a]	0.84	0.39	NS[a]
SAMDC	1.59	0.35	NS[a]	0.65	0.10	≤0.01[a]
BECLIN1	1.59	0.57	NS[a]	1.20	0.57	NS[a]
DND1	1.59	0.43	NS[a]	1.52	0.77	NS[a]
ABAKIN	1.49	0.55	NS[a]	0.87	0.20	NS[a]
NPR1	1.43	0.24	NS[a]	1.31	0.21	NS[a]
HSP90	1.17	0.56	NS[a]	0.77	0.12	NS[a]
SIPK	1.01	0.05	NS[a]	0.82	0.14	NS[a]
S26-PR6	0.95	0.23	NS[a]	2.09	1.33	NS
NtMEK2	0.91	0.07	NS[a]	0.68	0.14	≤0.05[a]
HSR203J	0.78	0.15	NS[a]	0.52	0.08	≤0.01[a]
Spermidine synthase	0.67	0.03	NS[a]	0.81	0.17	NS[a]
WIZZ	0.66	0.22	NS[a]	0.88	0.32	NS[a]
NOA1	0.53	0.09	≤0.05[a]	0.78	0.07	NS[a]
Polyamine oxidase	0.54	0.27	NS[a]	0.52	0.16	≤0.01[a]
PR1c	–	–	–	–	–	–
HSR515	–	–	–	–	–	–
2. Cell death induced by proteasome malfunction-related genes						
20S proteasome α subunit 3	1.34	0.26	NS[a]	1.38	0.54	NS[a]
Ubiquitin protease 6	1.35	0.56	NS[a]	1.12	0.13	NS[a]
Ubiquitin protease 12	1.03	0.15	NS[a]	1.38	0.44	NS[a]
BS2	1.17	0.42	NS[a]	1.06	0.32	NS[a]
NAM-like	0.88	0.42	NS[a]	1.14	0.11	NS[a]
ClpP	0.61	0.17	NS[a]	0.81	0.10	NS[a]
3. Both defence and proteasome malfunction cell death-induced genes						
PR2	21 585.51	18 447.37	≤0.01	1169.14	1116.96	≤0.01
HIN1	10.89	5.67	≤0.05	0.46	0.40	NS
NtCP-23	2.01	0.70	NS	1.54	0.57	NS[a]
SGT1	2.16	0.71	NS	2.27	0.88	NS
4. Apoptosis-related genes						
BI-1	1.71	0.46	NS[a]	0.93	0.36	NS[a]
DAD1	1.19	0.32	NS[a]	1.07	0.48	NS[a]
5. Leaf senescence-specific gene						
CP1	–	–	–	–	–	–
6. Oxidative stress-related genes						
APX	1.10	0.28	NS[a]	0.70	0.10	≤0.05[a]
SOD	0.69	0.07	NS[a]	0.58	0.06	≤0.05[a]
NbrbohA	2.32	0.98	NS	0.63	0.36	NS[a]

–, no expression detected; NS, not significant.
Genes indicated in bold underwent a significant change in expression in RLR1-1-1.
[a] A difference in expression lower than 2-fold up or 2-fold down was not considered in this study.

site. Although the function of these putative elements in the regulation of the expression of the *PtaRHE1* gene remains to be elucidated, the analysis of the expression of the *GUS* gene driven by *pPtaRHE1* upon biotic and abiotic stress and during development supported the function of some of the regulatory elements. Since a large number of putative stress-responsive elements are present in *pPtaRHE1* (such as W-boxes, GT-1s, ABREs, ACGTERD1, and MYB elements; see Supplementary Fig. S4 at *JXB* online), *pPtaRHE1::GUS* plants were confronted with various biotic and abiotic treatments (see Materials and methods). As shown in Fig. 4A, although 19-day-old plantlets co-cultured for 24 h with different bacteria (*A. tumefaciens*, *R. fascians*, and *P. syringae* pv *tabaci*) displayed a similar GUS pattern in the aerial parts, a clear induction of *pPtaRHE1* was visible in the root system. In the non-treated plants, GUS activity was not detected in the root, whereas following bacterial infection the promoter was clearly induced in the elongation and maturation zones of the root. To examine whether the response to pathogenic bacteria is linked to defence mechanisms, 12-day-old plants were treated with Cel and SA. Compared with the control, these two treatments (Cel in particular) strongly induced *pPtaRHE1* even in the aerial parts (Fig. 4B). Moreover, ABA induces *pPtaRHE1*, perhaps due to the occurrence of

ABREs in the promoter sequence. No obvious differences were observed for the other abiotic treatments (H_2O_2, NaCl, spermidine, or spermine; data not shown).

