Journal of Experimental Botany, Vol. 63, No. 2, pp. 785–795, 2012
doi:10.1093/jxb/err303 Advance Access publication 2 November, 2011
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

The expression pattern of the *Picea glauca Defensin 1* promoter is maintained in *Arabidopsis thaliana,* indicating the conservation of signalling pathways between angiosperms and gymnosperms*

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Received 17 June 2011; Revised 26 July 2011; Accepted 30 August 2011

Abstract

A 1149 bp genomic fragment corresponding to the 5' non-coding region of the *PgD1* (*Picea glauca Defensin 1*) gene was cloned, characterized, and compared with all *Arabidopsis thaliana* defensin promoters. The cloned fragment was found to contain several motifs specific to defence or hormonal response, including a motif involved in the methyl jasmonate reponse, a fungal elicitor responsive element, and TC-rich repeat *cis*-acting element involved in defence and stress responsiveness. A functional analysis of the *PgD1* promoter was performed using the *uidA* (GUS) reporter system in stably transformed *Arabidopsis* and white spruce plants. The *PgD1* promoter was responsive to jasmonic acid (JA), to infection by fungus and to wounding. In transgenic spruce embryos, GUS staining was clearly restricted to the shoot apical meristem. In *Arabidopsis*, faint GUS coloration was observed in leaves and flowers and a strong blue colour was observed in guard cells and trichomes. Transgenic *Arabidopsis* plants expressing the *PgD1::GUS* construct were also infiltrated with the hemibiotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000. It caused a suppression of defensin expression probably resulting from the antagonistic relationship between the pathogen-stimulated salicylic acid pathway and the jasmonic acid pathway. It is therefore concluded that the *PgD1* promoter fragment cloned appears to contain most if not all the elements for proper *PgD1* expression and that these elements are also recognized in *Arabidopsis* despite the phylogenetic and evolutionary differences that separates them.

Key words: *Arabidopsis*, *Ceratocystis*, defensin, jasmonic acid, *Picea glauca*, promoter, wounding.

Introduction

In order to ward off pathogens, plants have evolved a very robust, multi-layered and complex defence system. Once beyond a plant's physical barriers, a pathogen can first be detected by receptors located at the cell surface. Attempts by the pathogen to suppress this initial host response may then be detected by specific intracellular receptors leading to a rapid and robust response that culminates with the hypersensitive response (Jones and Dangl, 2006). Activation of the defence pathways also leads to the activation of pathogen response genes. Some of these pathogen response

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genes encode small proteinaceous molecules that possess antimicrobial activity and inhibit bacterial or fungal growth. Plant defensins are such small protein molecules, they have pathogen inhibiting properties and are ubiquitous throughout the plant kingdom and they are produced downstream in the defence response pathway. Defensins are not unique to plants; they are also found in vertebrates and invertebrates and appear to be ancient antimicrobial peptides that play a central role in innate immunity. They are conserved across the eukaryotic kingdom and possibly arose before the lineage separation between plants and animals occurred (Thomma *et al.*, 2002). Plant defensins possess a highly conserved structure characterized by the presence of eight cysteines that pair to form four disulphide bridges. This disulphide-bond stabilized proteinaceous structure possesses broad spectrum anti-fungal activity (Thevissen *et al.*, 1999; Pervieux *et al.*, 2004) and sometimes antibacterial activity (Moreno *et al.*, 1994; Segura *et al.*, 1998) that is generally believed to be caused by membrane permeabilization (Kagan *et al.*, 1990; Cociancich *et al.*, 1993; Maget-Dana and Ptak, 1997; Mello *et al.*, 2011).

Plant species genomes can contain a family of several defensin genes that may respond to different stresses. In *Arabidopsis thaliana*, for instance, 14 different defensins have been described. One of the most well-studied defensins, *PDF1.2 (plant defensin 1.2)*, is induced by methyl jasmonate (MeJa) and fungal pathogens but is not induced by salicylic acid (SA) (Manners *et al.*, 1998). Other members of the same family, namely *PDF1.1*, *PDF2.1*, *PDF2.2*, and *PDF2.3*, are constitutively expressed (Thomma *et al.*, 2002). Since defensin proteins are found in very high amounts in seeds and seedlings, it was initially speculated that they primarily protected the seeds and seedlings from soil-borne pathogens (Terras *et al.*, 1995). However, defensins have much broader expression patterns, being expressed in different organs, and they display localized expression in peripheral cells and guard cells (Kragh *et al.*, 1995; Terras *et al.*, 1995), the main entry point of pathogens.

Picea glauca does not yet have its genome fully sequenced; however, important EST resources (over 300 000; Rigault *et al.*, 2011) are available and only two defensin genes have been found. Similarly, *Picea sitchensis,* for which almost 200 000 ESTs are publicly available, also appears to contain only two defensin coding sequences. Whether this is caused by the fact that short cDNAs are lost when preparing libraries or whether it represents a biologically relevant phenomenon is unknown at this stage. It was previously demonstrated that *PgD1* (*P. glauca Defensin 1*) is up-regulated by wounding and jasmonic acid and, more importantly, that recombinant PgD1 displays antifungal activity against *Cylindrocladium floridanum*, *Fusarium oxysporum*, and *Nectria galligena* (Pervieux *et al.*, 2004). In this paper, the cloning and functional analysis of the *PgD1* promoter is reported. The *PgD1* promoter fragment was fused to the *uidA* gene (GUS) to analyse spatio-temporal promoter activity in *A. thaliana* and *P. glauca*. The transgenic plants were subjected to a variety of conditions and to two pathogens. It has been demonstrated that, despite the evolutionary divergence between *A. thaliana* and *P. glauca*, the *P. glauca* promoter appears fully functional and responsive in *Arabidopsis*, indicating that the regulatory mechanism and the defence signalling pathways between gymnosperms and angiosperms may be relatively well conserved.

