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Research Article

Localization of the Expression of the PtaRHE1 Gene, Coding for A RING-H2 Protein, in the Poplar Stem by *in Situ* Hybridization.

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Abstract

Background: *PtaRHE1* is a gene from poplar (*Populus tremula* × P. alba) encoding a RING (REALLY INTERESTING NEW GENE) domain-containing protein. RING proteins are widely represented in plants and play key roles in regulating developmental processes and responses to environmental stimuli. Previous work demonstrated that PtaRHE1 possesses E3 ubiquitin ligase activity, but its precise biological function and target proteins remain unknown. This study aims to further investigate the potential role of *PtaRHE1* in vascular development.

Methods: To this end, the PLACE database was used to identify potential cis-regulatory elements within the *PtaRHE1* promoter. In addition, RNA *in situ* hybridization was performed using a digoxigenin-labeled probe to localize *PtaRHE1* transcripts in stem tissues of poplar.

Results: Promoter analysis revealed the presence of several core promoter elements (CAAT and TATA boxes) and multiple putative cis-acting regulatory motifs, including: 2 ABREs, 8 W-boxes, 10 ARR1AT elements, 11 GT-1 binding motifs, 1 BS1 site, 10 POLLENLELAT52 elements, 3 ACGTERD1 sequences, 2 ACGTABOX elements, 6 ROOTMOTIFTAPOX1 motifs, 1 RAV1AAT sequence, 7 MYB recognition sites, 9 GTGANTG10 motifs, 8 NODCON2GM sequences, 12 DOF recognition sites, 3 MYC binding sites, and 1 HDZIP2ATATHB2 motif. *In situ* hybridization results showed that *PtaRHE1* transcripts are predominantly localized in ray parenchyma cells within the cambial zone of the stem.

Conclusion: These findings suggest that *PtaRHE1* may play a dual role, contributing both to plant defense mechanisms and to developmental regulation within the vascular cambial region.

Keywords: Poplar, RING-H2, *In Situ* Hybridization, *PtaRHE1*, Promoter Analysis

Introduction

Trees produce most of the Earth's biomass and dominate most terrestrial ecosystems. Faced with environmental variations such as high soil salinity, extreme temperatures or drought, plants have developed complex physiological and molecular mechanisms [19,13,23,17]. These mechanisms include signaling pathways linked to ionic stress, those induced by salt, changes in hormone levels and the activation of several genes associated with resistance to drought or salt stress [12,64,22,57,58,59,61]. In both Europe and North America, poplars are widespread and play an important ecological role. These trees provide a valuable source of fiber used in the manufacture of paper, lumber and plywood, and also represent a promising avenue for the production of biofuels [8]. Thanks to a high capacity for *in vitro* regeneration, the availability of the complete *Populus trichocarpa* genome, a relatively small genome size and high transformation efficiency, this genus has become a preferred model for research into the molecular biology of woody plants [53]. The phylogenetic relationship between Populus and Arabidopsis is particularly useful for comparative functional and genomic studies, facilitating research into the evolution of genomes and gene families in eudicots. Populus offers numerous opportunities for investigating problems that cannot be tackled with Arabidopsis or rice, the two main herbaceous models in plant biology and genomics. For example, the study of wood properties, such as the formation of parenchymatous rays, or the perennial life style, are areas where Populus is an indispensable model [26].

A previous study carried out at the Plant Biotechnology Laboratory focused on the identification and analysis of specific molecular markers linked to the formation and development of the vascular system in plants. Poplar was chosen as the biological model for this research. A cDNA-AFLP screen comparing gene expression in the upper and lower parts of six-month-old *Populus tremula* x P. alba hybrid poplar stems identified several genes encoding regulatory proteins potentially involved in the transition between primary and secondary growth [56]. Among these genes, *PtaRHE1* was identified and characterized as coding for a RING-H2 type protein. Numerous RING zinc finger proteins have been isolated and studied in various plant species, where they appear to play a variety of roles. For example, 508 RING domains have been predicted in *Arabidopsis thaliana* and divided into seven categories [51,47,27]. In *Populus trichocarpa*, *Tong, et al.,* (2019) [55] identified 288 genes encoding RING-H2 proteins. Recent advances on RING zinc finger proteins, including their structural characteristics, classification, subcellular localization and physiological functions, are now well documented *Li, et al.,* (2019) [49,50].

By focusing our research on the function of the *PtaRHE1* gene, we have shown that the recombinant *PtaRHE1* protein possesses functional E3 ligase activity, as demonstrated by its ability to self-ubiquitinate [33]. In this paper, we are describing our research results of the *PtaRHE1* promotor analysis and the localization of *PtaRHE1* gene by *in situ* hybridization.

Materials and Methods

PtaRHE1 Promoter Analysis

A sequence of 1207 base pairs located upstream of the ATG initiation codon of the *PtaRHE1* gene was isolated from *Populus tremula* x P. alba using the "genome walking" technique, then cloned into the pCR® 4-TOPO® vector (GenBank/EMBL accession number: GQ174438) [56]. Bioinformatic analysis of potential cis-elements present in the *PtaRHE1* promoter (*pPtaRHE1)* was performed using the PLACE database [24].