Histochemical GUS stainings were performed during plant development. As shown in Fig. 5A, 7 d post-germination, GUS activity was detected in the cotyledons and in the primary root but not within the root tips. A similar pattern of expression was observed in 12- and 33-day-old plantlets (Fig. 5B–D). In 3-month-old plants, *GUS* expression was also detected in anther, mainly in the degrading connective tissue, and in the stigma (Fig. 5E–H). A cross-section of the stem allowed the detection of GUS staining in ray parenchyma cells within the xylem (Fig. 5I). In conclusion, the expression of *PtaRHE1* is associated with specific tissues or cell types, is developmentally regulated, and is induced by environmental factors.

Discussion

Here, PtaRHE1, a poplar RING-containing protein, was shown to possess an E3 ligase activity since it is able to mediate its own ubiquitination in an *in vitro* assay (Fig. 1). The target substrate of PtaRHE1 has not yet been identified, but this E3 ligase was shown to function with the particular
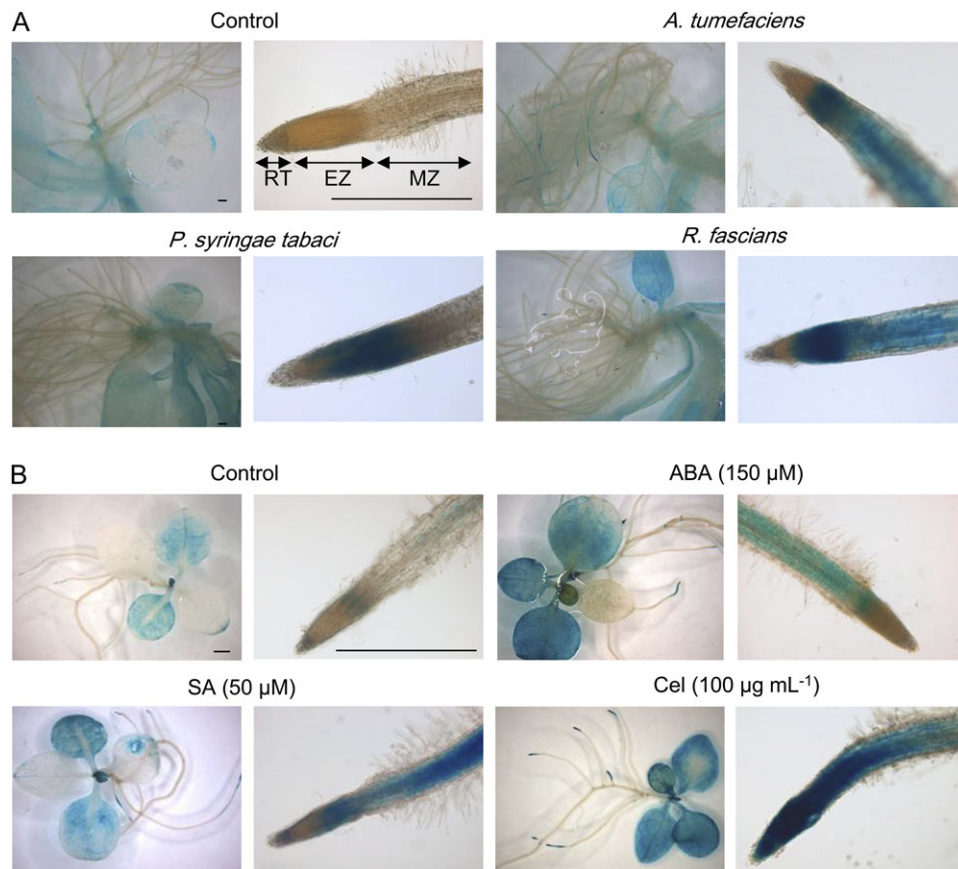