Materials and methods

Cloning of the PgD1 *promoter*

A genomic DNA fragment located 5′ of the defensin coding sequence (Pervieux *et al.*, 2004) was obtained by genome walking (Clontech). Genome walking banks were produced following the manufacturer's instructions, using genomic DNA isolated from white spruce embryonal tissue as previously described by Klimaszewska *et al.* (2001). Genome walking was conducted using the gene-specific primers def1 and def2 (see Supplementary Table S1 for primer sequences at *JXB* online) for the primary and nested PCR reactions, respectively. Amplified fragments were TA cloned (Invitrogen) and transformed into *E. coli* strain DH5α. Plasmid was isolated using Qiagen miniprep columns (Qiagen) and sequenced. A 1149 bp fragment upstream of the defensin start codon was obtained (accession number JN098426). Motif search in this putative *PgD1* promoter and in all *Arabidopsis* defensin promoters was performed using Plant Care (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). *Arabidopsis* promoters were retrieved from the TAIR website (http://www.arabidopsis.org/).

Generation of transgenic white spruce and Arabidopsis

The putative defensin promoter was fused to the *uidA* coding sequence and used to transform *Arabidopsis* and white spruce. For *Arabidopsis*, single insertion homozygous lines were selected using Mendelian segregation analysis for the selectable marker. To be sure that our spruce transgenic lines were independent, they were selected from independent Petri dishes. The number of insertions was not assessed. Primers def*Eco*RI and def*Bam*HI (see Supplementary Table S1 at *JXB* online) were designed to incorporate an *Eco*RI site at the 5′-end of the promoter sequence and a *Bam*HI site at the 3′-end. The promoter was re-amplified from genomic DNA, then the amplified band was purified by gel extraction, double digested with *Eco*RI and *Bam*HI, and directionally inserted into *Eco*RI/*Bam*HI digested pCambia 1381Xc (ATG in phase with the GUS coding sequence, confirmed by sequencing). The construct was then transferred into *Agrobacterium* strain C58pMP90 and used to transform white spruce (*P. glauca* Moench (Voss)) embryogenic tissue (genotype PG653) as previously described by Klimaszewska *et al.* (2001) with the sole modification being the use of hygromycin (10 μg ml^{-1}) as the selective agent. Seedlings were produced by somatic embryogenesis for individual lines as previously described by Klimaszewska *et al.* (2001). *Arabidopsis* (ecotype Wassilewskija) was transformed using the flower infiltration method (Clough and Bent, 1998). T1 transformants were selected on 50 μg ml^{-1} hygromycin from which single insertion T_3 lines were subsequently produced and used for all histochemical analysis. The MUG assays were performed as described in Klimaszewska *et al.* (2001, 2003). Briefly, transgenic needles (100 mg) were ground and macerated in extraction buffer and clarified by centrifugation. Protein concentrations were determined using the Bio-Rad Bradford Protein Assay kit. MUG assays were performed in 200 μl volumes containing 5 μl extract and 1 mM MUG in extraction buffer at 37 °C. 20 μl samples were removed at regular time intervals and the reaction was stopped by adding 180 μl 0.2 M sodium carbonate stop buffer, and florescence was measured using a Fluorolite1000 microtiter plate reader (Dynatech Laboratories).

GUS histochemical assays were performed as described by Jefferson (1987) with the following modifications: after submerging

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tissue in GUS buffer, samples were vacuum infiltrated for 10 min, then incubated at room temperature for up to 16 h. Tissues were then cleared in 70% ethanol for 2–3 d before the photographs were taken.

Plant treatments: spruce cell suspensions

Line 55 was arbitrarily selected, put into suspension culture and maintained by weekly sub-culturing with fresh MLV liquid media (Klimaszewska *et al.*, 2001) containing 2% sucrose, at a ratio of 10 ml culture to 30 ml fresh media. JA induction of the suspension culture was accomplished by adding JA directly to the culture, to a concentration of 50 μM (0.1% ethanol). A control culture containing no JA was also prepared. Two millilitres of culture was then removed from each culture at each of the following time points: 0, 6, 24, 48, and 96 h, and then briefly vacuum-filtered to remove the liquid media before detecting GUS activity by histochemical staining.

Plant treatments: white spruce

Transgenic white spruce trees of lines 55 and A were used for RT-qPCR analysis following induction with JA, wounding, and infection with *Ceratocystis resinifera*. The plants had just completed their fifth growth cycle (plants had set buds). One plant was used for each treatment and the experiment was conducted in duplicate. For a given tree, five fully elongated terminal buds representing current year growth were selected, one of which was to be harvested at each of the following time points: 0 (just prior to treatment) and 6, 12, 24, and 72 h post treatment. All buds selected were from the top third of the plant and from the same whorl. The JA treatment consisted of spraying the entire seedling to dripping with 500 μM JA (in 0.1% ethanol). Control plants were sprayed with 0.1% ethanol. For the wounding and *Ceratocystis* treatments, only the selected buds were treated. Wounding consisted of cutting back the bark with a scalpel at the base of each bud to about 1 cm before repositioning it and wrapping with parafilm. The *C. resinifera* treatment consisted of wounding and then positioning the agar containing the pathogen along the wound before repositioning the bark and sealing it with parafilm. Control plants of the same line were untreated. One of the selected buds was removed at each time point, immediately frozen in liquid nitrogen, and stored at –80 °C until RNA was extracted.