In Situ Hybridization

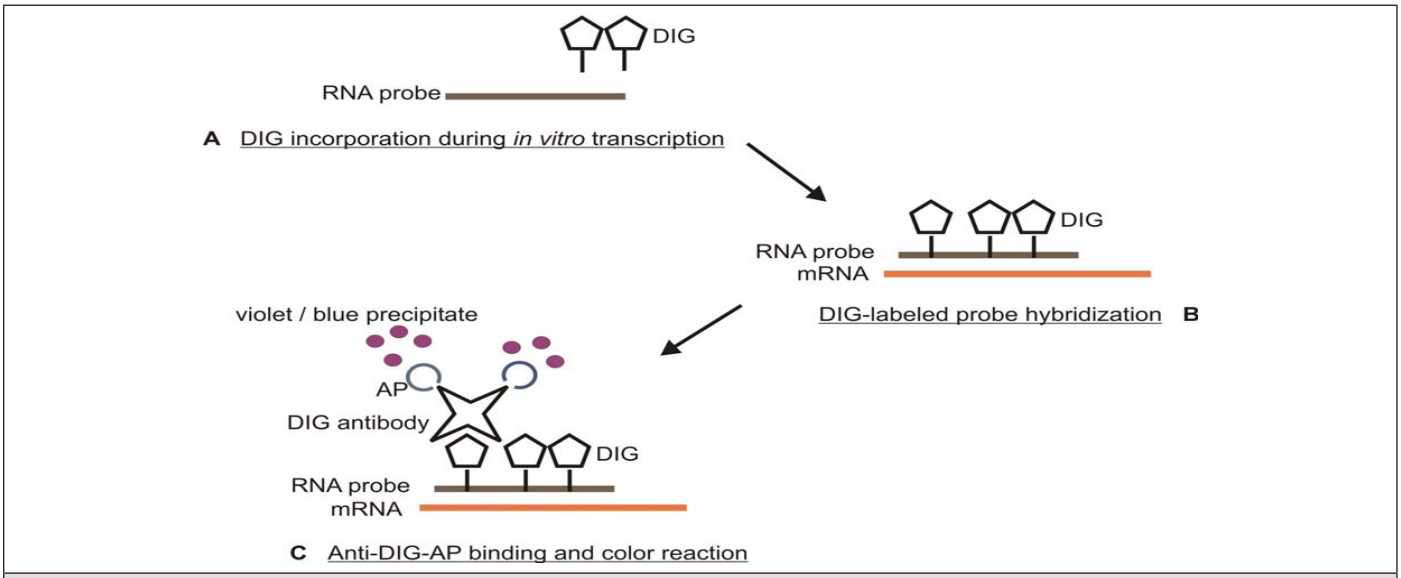

Figure 1: Probe synthesis and *in situ* hybridization reaction scheme. Digoxigenin (DIG) incorporation in RNA strands during *in vitro* transcription (A), DIG-labeled probe binding during hybridization reaction (B) and anti-DIG-Alkaline Phosphatase (AP) binding with color reaction (C) are shown *Roche Diagnostics*, (2020) [42].

In Situ Hybridization (ISH) is a technique for locating specific mRNA transcripts at the cellular level. It is based on the use of a labelled probe, complementary to the target sequence, applied to tissue sections where the integrity of the RNA is preserved [32,6]. To detect *PtaRHE1* transcripts in poplar stem tissue at the cellular level by *in situ* hybridization, a labelled RNA probe was used. The principle of this technique is illustrated in Figure 1. All the experiments were carried out according to the protocol described by [7]. All steps were performed using ultrapure water (H_2 Odd), previously demineralized using a purification system (Micro-TKA, Niederelbert, Germany), then autoclaved for 20 minutes at 120°C after treatment with DEPC (0.1%) for one hour (Figure 1).

Plant Material

Samples were obtained from 4-week-old poplar seedlings (*Populus tremula* × P. alba, clone INRA 717 1B-4) grown in a growth chamber under controlled environmental conditions: photoperiod of 16 hours of light, temperature of 23°C during the day and 19°C at night, with a relative humidity of 60±10%.

Tissue Fixation and Embedding in Paraffin

Tissue preparation was carried out according to the method described by *Regan, et al.,* [40], with modifications made by *Brunel, et al.,* (2002) [7]. Fresh fragments of poplar stem (2-3mm) were transferred into 10 ml glass vials fitted with plastic caps (VWR, Darmstadt, Germany). The samples were immediately immersed in a fixation solution containing 3.7% Formalin-Acetic Acid (FAA), which was placed on ice. The purpose of this solution is to stabilize the RNA transcripts *in situ* while facilitating access to the probes during hybridization. Vacuum infiltration was performed using a pump to remove air from the intercellular spaces by means of negative pressure. Samples were then stored at 4°C for 3 to 5 hours, or even overnight. Tissue samples were progressively dehydrated using an increasing series of ethanol baths (30,50,70,80,95 and 100%). Each step was carried out at 4°C for 30 minutes. As a final step, the samples were incubated overnight at 4°C in fresh 100% ethanol.