Fig. 4. *pPtaRHE1* response to various biotic and abiotic stresses. (A) Nineteen-day-old *pPtaRHE1::GUS* transgenic tobacco plants treated for 8 h with phytopathogens. (B) Twelve-day-old *pPtaRHE1::GUS* transgenic tobacco plants treated with various abiotic stresses. EZ elongation zone; MZ, maturation zone; RT, root tip. Scale bars represent 1 mm.
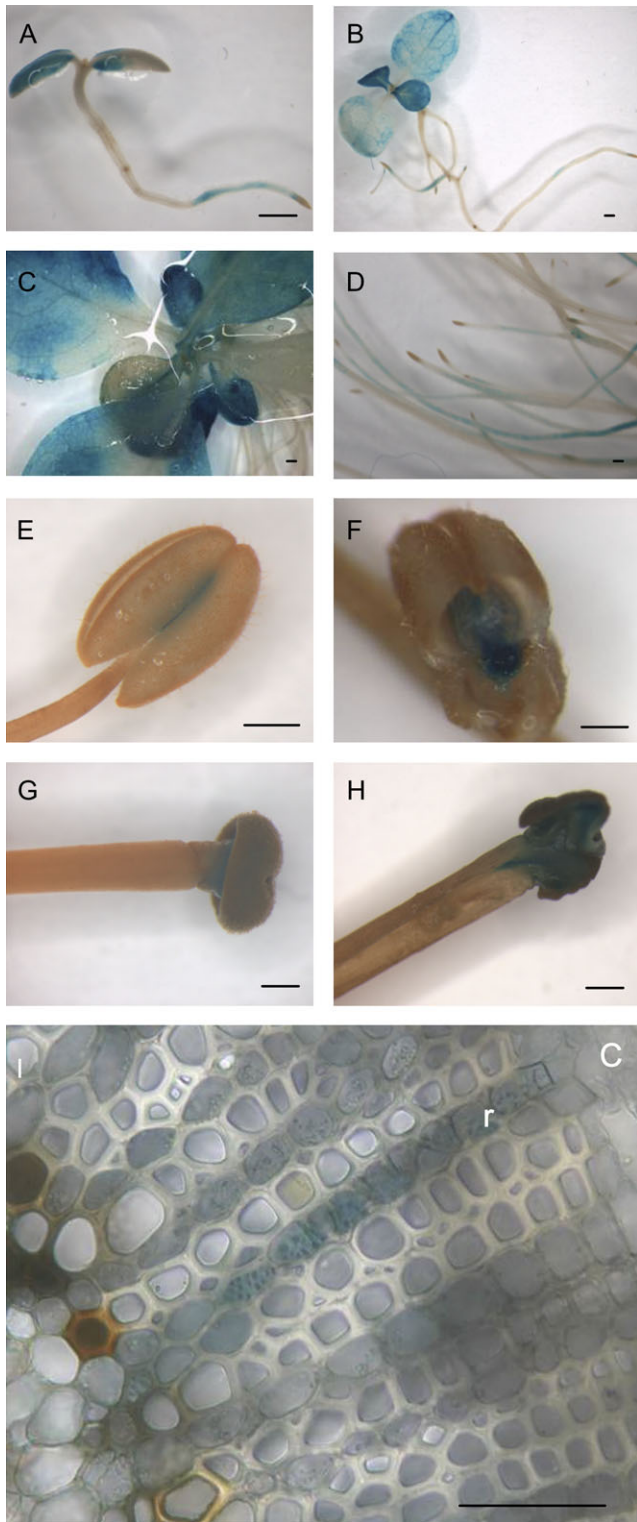
Fig. 5. *pPtaRHE1*-driven expression during plant development. (A) Seven-day-old seedling. (B) Twelve-day-old plant. (C) Aerial part of a 33-day-old plant. (D) Roots of a 33-day-old plant. (E–I) Three-month-old plants. (E) Anther. (F) Section in an anther showing expression in connective tissue. (G) Style. (H) Expression in the stigma. (I) Secondary xylem. c, cambium; r, ray. Scale bars represent 1 mm in A–H and 100 μm in I.