Plant treatments: Arabidopsis

Arabidopsis plants were grown in a 16/8 h light/dark regime at 22 °C. Induction by spotting consisted of pipetting a drop of JA directly onto a rosette leaf at a concentration of 500 μM (two drops per rosette leaf on either side of the mid-vein). The wounding treatment consisted of puncturing a rosette leaf multiple times with a scalpel. Treatment with the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 consisted of infiltrating select rosette leaves with bacteria in a 10 mM $MgCl_2$ solution at a concentration of OD_{600}=0.001 with a needleless syringe.

RNA extraction

Needle tissue was ground in liquid nitrogen and total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) with on-column DNase treatment (RNase-Free DNase Set) as per the manufacturer's instructions with the exception that the lysis buffer described in MacKenzie *et al.* (1997) was used. RNA was quantified using the Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies).

Reverse transcription

200 ng of total RNA was reverse-transcribed using the Quantitect Reverse Transcription Kit (Qiagen). cDNA reactions were diluted to a final concentration of 5 ng μl^{-1} using 10 mM TRIS, pH 8.0, and aliquoted into three separate tubes to avoid repeated freeze–thaw cycles. No RT controls were performed on the samples.

Primer design

Primers were designed using the Oligo Analyzer/Oligo Explorer program (http://www.genelink.com/tools/gl-oe.asp). Tm values were selected at 65 °C using the following parameters: 50 mM salt concentration and 250 pM DNA. No template control reactions were run on the primer pairs to check for dimer formation.

RT-qPCR analysis

Gene expression was analysed for each of the treatments (*C. resinifera*, wounding, and JA) using the Stratagene Mx3000P system (Agilent Technologies Inc.). Each reaction consisted of 0.6 μM of both forward and reverse primers, 5 ng of cDNA as template, and 1× QuantiTect™ SYBR Green mix (Qiagen) in a final volume of 10 μl. Sequence and Tm information for each primer set can be found in the supplementary material. PCR cycling conditions were 15 min incubation at 95 °C, followed by 40 amplification cycles (94 °C, 15 s; 64 °C, 30 s; 65 °C, 90 s). Fluorescent readings were taken at the end of each cycle and the specificity of amplification as well as the absence of primer dimers were confirmed with a melting curve analysis at the end of each reaction. Fluorescence and Ct values were exported and analyzed in MS Excel. The relative number of transcripts ($1/2^{Ct}$) was then averaged for technical RTqPCR duplicates and used for subsequent normalization.

To correct for technical variation in RNA extraction, total RNA quantification, reverse transcription, and RT-qPCR reactions (e.g. inhibitors of PCR) as well as biological variation expression data were normalized against the geometric mean of three reference genes, PTSR, EF, and Actin 2 (Stefani *et al.*, 2010) (see Supplementary Table S1 at *JXB* online), by geNORM VBA applet for Microsoft Excel (http://medgen.ugent.be/~jvdesomp/genorm) (Vandesompele *et al.*, 2002).

For all experiments, fold change is expressed as treatment relative to the control calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and, finally, normalized by the control experiment (Bustin *et al.*, 2009). Standard deviation related to the biological variation within one line was calculated in accordance with error propagation rules.

Results

Motif analysis in the PgD1 promoter

In order to gain insight into how the *PgD1* gene is regulated, a genomic DNA fragment of 1149 bp 5' of the *PgD1* start codon was obtained. A motif search was conducted on our putative promoter as well as on all the 5' non-coding sequences (–1200 bp) of each *Arabidopsis* defensin. Retained *Arabidopsis* sequences of less than 1200 bp are due to the presence of an adjacent gene located within 1200 bp.

It had previously been noted that *PgD1* mRNA strongly accumulates following JA treatment (Pervieux *et al.*, 2004), so a search was made for the presence of a JA response motif. The putative *PgD1* promoter was found to have the JA response element CGTCA (Fig. 1) almost in the centre of the cloned fragment. Most of the defensin promoters from *Arabidopsis*, including *PDF1.2* (*a* and *b*), also contained this motif *(10/14)*. Among the promoters that did not contain this motif were the promoters of *PDF1.2c*, *PDF*

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2.5, *PDF3.1*, and *PDF3.2*. Genevestigator (www.genevestigator.com) was then used to look into gene expression specificity in relation to the respective motif found. Among these four genes, *PDF1.2c* and *PDF3.2* did not show a positive and significant (above 2-fold) induction following MeJa treatment. However, *PDF 2.5* and *PDF3.1* did show an induction (2.1-fold and 4.9-fold, respectively) when the MeJa treatment was performed on cell culture, but not on plants.

It had also been shown that PgD1 has antifungal properties and was induced upon wounding, so the presence of fungal elicitor responsive elements was anticipated and TC-rich repeats were expected to be involved in the stress response. The *PgD1* promoter was found to contain three copies of the TC-rich motif and one copy of the fungal elicitor responsive element (Fig. 1). A motif involved in the SA response was also found in our promoter, although it was located very near the 3'-end of the promoter between the TATA box and the ATG, and in eight out of the 14 *Arabidopsis* promoters. It is well established that, in contrast to most pathogen-induced genes, defensin induction, namely *PDF1.2,* is independent of the SA pathway (Manners *et al.*, 1998).