For clarification, ethanol was replaced with Histoclear II™ (Elvetec) for 2 hours at room temperature. An increasing series of Histoclear II™/ethanol mixtures (25,50,75%) was then applied, followed by pure Histoclear II™, each step lasting 2 hours at room temperature. Subsequently, the samples were incubated overnight at room temperature in a fresh solution of paraffin-saturated Histoclear II™, obtained by adding one Paraplast chip (Oxford Labware, St. Louis, MO, USA) to 1 ml of solvent. The paraffin was melted at 60°C. Finally, the solution was replaced with fresh paraffin-saturated Histoclear II™, and the tissues were incubated at 50°C for 3 hours. The temperature of the water bath was raised to 60°C, and Paraplast chips (30 to 40 units) were gradually added. The solution was then incubated overnight at 60°C in an oven. All subsequent steps were carried out at this temperature, and all equipment used was pre-warmed to 60°C to avoid immediate solidification of the paraffin at lower temperatures. The Histoclear II™ was replaced by replenishing the melted paraffin twice a day (morning and evening), for two days. Samples were then individually transferred to 19×16 mm Petri dishes (Sarstedt, Numbrecht, Germany) and embedded in the melted paraffin. The dishes were placed on ice to allow solidification. The embedded plant fragments were stored at 4°C in the presence of silica gel.

Preparation of Tissue Slices

Paraffin-embedded tissues were sectioned into thin sections using a microtome (HM355, Microm, Walldorf, Germany), producing serial sections 8-10µm thick. To avoid folding of the sections during cutting, the paraffin blocks were kept on ice. The sections were then spread on the surface of ultrapure water (H_2 Odd) on treated microscope slides (SuperFrost® Plus, Fisher Scientific). These slides, which have a positively charged coating, favour the adhesion of sections whose protein-rich surface is generally negatively charged. The slides were dried on a hot plate at 42°C for 2 hours, then stored at 4°C in a dust-free box.

Synthesis of DIG-Labeled RNA Probes

RNA probes specific to the *PtaRHE1* sequence were generated by standard PCR amplification using Taq polymerase (Fermentas GmbH, St. Leon-Roth, Germany). For probe synthesis, the sense primers (used for control hybridization) were tagged 5' with the SP6 polymerase promoter sequence (*5'-CATACGATTTAGGTGACACTATAG-3'*), while the antisense primers (used for the specific antisense probe) carried the T7 polymerase promoter sequence (*5'-CTAATACGACTCACTATAGGGAGA-3'*) (New England Bio Labs). After PCR amplification, the products were separated by 1% agarose gel electrophoresis. The corresponding DNA fragments were extracted from the gel and used as a template for a second PCR reaction, designed to increase the amount of DNA obtained. This amplification was carried out in a volume of 200µl, using the same primer pairs as before, according to a standard protocol. The amplified PCR products were then cloned into the pCR® 2-TOPO® vector (Invitrogen). To prepare the RNA probes, high-purity plasmid DNA was isolated using the NucleoSpin kit (Clontech) in mini-preparations. The plasmid DNA was then linearised by digestion with the appropriate restriction enzymes (NcoI and SpeI).

After linearisation, the antisense and sense probes were synthesized by *in vitro* transcription from the SP6 or T7 promoters respectively, incorporating Digoxigenin (DIG) during the transcription reaction Figure 2. Digoxigenin (DIG), a plant steroid extracted from Digitalis lanata, is coupled to Uridine Triphosphate (UTP) and can only be detected once bound to the probe. Labelled RNA was precipitated and then separated by centrifugation at 16,000×g for 30 minutes at 4°C (5402 centrifuge, Eppendorf, Hamburg, Germany). The pellet was washed twice with 80% (v/v) ethanol. After drying, the quality of the DIG-labelled RNA probe was assessed by comparison with a DIG control probe supplied in the DIG RNA labelling kit (Roche Diagnostics, Mannheim, Germany). The concentration of the experimental probes was then estimated calorimetrically using the same kit.