E2 UbcH5a. The interaction of ATL proteins, which are closely related to PtaRHE1, with members of the Ubc4/5 subfamily of E2s has already been reported. The rice EL5 functions in co-operation with UbcH5a and Ubc4, and their rice counterparts, OsUBC5a and OsUBC5b (Takai *et al.*, 2002; Katoh *et al.*, 2003, 2005). In accordance with this, most of the residues crucial for EL5–OsUBC5b binding, as identified by mutation analysis (Katoh *et al.*, 2005), including V136, L138, D163, W165, L174, and R176, are conserved in the PtaRHE1 RING-H2 finger domain and correspond to V113, L115, D140, W142, L151, and R153 (Supplementary Fig. S2 at *JXB* online). Aguilar-Henonin *et al.* (2006) showed that the toxicity of the expression of *A. thaliana ATL2* in *Saccharomyces cerevisiae* (Martínez-García *et al.*, 1996) was alleviated when components of the yeast Ub–26S proteasome pathway were mutated, among others the E2 Ubc4. These data suggest that ATL2 also interacts with the Ubc4/5 E2 family to mediate ubiquitination of the target substrate. Performing *in vitro* ubiquitination assays with poplar homologues of these E2s could further validate the interaction of the Ubc4/5 subfamily with PtaRHE1.

Overexpressing *PtaRHE1* in transgenic tobacco plants resulted in a marked alteration of plant development, characterized by a slower growth, a precocious inward curling of the leaves, the formation of necrotic lesions on leaf blades, and a delay in floral transition (Fig. 3). Increasing evidence supports a role for the Ub–proteasome system and protein degradation in plant development (Stone and Callis, 2007), in the regulation of PCD (Kim *et al.*, 2003, 2006), and in plant defence against pathogens (reviewed by Zheng *et al.*, 2006; Craig *et al.*, 2009). The pleiotropic phenotype observed in *PtaRHE1* overexpressors may therefore result from alterations of processes linked to ubiquitination. Alteration of plant development has been observed following the overexpression of several RING-H2 proteins belonging to the ATL family. For instance, the overexpression of *MsRH2-1* in alfalfa and in *A. thaliana* caused a shortening of plant stature, increased apical dominance, leaf hyponasty, inhibition of leaf venation and lateral root development, delayed nodulation in the case of alfalfa, and abnormal flower development, probably due to a disruption of auxin signalling pathway(s) (Karlowski and Hirsh, 2003). In contrast, transgenic tobacco overexpressing *OsBIRF1* were characterized by an increased growth and had more leaves than the WT plants (Liu *et al.*, 2008b). Finally, the overexpression of *EL5* in rice resulted in root growth arrest, due to the possible role of *EL5* in the maintenance of cell viability after the initiation of root formation (Koiwai *et al.*, 2007).

The phenotype described for *PtaRHE1* tobacco over-expressors does not seem to be unique to RING-H2 proteins since similar phenotypes have also been reported in plants where other genes are up-regulated. For instance, the overexpression of the *N. plumbaginifolia* gene encoding the ankyrin repeat protein glucanohydrolase-binding protein 1 (GBP1) resulted in downward curling of leaves accompanied by necrotic lesions (Wirdnam *et al.*, 2004). By performing grafting experiments, these authors reported

that the signal inducing leaf curling was transmissible acropetally, suggesting that the sugar transport through the phloem was altered, with disturbances in carbohydrate metabolism leading to leaf curling as a consequence. In the case of *PtaRHE1*-overexpressing lines, grafting experiments did not show signal transmission and comparative analysis of carbohydrates did not reveal significant qualitative and quantitative differences between stems of WT and RLR1-5-7 lines (data not shown).

The HR-like cell death phenotype observed in leaves of *PtaRHE1*-overexpressing lines may be related to an alteration of the Ub–proteasome pathway. For instance, the overexpression of a variant form of Ub, where K48 is replaced by an R, inhibits proteolysis and induced a phenotype in tobacco similar to the one observed in *PtaRHE1* overexpressors, characterized by shorter internode length, leaf curling, abnormalities in vascular tissues, and formation of necrotic lesions on leaves (Bachmair *et al.*, 1990). In the present study, none of the selected genes specifically related to proteasome malfunction (Kim *et al.*, 2003, 2006; Table 1) was up-regulated in *PtaRHE1*-overexpressing lines, suggesting that proteasome functioning is not affected in these lines. This hypothesis is supported by Kim *et al.* (2003, 2006) who showed that the silencing of two different subunits of the 26S proteasome, the α6 subunit of the 20S proteasome and the RPN9 subunit of the 19S regulatory complex, activated the PCD process. As shown by these authors, this proteasome-mediated cell death stimulated the expression of only a subset of transcripts that are highly induced during *P. syringae* pv *syringae*-mediated HR, indicating that diverse PCD pathways co-exist in plant cells with differential regulation mechanisms.