GUS expression in transgenic spruce

In order to analyse the *PgD1* promoter-related expression and stress response, transgenic white spruce expressing the *uidA* gene (GUS) driven by our 5' *PgD1* fragment were generated. Since it had previously been established that *PgD1* was strongly induced following JA treatment, it was first verified if GUS activity could be induced in embryogenic tissue. Line 55 was arbitrarily selected and put into cell suspension culture. Induction of GUS expression, and therefore responsiveness of the promoter to JA, was confirmed by histochemical staining (Fig. 2A). Nine transgenic white spruce lines were then selected, showing a range of basal GUS expression (histochemical analysis) in embryogenic tissue (data not shown), and seedlings were generated by somatic embryogenesis. Following maturation of the embryonal tissue, since it has been established that defensins are strongly expressed in seeds and seedlings (Terras *et al.*, 1995), GUS histochemical staining was performed on the somatic embryos generated. For all lines, a very specific pattern of expression was observed, showing it to be restricted to the shoot apical meristem (Fig. 2B, panel i). No blue colour was visible in the root meristem (Fig. 2B, panel i). Since the absence of blue coloration could have been caused by a lack of substrate penetration in the radicle, *CaMV35S::GUS* and *Ubiquitin::GUS* transgenic lines were generated as controls and stained as for the *PgD1::GUS* lines. It is clear from panels (ii) and (iii) that GUS substrate penetration is not problematic since the entire embryo turns dark blue when using these promoters. Therefore, it is concluded that the GUS staining observed in panel (i) is specific. Following germination of the somatic embryos and transfer to the greenhouse, MUG assays were performed on the needles during the first growing season to determine basal levels of GUS expression. As expected, a relatively narrow gradient of GUS expression was observed (Fig. 2C). Two lines showing intermediate levels of expression were selected for future experiments (see arrows in Fig. 2C).

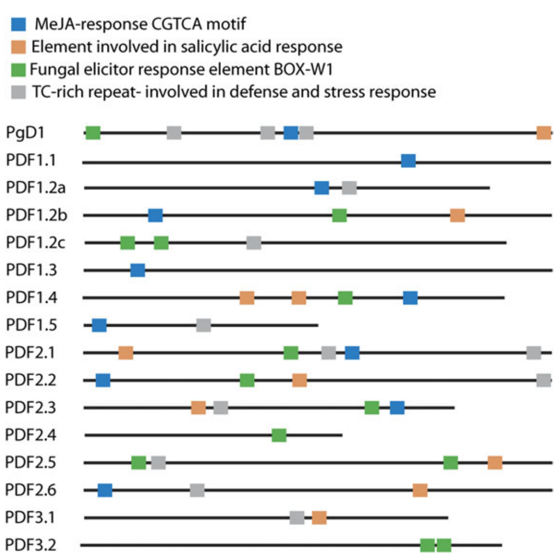

Fig. 1. The solid black line represents the promoter DNA sequence; the coloured boxes represent the various motifs found with PlantCare.

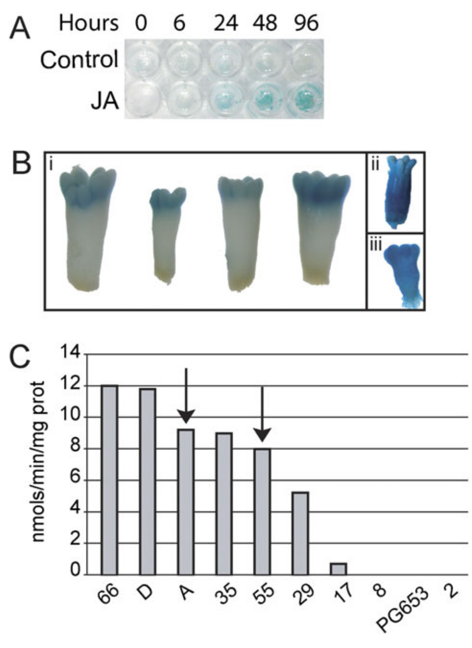

Fig. 2. (A) White spruce cellular suspension treated with ethanol 0.1% (control) or JA (in ethanol 0.1%) harvested at 0, 6, 24, 48, and 96 h and placed in GUS reagent. (B) Somatic white spruce embryos treated with GUS reagent and displaying the characteristic GUS staining. Panel (i) *PgD1::GUS*; panel (ii) CaMV *35S::GUS* panel; (iii) *Ubiquitin::GUS*. (C) Needles of lines of transgenic white spruce seedlings containing the *PgD1::GUS* construct were used for MUG assays.

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JA, wounding, and pathogen treatments on transgenic white spruce trees

To assess whether the transgenic plants were responding to our treatments, real-time quantitative RT-PCR was used instead of MUG assays since it made it possible to monitor the transcript level of GUS, control genes, and native defensin genes all at the same time in a comparable manner.

The expression level of the genes encoding for a peroxidase and a chitinase, which are known to be induced by pathogens and wounding in spruce (Conrads-Strauch *et al.*, 1990; Liang *et al.*, 1989) were quantified. Both genes showed a quick induction of mRNA expression in the needles following treatments, thus confirming the activation of a defence response (Fig. 3E, F).

In order to evaluate if the promoter fragment that had been cloned was driving GUS expression in a manner similar to the native *PgD1* expression, *GUS* and *PgD1* expression was then compared. To our surprise, the induction of GUS mRNA was not observed following treatment with JA (Fig. 3A, C), while an induction of 20-fold and 15-fold was observed for the native defensin (PgD1) in lines A and 55, respectively (Fig. 3B, D). However, the induction of GUS expression was observed in both lines following wounding, although it was much weaker than the induction observed for the endogenous defensin (see line A with GUS expression at 4.4-fold versus 660-fold for defensin), and a similar pattern was observed following infection with *C. resinifera* (the same trend was observed for line 55). To gain an insight into the difference in amplitude induction between the *GUS* transgene and the native defensin, the relative constitutive expression levels of both genes was calculated at time 0. In line A, the *GUS* gene was constitutively expressed 17 times more than defensin, while in line 55 a 20-fold difference was observed (GUS/defensin). This very strong constitutive expression observed for *GUS* is likely to be due to the genomic effect of the construct insertion site, which could result from insertion in a more active transcriptional region. Alternatively, it cannot be excluded that the promoter fragment cloned here may be incomplete and does not contain all the regulatory elements required for proper *PgD1* expression.