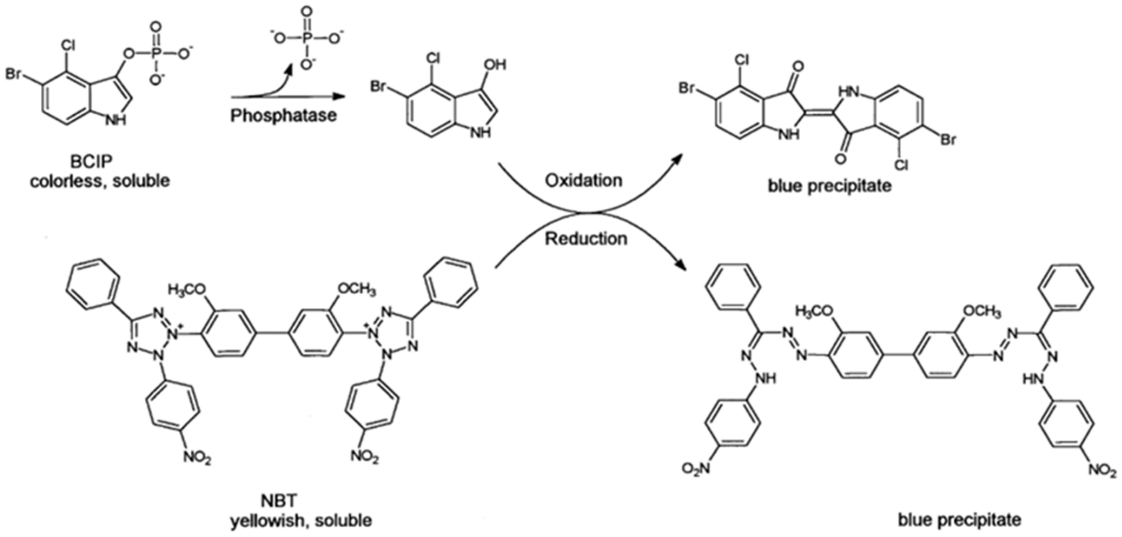

Figure 2: Redox reaction of BCIP and NBT. The alkaline phosphatase cuts a phosphate residue from the substrate 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) which results in a blue/violet precipitate by redox reaction with Nitroblue Tetrazolium Chloride (NBT) *Roche Diagnostic* (2020) [42].

Pre-Hybridization

The various steps were carried out in staining baths equipped with slide holders. The pre-hybridization treatment began with the deparaffinization of sections fixed on treated microscope slides, by immersing the samples twice in pure Histoclear II™ for 10 minutes. The sections were then rehydrated by passing through a decreasing series of ethanol prepared in DEPC-treated water at room temperature, with steps of one minute each at the following concentrations: 100,100,95,90,80,70,50,30 and then 0% (v/v). The samples were then incubated for 5 minutes at room temperature in PBS buffer. Partial digestion of tissue proteins was performed by incubation with proteinase K (Sigma-Aldrich) for 15 minutes at 37°C, to improve probe accessibility during hybridization. The enzymatic action was stopped by treatment with 0.2% (w/w) glycine at room temperature, followed by 10 minutes post-fixation with 4% (v/v) Paraformaldehyde (PFA) in PBS. The slides were then washed twice in PBS at room temperature to remove the paraformaldehyde. The samples were subjected to an acetylation step at room temperature in the presence of freshly prepared 0.5% acetic anhydride in 0.1 M triethanolamine buffer (pH 8) for 10 minutes to block the positive charges on the sections. Finally, before hybridization, the sections were dehydrated by successive immersion in an increasing series of ethanol (30,50,70,80,90,95,100,100%), for one minute per stage, at room temperature.

In Situ Hybridization with DIG Labeled Probes

For hybridization, 120µl of hybridization buffer was applied to the slides. This buffer contained: 5 M NaCl, 1 M Tris-HCl (pH 6.8), 0.5 M EDTA, 100% formamide, 50% dextran sulphate, 100X Denhardt's solution, 50 mg/ml transfer RNA (tRNA), and DEPC-treated water. To this mixture was added a 10µl volume of probe solution, composed of formamide, antisense or sense labelled DIG probe (0.1 to 3ng/µl), and DEPC-treated water. The probe solution was heated to 80°C for 2 minutes to denature the RNA, thereby facilitating access to the RNA molecules present in the tissue, and then rapidly cooled on ice for 5 minutes before application. The prepared slides were covered with a coverslip and incubated overnight at 50°C.

Post-hybridisation washes were performed according to the protocol of *Regan and Sundberg* (2002) [41], including an RNAse step to remove non-specifically bound probes. The detailed protocol is as follows: 1) Wash in 2X SSC for 15 minutes at 42°C, 2) Wash in 2X SSC for 15 minutes at 42°C, 3) Wash in 0.1X SSC for 20 minutes at 42°C, 4) Wash in 0.1X SSC for 20 minutes at 42°C, 5) Wash in 0.1X SSC for 20 minutes at 42°C, 6) Incubation in 1X NTE buffer for 5 minutes at 37°C, 7) Incubation in 1X NTE buffer for 5 minutes at 37°C, 8) Treatment with 1X NTE buffer containing 10µg/ml RNAase for 30 minutes at 37°C (on the slide, outside the staining bath), 9) Rinse in 1X NTE buffer for 5 minutes at 37°C, 10) Rinse in 1X NTE buffer for 5 minutes at 37°C, 11) Wash in 0.1X SSC for 20 minutes at 42°C, 12) Wash in 0.1X SSC for 20 minutes at 42°C, 13) Wash in 0.1X SSC for 20 minutes at 42°C, 14) Rinse in 1X PBS for 10 minutes at room temperature, 15) Rinse in 1X PBS for 10 minutes at room temperature.