Numerous defence-related genes are strongly induced in *PtaRHE1* overexpressors, suggesting a role for *PtaRHE1* in defence mechanisms (Table 1). The most strongly increased gene in *35S::PtaRHE1* lines is *PR-1a*, which encodes an acidic protein widely represented in plants that is considered as a marker for systemic acquired resistance (SAR) and as the most abundant pathogenesis-related (PR) protein in infected plant tissue (Ward *et al.*, 1991; van Loon *et al.*, 2006). Other *PR* genes are up-regulated in *PtaRHE1* overexpressors, including genes encoding PR2, a β-1,3-endoglucanase; PR3, a class I basic chitinase CHN50; PR4, an endochitinase; and PR5, an osmotin (van Loon *et al.*, 2006). In addition, diverse genes known to be preferentially expressed during plant defence were induced, such as *Hin1* (Gopalan *et al.*, 1996), *SAR8.2A* (Alexander *et al.*, 1992), *HSR201* (Czernic *et al.*, 1996), and *HMGR2* (Genschik *et al.*, 1992). In accordance with this, increased expression of pathogenesis-related and SA-responsive genes upon overexpression of *ATL* genes has recently been reported (Serrano and Guzman, 2004; Hondo *et al.*, 2007; Liu *et al.*, 2008*b*). Another key element in the signalling cascade leading to HR downstream of R-gene-mediated pathogen recognition, *EDS1*, has been found to be up-regulated in the *PtaRHE1* overexpressors. *EDS1* encodes a lipase-like protein required for disease resistance mediated by the tobacco N protein, a TIR-NBS-LRR protein (Peart *et al.*, 2002).

Two genes coding for WRKY transcription factors were induced in the *PtaRHE1* overexpressors, *TIZZ* (a homologue of *AtWRKY40*) and *NtWRKY12* (a homologue of *AtWRKY51*). WRKY proteins are a family of transcription factors that are strongly and rapidly up-regulated in response to wounding, pathogen infection, or abiotic stresses in numerous plant species (Ülker and Somssich, 2004). Moreover, WRKY proteins have been associated with the regulation of developmental processes such as trichome and seed development (Eulgem and Somssich, 2007) as well as with leaf senescence (Balazadeh *et al.*, 2008). WRKY factors, which ensure proper cellular responses to internal and external signals, bind to particular *cis*-elements found in various gene promoters and called W-boxes (Eulgem *et al.*, 2000). W-boxes are considered to be major *cis*-acting elements responsible for the pathogen inducibility of many plant genes and have been found in the promoters of various wound- and pathogen-responsive genes, including several *PR* genes (Rushton and Somssich, 1998; Yang *et al.*, 1999; Yu *et al.*, 2001). Two binding sites for NtWRKY12 have been identified in the *PR-1a* promoter, and NtWRKY12 has been shown to activate *PR-1a::GUS* expression in *A. thaliana* protoplasts, providing evidence that NtWRKY12 is a transcriptional activator of *PR-1a* (van Verk *et al.*, 2008). Therefore the activation of *PR-1a* may be due to the overexpression of *PtaRHE1* either directly or via the induction of NtWRKY12. Interestingly, the isolated *PtaRHE1* promoter contains eight putative W-boxes (Supplementary Fig. S4 at *JXB* online), suggesting that this gene could itself be regulated by particular WRKY factor(s). The identification of the poplar WRKY(s) binding to the W-boxes in *pPtaRHE1* would indicate whether *PtaRHE1* is a target for a particular WRKY and provide information on the biological function of PtaRHE1. Induction of WRKY factors and defence genes was also observed during the activation of a tobacco mitogen-activated protein kinase (MAPK) cascade (Kim and Zhang, 2004). Besides, several MAPKs, including SA-induced protein kinase (SIPK), wound-induced protein kinase (WIPK), and their upstream kinase NtMEK2, have been shown to be involved in the perception of pathogens and pathogen-derived elicitors (Zhang *et al.*, 2000; Jin *et al.*, 2003; del Pozo *et al.*, 2004). In the *PtaRHE1*-overexpressing plants, *WIPK* was >2-fold induced, but the expression of *NtMEK2* and *SIPK* was not significantly altered (Table 1). These data suggest that PtaRHE1 possibly acts upstream of WIPK in the activation of WRKYs, or in an independent pathway to trigger plant defence.