Transgenic Arabidopsis plants react to wounding and JA

Since limited information exists about defence pathways in conifers and, more generally, in gymnosperms, it was thought that transforming *Arabidopsis* with our construct would, indirectly, provide valuable information about the *P. glauca* defence pathway. The rationale behind this is that if *Arabidopsis* can express a spruce construct in a manner similar to its own defensin expression profiles, it would be concluded that the *Arabidopsis* transcription factors and signalling pathways that regulate defensin expression share some conserved elements between both species.

A basal level of reporter gene expression was observed in *Arabidopsis PgD1::GUS* plants. A closer analysis of the leaves revealed promoter expression in the vascular tissue and in the trichomes (Fig. 4A, I, II). Blue coloration, albeit weak, was also observed in the flowers, mostly on the stigma and in the sepals (Fig. 4A, III). When entire seedlings were stained, GUS expression was predominantly observed in the aerial part of the plant (Fig. 4A, IV). At higher magnification, very strong expression was observed in the guard cells of the leaves (Fig. 4A, V, VI), which are the main entry point of pathogens. It was also investigated whether the *PgD1* promoter was responsive to wounding by puncturing *Arabidopsis* rosette leaves with a scalpel blade. Four hours after wounding, the leaf was immersed in GUS reagent and a clear blue coloration was observed around the puncture point (Fig. 4B) following tissue clearing. It was then investigated whether our spruce promoter construct was responsive to jasmonic acid in *Arabidopsis* by placing a drop of jasmonic acid on a rosette leaf. Reporter gene activity became visible after 30 min with a clear delimitation line that appeared dark blue at the edge of the drop after 4 h (Fig. 4C).

Arabidopsis infiltration with Pst DC3000 suppresses PgD1 expression

It is a well-known fact that the SA pathway has the capacity to negatively regulate the JA pathway, which is upstream of *PDF1.2* and necessary for its expression (Ndamukong *et al.*, 2007). Thus, it was investigated whether the infection of our transgenic *Arabidopsis* plants with the hemibiotrophic virulent pathogen *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) would repress the expression of the *PgD1::GUS* construct. *Arabidopsis* infiltration was performed with a sterile 10 mM $MgCl_2$ solution or with a fresh culture of *Pst* DC3000 resuspended in 10 mM $MgCl_2$, and leaves were collected at times 0 h (control) and 24 h. The leaf being infiltrated was identified with a marker on the leaf surface on one side of the mid vein and infiltrated on the opposite side; it was necessary to ensure that the *Pst* solution or the $MgCl_2$ dispersed only on the side of the mid-vein that was being infiltrated. $MgCl_2$-infiltrated plants showed blue staining on both sides of the mid-vein at a similar intensity (Fig. 4D). However, when the plants were infiltrated with the hemibiotrophic pathogen, considerably less blue was observed after 24 h (Fig. 4D). Thus, a clear suppression of *PgD1::GUS* expression was observed when *Pst* DC3000 was infiltrated. These results seem to indicate that a stimulation of the SA pathway inhibits the expression of the JA pathway and abolishes the expression of our construct.

Discussion

Early work on defensins established that some of them are quickly induced upon pathogen infection (Bowles *et al.*, 1991; Brederode *et al.*, 1991) and that some display potent antimicrobial activity (Terras *et al.*, 1992). For these reasons, enhanced disease resistance following defensin over-expression has been reported in potatoes, tomatoes, carrots, wheat,