Prior to immunological detection of hybridized DIG probes, non-specific binding sites in tissues were blocked by incubating samples in a blocking solution containing 1% (w/w) blocking reagent (bovine serum albumin, BSA, Sigma-Aldrich) in buffer 1 (100mM Tris-HCl pH 7.5, 150mM NaCl) for 45 minutes at room temperature. This was followed by a 45-minute incubation at room temperature in a 1% BSA solution prepared in buffer 2 (100mM Tris-HCl pH 7.5, 150 mM NaCl, 0.3% Triton X-100) to improve

penetration and binding of the alkaline phosphatase-coupled anti-digoxigenin antibody (Fab fragments, Roche Diagnostics). The reaction with the antibody was carried out for 2 hours at room temperature in the dark. Each slide was incubated with 100µl of antibody diluted 1:500 in buffer 2. The slides were then washed four times, for 20 minutes each, with the same buffer 2. Finally, the samples were equilibrated once in detection buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50mM $MgCl_2$) for immunological detection of probe-incorporated DIG using the antibody. The antibody was visualised using the Alkaline Phosphatase (AP) enzymatic reaction using 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP, Biorad) and Nitroblue Tetrazolium Chloride (NBT, Biorad), according to the method described by *Poupard, et al.,* (2001) [37]. For the colorimetric reaction, samples were incubated at room temperature for 10 min in detection buffer (100mM Tris-HCl, pH 9.5, 100mM NaCl, 50mM $MgCl_2$), to which the two substrates NBT and BCIP were added at a final concentration of 0.2 mM each. The reaction took place in the detection buffer, at room temperature, protected from light and without agitation. Alkaline Phosphatase (AP), bound to the anti-DIG antibody, cleaves a phosphate group from BCIP, triggering a redox reaction between dephosphorylated BCIP and NBT (Figure 2).

This reaction produces an insoluble blue/purple precipitate. The development of the coloration can last from 1 to 3 days. The reaction was stopped by rinsing the slides for 5 minutes with water. The samples were then air-dried before being mounted with a mounting medium (Eukitt, Euromedex, Mundelsheim, Germany) and covered with a coverslip. This mounting medium effectively preserves the product of the alkaline phosphatase reaction, enabling the slides to be preserved for a long time.

Microscopic Observations

The preparations were observed using a light microscope (Axio plan 2, Zeiss, Jena, Germany). Images were captured using a digital camera (Axio Cam HR, Zeiss) and Axio Vision 4.6 software (Zeiss GmbH, Göttingen, Germany).

Results

PtaRHE1 Promoter Analysis

A 1207 bp sequence upstream of the ATG initiation codon of the *PtaRHE1* gene was isolated from *Populus tremula* ×P. alba by genome walking. Bioinformatics analysis of this promoter region (*pPtaRHE1*) was performed using the PLACE database to identify possible cis-regulatory elements. As shown in Figure 3, several putative CAAT and TATA boxes were detected. Numerous potential cis-acting elements were also identified, including : - 2 ABREs (abscisic acid response elements), - 8 W-boxes (WRKY protein binding sites), - 10 ARR1AT elements (linked to cytokinin signalling), - 11 GT-1 binding motifs, - 1 BS1 site, - 10 POLLENLELAT52 elements (linked to pollen-pecific expression), - 3 ACGTERD1 sequences, - 2 ACGTABOX elements, - 6 ROOTMOTIFTAPOX1 motifs (associated with expression in roots), - 1 RAV1AAT sequence, - 7 MYB recognition sites, - 9 GTGANTG10 motifs, - 8 NODCON2GM sequences, - 12 DOF transcription factor binding sites, - 3 MYC recognition sequences, - and 1 HDZIP2ATATHB2 site (associated with HD-ZIP-type transcription factors). The detailed annotation and putative functions of these elements are presented in (Figure3) (Table 1).

Table 1: Annotation and functions of putative *Cis*-acting elements identified in *pPtaRHE1*