Members of the ATL gene family have already been proposed to be involved in the defence response (Craig *et al.*, 2009). For instance, the *A. thaliana ATL2* is induced following treatment by Cel, chitin, chitooctaose, and flagellin (Salinas-Mondragón *et al.*, 1999; Navarro *et al.*, 2004; Libault *et al.*, 2007), the rice *EL5* is up-regulated after treatment with *N*-acetylchitoheptaose (Takai *et al.*, 2001), while the expression of the tobacco *ACRE132* is triggered during Avr9- and Cf-9-mediated defence responses (Durrant *et al.*, 2000). Histochemical GUS staining revealed

that *PtaRHE1* is induced by pathogens, SA, and Cel, suggesting that this gene might be part of the genetic network activated during plant responses to pathogens and elicitors (Fig. 4). These results are in accordance with the occurrence of W- and GT-boxes in the *pPtaRHE1* sequence (see the legend of Supplementary Fig. S4 at *JXB* online). In addition, this promoter is activated by ABA, possibly due to the occurrence of ABREs in *pPtaRHE1*. Similar results have been observed in poplar leaves where PtaRHE1 was found to be clearly induced by Cel and ABA, as shown by RT-PCR analysis (Supplementary Fig. S5), indicating a comparable response of this gene to these stress conditions in both poplar and tobacco.

The GUS histochemical analysis also revealed that *pPtaRHE1* is expressed in the connective tissue of anthers and in the stigma (Fig. 5). Recently, an analysis of the *N. tabacum* stigma/style transcriptome revealed that highly expressed genes in these tissues are associated with defence mechanisms or pollen–pistil interactions (Quiapim *et al.*, 2009). Connective tissue has been shown to undergo PCD during development and dehiscing of the anther (Senatore *et al.*, 2009). The spatio-temporal expression pattern of the *PtaRHE1* promoter showed that *PtaRHE1* is developmentally regulated, being high in young leaves and roots (Fig. 5A–D). At later stages of development and in tissues undergoing the secondary growth phase, GUS staining was localized in ray parenchyma cells (Fig. 5I). This observation is in agreement with the previously reported expression of *PtaRHE1* in poplar stem, as analysed by *in situ* RT-PCR (van Raemdonck *et al.*, 2005). Likewise, in zinnia, the RING-encoding gene *ZeRH2.1* was shown to be expressed within vascular bundles of the mature stem in xylem parenchyma cells and in the phloem (Dahiya *et al.*, 2005). Therefore, these authors suggested a role for ZeRH2.1 during active transport. A role for PtaRHE1 in transport could also be proposed since ray parenchyma cells are involved in the transport of water and nutrients as well as of signalling molecules between the phloem and the xylem.

Characterizing the exact function(s) of PtaRHE1 requires the identification of PtaRHE1's target(s). Nevertheless, the pleiotropic effects observed in tobacco overexpressors suggest that PtaRHE1 targets (a) protein(s) involved in signalling cascades/pathways regulating important developmental processes and the interaction of plants with their biotic and abiotic environments. The increased expression of pathogenesis-related genes, the responsiveness of the promoter *PtaRHE1* to pathogens and elicitors, as well as the HR-like phenotype induced in transgenic tobaccos suggest that PtaRHE1 might target (a) protein(s) that is/are linked to defence mechanisms but that might also regulate developmental processes at a point where both genetic networks intersect each other.

Supplementary data

Additional supporting information may be found at *JXB* online.

Figure S1. Alignment of the PtaRHE1 amino acid sequence with the *Arabidopsis* ATL2 amino acid sequence (At3g16720).

Figure S2. Sequences of full-length PtaRHE1 and PtaRHE1-Ct (where the TM domain and the basic domains were deleted).

Figure S3. RT-PCR expression analysis of *PtaRHE1* in three individual plants of the WT, RLR1-1-1, and RLR1-5-7.

Figure S4. Nucleotide sequence of the 5′-flanking promoter region and putative *cis*-acting elements of the *PtaRHE1* promoter.

Figure S5. RT-PCR expression analysis of *PtaRHE1* in poplar leaves treated during 8 h with Cel (100 μg ml^{-1}) and ABA (150 μM). *18S* was used as a loading control.

Table S1. Primer sequences and amplicon size for RT-PCR and RT-qPCR. The gene classification was based on literature data.

Acknowledgements

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