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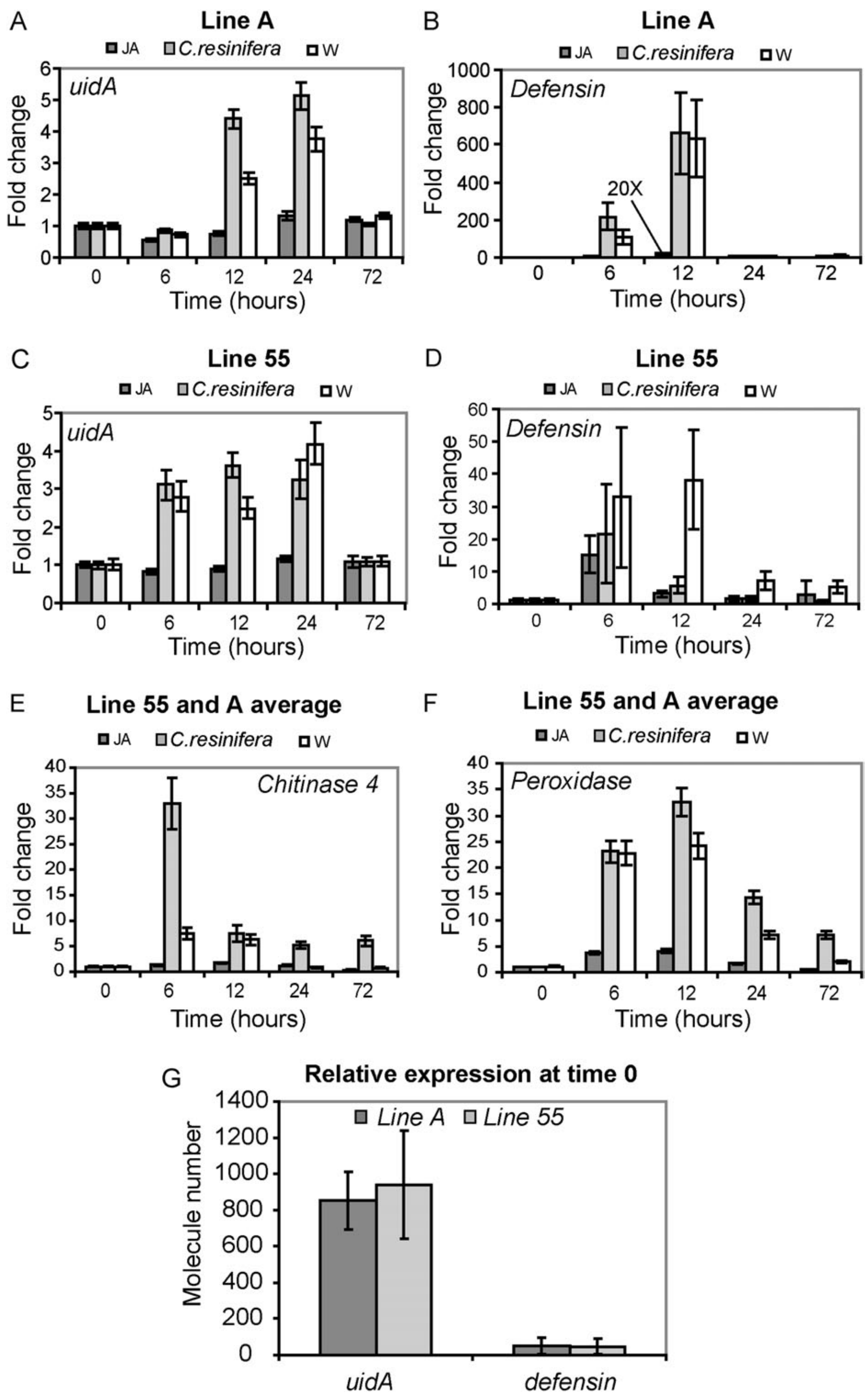

Fig. 3. RNA expression analysed using RT-PCR of plants treated with JA, wounded or infected with *C. resinifera*. (A) GUS expression in line A. (B) Defensin expression in line A. (C) GUS expression in line 55. (D) Defensin expression in line 55. (E) Chitinase gene expression, average of lines A and 55. (F) Peroxidase gene expression, average of line A and 55. (G) Expression at time 0 of GUS/defensin in lines A and 55.

tobacco, melon, rice, Norway spruce, pine, and *Arabidopsis* (Terras *et al.*, 1995; Wang *et al.*, 1999; Gao *et al.*, 2000; Parashina *et al.*, 2000; Elfstrand *et al.*, 2001; Kim *et al.*, 2004; Choi *et al.*, 2009; Ntui *et al.*, 2010; Portieles *et al.*, 2010; Li *et al.*, 2011); this list may not be exhaustive. Plant defensin expression in various organs and treatments has been well studied in *Arabidopsis* (Kragh *et al.*, 1995; Terras *et al.*, 1995; Manners *et al.*, 1998; Thomma and Broekaert, 1998). Manners *et al.* (1998) demonstrated that *PDF1.2* was highly responsive to JA but insensitive to SA; hence, *PDF1.2* is now frequently used as a marker for the ethylene/jasmonate-mediated signalling pathway.

However, the regulation and knowledge of the gymnosperm defence pathway and the role of defensin in conifers

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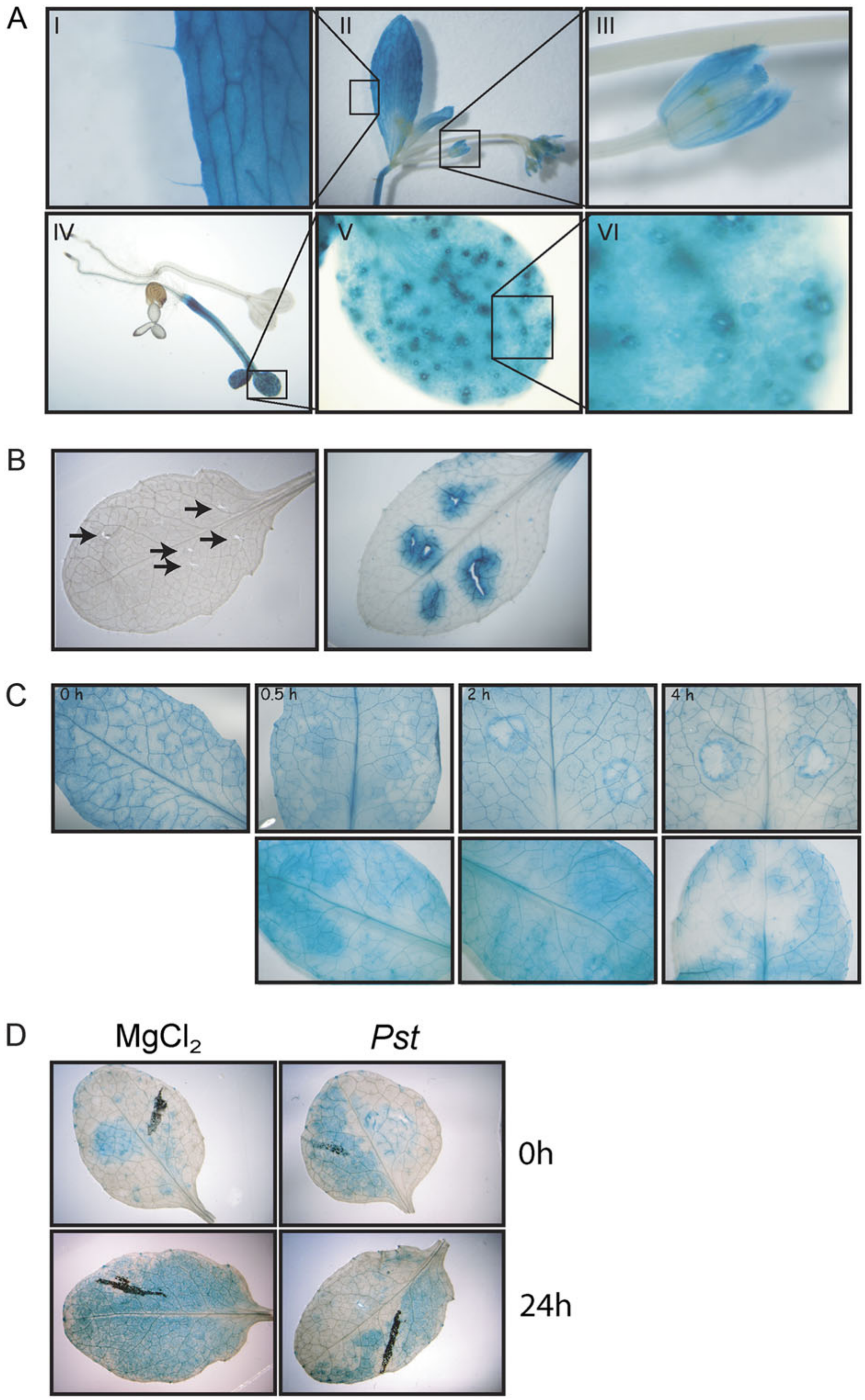