Cis-Acting Elements	Function	Number	Reference
ABREs	ABA-responsive elements. They are found in the promoter of ABA and/or stress-regulated genes	2	[34]
ACGTABOX	"A-box" ACGT (nucleotide sequence required for protein/DNA interactions and transcriptional activation) elements which are binding sites for bZIP (basic leucine zipper type of transcription factor) proteins.	2	[18,10]
ACGTERD1	ACGT sequence required for induction of expression of erd1 in A. thaliana by dehydration stress and dark-induced senescence	3	[48]
ARR1AT	"ARR1 (Arabidopsis response regulator)-binding element" found in A. thaliana. They act as transcriptional activators.	1	[44]
BS1	Binding site 1 found in Eucalyptus gunnii cinnamoyl-CoA reductase gene promoter; it is required for vascular expression	1	[30]
DOF	Core site required for binding of Dof proteins (DNA binding with one finger), regulating the expression of plant genes in response to light, developmental stage and hormone treatment such as gibberellins.	12	[62]
GT-1	GT-1 binding motif found in the promoter of many light-regulated genes; Binding of GT-1-like factors to the PR-1a promoter influences the level of SA-inducible gene expression;	11	[9]
GTGANTG10	"GTGA motif" found in the promoter of the tobacco late pollen gene g10	9	[43]
HDZIP2ATATHB2	Binding site of the A. thaliana homeodomain leucine-zipper gene ATHB-2 found in its own promoter. The HD-Zip genes encode plant specific transcription factors.	1	[36,38]
MYB	MYB1(AT) sites are MYB recognition sites found in the promoters of the dehydration-responsive gene rd22 and many other genes in A. thaliana. MYB (derived from myeloblastosis) genes act as transcriptional activators.	7	[1]

MYC	Binding site of ATMYC2 which function as transcriptional activator in ABA signaling. MYC (derived from myelocytomatosis) recognition sequence (from -466 to -461) are necessary for expression of erd1 in dehydrated Arabidopsis.	3	[1,48]
NODCON2GM	One of two putative nodulin consensus sequences in soybean (Glycine max).	8	[52]
POLLENLELAT52	One of two co-dependent regulatory elements responsible for pollen specific activation of tomato lat52 gene	10	[5]
RAV1AAT	Binding consensus sequence of A. thaliana transcription factor, RAV1 (Related to ABI3/VP1).	1	[28]
ROOTMOTIFTAPOX1	Motif found in both promoters of rolD, a root-specific promoter.	6	[15]
W-boxes	Binding sites for WRKY transcription factors; WRKYs are involved in elicitor-responsive transcription of defence genes in tobacco.	8	[16,63]