Fig. 4. (A) *Arabidopsis* transgenic plants containing *PgD1::GUS* were either grown in soil for 3 weeks (panels I, II, III) or on MS media for 1 week (panels IV, V, VI) and stained with GUS reagent. Panel IV shows both a GUS-stained *PgD1::GUS* (blue) and wild-type seedling (white) for comparison. (B) Three-week-old *Arabidopsis* leaves were punctured with a scalpel blade and stained for 4 h. (C) Drops of JA were put on either side of the mid-vein, and the leaves were collected at 0, 0.5, 2, and 4 h. (D) Plants were infiltrated with $MgCl_2$ or with a solution of *Pseudomonas syringae* pv. *tomato* DC3000 at OD_{600}=0.001, and harvested at 0 h or 24 h. The leaf being infiltrated was identified with a black line on one side of the mid-vein and infiltrated on the opposite side.

is much less understood. It was previously demonstrated that PgD1 displays anti-fungal activity and that its corresponding mRNA accumulates after wounding and JA treatments, but not after treatment with SA (Pervieux *et al.*, 2004). SPI1, a defensin from Norway spruce (*Picea abies*), was shown to be induced by the root rot fungus *Heterobasidion annosum* (Fossdal *et al.*, 2003) and it increases resistance to *H. annosum* when over-expressed in transgenic

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Norway spruce (Elfstrand *et al.*, 2001). In addition, a second Norway spruce defensin SPI1B (AF548021) was found in needles (Fossdal *et al.*, 2003), but the expression pattern of SPI1B in embryos or in response to infections has not been examined. Furthermore, no studies to date have focused on the regulatory elements of spruce defensin promoters. The cloning of the *PgD1* promoter and its responsiveness to JA, wounding, and pathogen treatments in both spruce and *Arabidopsis* is reported here.

A MeJa response element (CGTCA) was identified in the *PgD1* promoter and in several *Arabidopsis* defensin promoters. However, a GCC box was not found, such as the one described (GCCGCC) by Brown *et al.* (2003) which is present in the *PDF1.2* promoter (Brown *et al.*, 2003; Zarei *et al.*, 2011). It should be noted that the PgD1 protein is more closely related to PDF1 than to PDF2 but a high similarity level precludes higher discrimination (see Supplementary Fig. S1 at *JXB* online). Nevertheless, the spruce defensin promoter does contain several GCC sequences, but no occurrence of the doublet GCCGCC was found. On the other hand, a clear response to JA was observed in *P. glauca* cell suspension culture and in *Arabidopsis,* indicating that the CGTCA motif may be sufficient for the response to JA.

The *PgD1* promoter also contained a fungal elicitor responsive element (GGTCAA). It had previously been observed that PgD1 had antifungal activity, but it had not been investigated whether it was induced by pathogens. In spruce, the native defensin gene *PgD1* was strongly induced upon infection with the necrotrophic pathogen *C. resinifera*; however, the level of induction observed for our transgenic GUS lines was much lower. In Norway spruce, the SPI1 protein was shown to accumulate following *Heterobasidion annosum* infection. Following *Pythium dimorphum* infection, the *SPI1* mRNA level increased 2-fold and reached a maximum at 24 h post-infection followed by a dramatic reduction, indicating that this pathogenic oomycete may have the ability to suppress its expression (Fossdal *et al.*, 2003). *PDF1.2* accumulation also occurs in *Arabidopsis* via a SA-independent pathway upon infection with fungal pathogens (Manners *et al.*, 1998).

Wounding treatment of the *PgD1::GUS* transgenic spruce resulted in strong induction of the native defensin gene. A similar trend was also observed for the induction of the GUS reporter gene, but the amplitude of the response was much weaker. Interestingly, the *Arabidopsis PDF1.2* promoter is also up-regulated by pathogen and jasmonate, but not by wounding or salicylate (Manners *et al.*, 1998). The *PgD1* promoter response observed is fairly consistent since three motifs corresponding to the TC-rich repeat involved in stress are present versus only one in the *Arabidopsis PDF1.2a* promoter. A study on specific wheat and rice defensin promoters also found that they were all strongly induced by wounding (Kovalchuk *et al.*, 2010).

Interestingly, the GUS reporter induction observed following wounding or *C. resinifera* infection was more than one order of magnitude weaker than that of the native defensin gene. This difference can be explained by the fact that the basal level of expression of the *PgD1::GUS* transgene at time 0 h was nearly 20-fold higher than that of the native endogenous *PgD1*. Defensin expression seems to be under tight transcriptional control, probably due to a fitness cost associated with aberrant or constitutive expression. Post-transcriptional gene silencing may also affect defensin mRNA accumulation in the absence of stress, which would not impact our construct since the mRNA that is expressed is the one encoding GUS and not defensin. The very high level of GUS expression even at time 0 (basal level) may preclude a further increase in transcriptional activity. Finally, it cannot be excluded that the 1149-bp fragment cloned did not cover the entire *PgD1* promoter and that a negative regulator that would keep expression lower in the absence of stress may be located upstream from the genomic region cloned here.

In order to gain an indirect insight into the *P. glauca* defensin signalling pathway, our construct was also transformed in *A. thaliana,* a plant species with an extensive dataset on defensin gene expression. The conserved *PgD1::GUS* expression pattern in *A. thaliana* and spruce, as well as for other known *Arabidopsis* defensins, would suggest the presence of conserved regulatory element and signalling pathways. Knowledge of transcription factors affecting *PDF1.2* mRNA accumulation in *Arabidopsis* has been the subject of many studies. The induction of *PDF1.2* has been observed upon the over-expression of ERF1 (Pré *et al.*, 2008), ERF2 (Brown *et al.*, 2003), ORA59 (Pré *et al.*, 2008), and TGA5 (Zander *et al.*, 2010). Over-expression or knock-out of other transcription factors were also shown to affect *PDF1.2* expression (Boter *et al.*, 2004; Li *et al.*, 2004; Lorenzo *et al.*, 2004; McGrath *et al.*, 2005).

Our results demonstrate that, both in transgenic spruce embryos and in *Arabidopsis* seedlings, the blue coloration corresponding to GUS expression is restricted to the aerial part of the plant. This is partly in opposition to the results obtained for SPI1 expression in Norway spruce, which was only detected in the radicles, roots, stem, and aerial part of seedlings, but was not detectable in the embryo using antibodies (Fossdal *et al.*, 2003). In more mature plants, expression in leaves is predominant in guard cells, which is supported by the literature (Kragh *et al.*, 1995) and is coherent since stomata are the main entryway used by leaf-infecting fungal pathogens. Similarly, defensin expression in mammalians has been reported in several types of epithelial tissue (Wong *et al.*, 2007).

When our transgenic *A. thaliana* plants were subjected to JA, a clear induction of expression was observed along the margins of the drop. This is in line with the literature on some *Arabidopsis* defensins, the archetype of which is *PDF1.2* (Manners *et al.*, 1998). *PDF1.2* is induced by JA in wild-type plants but not in *ein2* and *coi1* mutant plants, which are impaired in their response to ethylene and methyl jasmonate, respectively (Penninckx *et al.*, 1996). However, neither the radish defensin *Rs-AFP* (Terras *et al.*, 1998) nor *PDF1.2* (Penninckx *et al.*, 1996) are induced by wounding, while a strong induction was observed with similar treatments with the *PgD1::GUS* construct in both spruce and

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A. thaliana. Using Genevestigator, it was verified whether less studied *Arabidopsis* defensins were induced following wounding. As it turns out, most defensins are not induced following wounding or if they are it is at a very low level. However, 24 h after wounding, *PDF2.1*, *PDF1.4*, and, notably, *PDF1.2a* were induced at levels of 3.1, 11.9, and 5.8-fold, respectively (www.genevestigator.com). Interestingly, the seven rice and wheat defensin promoters studied by Kovalchuk *et al.* (2010) were all strongly up-regulated after wounding.

Lastly, the impact of infection by the hemibiotrophic pathogen *P. syringae* pv *tomato* DC3000, *PgD1::GUS* expression, and the related accumulation of GUS protein was assessed. A clear reduction was observed in GUS reporter protein accumulation upon pathogen treatment, while control leaves that received only $MgCl_2$ remained blue. Infiltration with the hemibiotrophic pathogen *P. syringae* resulted in endogenous SA accumulation which, in turn, caused a decrease in JA levels and strongly suggested a dependence on JA for *PgD1* expression. This correlates with the results obtained showing an up-regulation of both endogenous defensin and *PgD1::GUS* expression following infection with the necrotrophic pathogen *C. resinifera* and wounding.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. A phylogram was inferred from a Neighbor–Joining alignment of protein sequences from PgD1 sequences and *Arabidopsis* defensin.

Supplementary Table S1. Sequence of primers used in this study.

Acknowledgements

We wish to thank Dr Louis Bernier (Département des sciences du bois et de la forêt, Université Laval) for the *Ceratocystis resinifera* strain and Isabelle Lamarre for editorial work. We also wish to express our gratitude to Dr Philippe Tanguay for critical comments on the manuscript and Frédéric Vigneault for scientific discussions. HG is the recipient of a postdoctoral Visiting Fellowship in Canadian Government Laboratories from the Natural Sciences and Engineering Research Council of Canada. This work was supported by a grant from the Canadian Genomics R&D Initiative to AS.

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