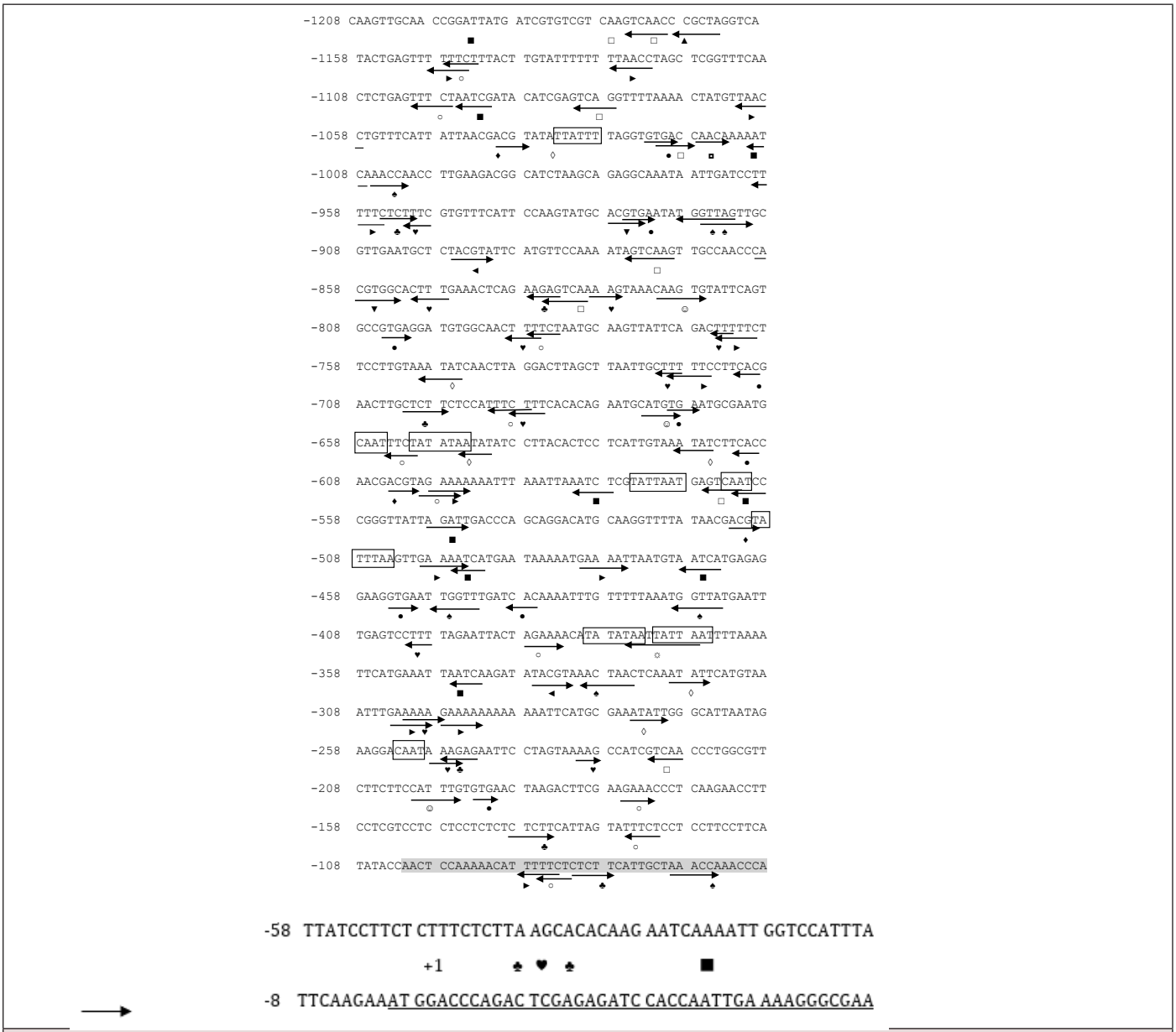

Figure 3: Nucleotide sequence of the 5′-flanking promoter region and putative *cis*-acting elements of the *PtaRHE1* promoter. The 5′UTR region is marked in grey and the coding sequence is underlined. The translational start site is denoted as +1. Putative CAAT- and TATA- boxes are boxed. Putative *cis*-acting elements are represented by a symbol and underlined by an arrow. □, W-box); ▲, BS1; ►, GT-1; ▼, ABRE; ♦, ACGTERD1; ◄, ACGTABOX,; ■, ARR1AT; ●, GTGANTG10;; ☼, HDZIP2ATATHB2; ♠, MYB1; ☺, MYC; ○, POLLEN1LELAT52; ◘, RAV1AAT; ◊, ROOTMOTIFTAPOX1; ♣ NODCON2GM; ♥, DOF.

Localization of *PtaRHE1* by *In Situ* Hybridization in Poplar Stem

To localize *PtaRHE1* mRNA in poplar, transverse stem sections (8-10µm thick) from one-month-old *Populus tremula* × P. alba plants were hybridized with digoxigenin-labeled sense and antisense probes specific to *PtaRHE1*. Detection was carried out via immunological recognition of the digoxigenin label using an anti-digoxigenin antibody conjugated to alkaline phosphatase, followed by enzymatic reaction with BCIP and NBT substrates, as previously described. The analysis revealed that *PtaRHE1* transcripts are predominantly localized in ray cells within the cambial zone (Figure 4).

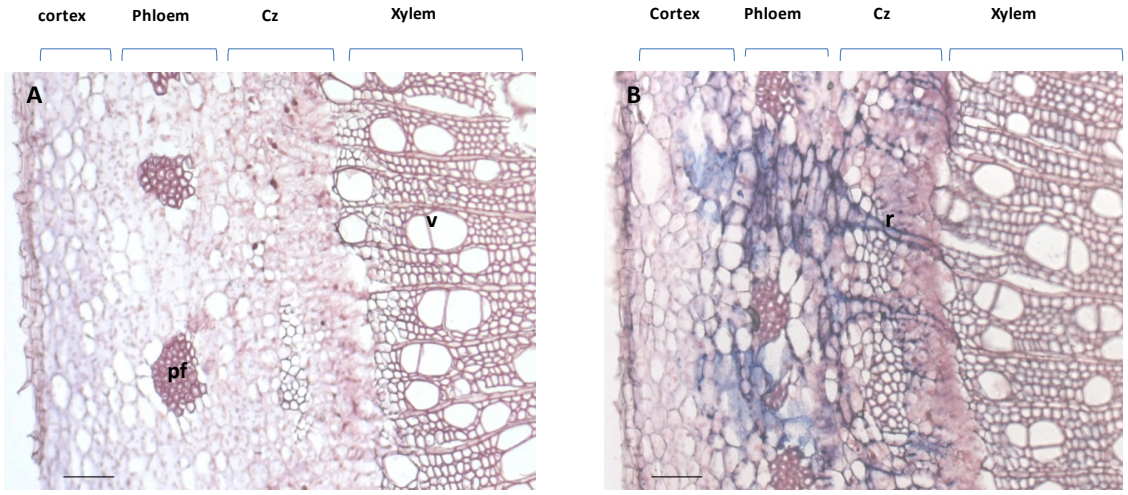

Figure 4: Localization of *PtaRHE1* mRNA by *in situ* hybridization. *In situ* localization of *PtaRHE1* transcripts in cross sections of one-month old *P. tremula x* P. alba. Cross sections were hybridized with a *PtaRHE1* sense mRNA probe used as negative control (A) or with a *PtaRHE1* antisense mRNA probe (B). Positive hybridization signals are dark purple. pf, phloem fiber; r, ray; v, vessel. Scale bars = 100µm.

Discussion

The analysis of the *PtaRHE1* promoter revealed the presence of multiple cis-regulatory sequences and a wide array of putative cis-acting elements. The putative functions of these elements are summarized and discussed in Table 1. Notably, several of them are clearly associated with plant defense responses, as mentioned earlier. These findings are consistent with the results reported by *Tong, et al.,* (2019) [55], who, through promoter analysis, observed that many RING-H2 genes preferentially expressed in xylem tissues contained cis-elements such as SNBE, TERE, M46RE, AC, and SMRE-motifs known to be implicated in secondary cell wall formation and programmed cell death.

As previously reported *Mukoko Bopopi, et al.,* (2010) [33], histochemical GUS staining demonstrated that *PtaRHE1* expression is induced by pathogen infection, Salicylic Acid (SA), and cellulase treatment. The inducibility of several ATL family genes, which encode RING finger proteins, has been well documented. In Arabidopsis, for instance, ATL2 and ATL6 are rapidly upregulated following exposure to chitin or cellulases during seedling development. [31,45] Conversely, ATL2 expression was not triggered by other fungal-derived enzyme preparations such as Zymolyase 20T, Lyticase, or Novozyme 234 [4,45]. In rice, the ATL member EL5 encodes a RING-H2 finger protein responsive to N-acetylchitooligosaccharide elicitors [54]. Similarly, in tomato, LeATL6, an ortholog of *Arabidopsis* ATL6, is induced by a cell wall-derived protein elicitor from the biocontrol fungus *Pythium oligandrum* [25]. Moreover, a RING-H2-type E3 ubiquitin ligase gene from Vitis pseudoreticulata has been shown to enhance resistance to powdery mildew [60]. Notably, ATL gene activation is not limited to fungal elicitors; significant upregulation of seven ATL genes has also been observed in response to bacterial *flagellin* [35, 66].

GUS histochemical analysis also revealed that the *pPtaRHE1* promoter is active in the connective tissue of anthers and in the stigma. Transcriptomic profiling of Nicotiana tabacum stigma and style tissues has shown that highly expressed genes in these regions are often associated with defense responses or pollen pistil interactions [39]. Notably, the connective tissue within anthers has been reported to undergo Programmed Cell Death (PCD) during anther maturation and dehiscence [46]. Furthermore, the spatial and temporal expression pattern of the *PtaRHE1* promoter suggests that its activity is developmentally regulated, with high expression levels observed in young leaves and roots [33]. Collectively, these findings support the idea that certain ATL family genes, including *PtaRHE1,* may play a role in the early phases of plant defense responses following pathogen attack. Our in-situ hybridization experiments in poplar stems further confirmed the expression of *PtaRHE1* in the cambial zone, consistent with previous observations made using *in*

situ RT-PCR [56]. At later developmental stages, particularly during the phase of secondary growth, GUS staining was predominantly observed in ray parenchyma cells. This spatial pattern suggests a possible regulatory function of *PtaRHE1* in secondary phloem fiber development [3]. Supporting this, microarray analysis using Affymetrix poplar genome arrays identified 64 out of 249 RING-H2 genes as being highly or preferentially expressed in stem xylem, highlighting their potential involvement in wood formation [55].

In addition, RING-H2 gene expression has been reported in various tissues-including roots, stems, leaves, flowers, and fruits-of *Malus hupehensis* [65], *Malus domestica* [29], and Chinese white pear (*Pyrus bretschneideri*) [58], suggesting that members of this gene family fulfill diverse physiological roles across different plant species and organs. In *Zinnia*, the *RING-H2* gene *ZeRH2.1* has been reported to be expressed in vascular bundles of mature stems, specifically in xylem parenchyma cells and phloem tissues [11]. Based on this expression pattern, the authors proposed a role for *ZeRH2.1* in active transport processes. A similar hypothesis could be considered for *PtaRHE1*, given its expression in ray parenchyma cells, which are known to mediate radial transport of water, nutrients, and signaling molecules between xylem and phloem. In silico promoter analysis combined with *in situ* hybridization results suggests that *PtaRHE1* may regulate proteins involved in plant defense, while also contributing to developmental processes at the interface of genetic regulation and environmental responsiveness. This dual role-linking defense mechanisms with development-could be particularly relevant in the cambial zone, a dynamic region central to both growth and response to biotic stress.

Conclusion

Our findings support the hypothesis that *PtaRHE1* is involved in plant defense responses and may also participate in the regulation of developmental events, particularly in the cambial zone. The specific expression in ray parenchyma cells and response to elicitors further point to a functional role in the coordination of physiological processes associated with vascular development and stress signaling. Identifying the molecular targets of *PtaRHE1* would provide valuable insights into its precise biological function and its place within the broader regulatory network of RING-H2-type proteins in woody species.

Author Contributions

MBJ, BM and EJM conceptualized the idea.

MBJ, BN and MA did laboratory work.

MBJ, BM and EJM participated in the writing and modification of this manuscript.

All authors read and approved the final manuscript.

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Conflict of Interest

We declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

Ethical Concern and Informed Consent

No human patient nor animal were used in this work.

AI Tool Usage Declaration

We declare that no AI and associated tools were used for writing scientific content in this article.

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