Phytochemistry 69 (2008) 3032–3037

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Relationship of dirigent protein and 18s RNA transcript localization to heartwood formation in western red cedar

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ARTICLE INFO

Article history:
Received 28 May 2008
Accepted 18 June 2008
Available online 11 September 2008

Keywords:
Western red cedar (*Thuja plicata*)
Cupressaceae
In situ hybridization
Dirigent protein mRNA
Lignans
Plicatic acid
18s rRNA
Sapwood
Heartwood

ABSTRACT

Western red cedar (*Thuja plicata*) heartwood contains abundant amounts of structurally complex plicatic acid-derived lignans that help confer protective properties and longevity to this tissue type. Although the lignan biochemical entry point is dirigent protein-mediated, the formation of heartwood and its associated lignans in some species remains poorly understood due to technical difficulties of working with the former. To begin to address such questions, this study therefore focused on the anatomical localization of dirigent protein and 18s rRNA (control) gene transcripts within recalcitrant woody tissues, including heartwood.

This *in situ* mRNA hybridization approach enabled detection of dirigent protein transcripts in cork cambia, vascular cambia and ray parenchyma cells of the sapwood, but not the heartwood under the conditions employed. By contrast, the hybridization of the 18s rRNA (control) transcript resulted in its detection in all tissue types, including radial parenchyma cells of apparently preformed heartwood. Application of *in situ* hybridization to such recalcitrant tissues thus demonstrates the utility of this technique in identifying specific cell types involved in heartwood formation, as well as the relationship of dirigent protein localization to that of heartwood metabolite generation.

1. Introduction

Heartwood in vascular (woody) species represents perhaps the largest repository of organic carbon in the terrestrial habitat, particularly via deposition of species-specific heartwood metabolites, such as lignans, tannins, phlobaphenes, alkaloids, and so forth (Hillis, 1999). Such metabolites constitute the so-called wood "extractives". Yet little is known about how heartwood generation is biochemically induced and ultimately formed from its sapwood origins (Gang et al., 1998; Magel, 2000; Taylor et al., 2002). It has long been known, though, that heartwood "extractives", whose chemical compositions can vary enormously between species, differentially impart distinct properties of durability and protection to woody plants (see Taylor et al., 2002).

In the case of western red cedar (*Thuja plicata* Donn ex. D. Don), the bulk of the metabolites formed in heartwood are the 8–8′ linked lignans, derived from plicatic acid (**1**) (Gardner et al., 1960, 1966; Barton and MacDonald, 1971; Johansson et al., 2000) (Fig. 1). These can account for up to 10–15% of the heartwood mass and, together with other metabolic products, help confer remarkable pest and disease resistance, making western red cedar very highly valued for lumber.

Entry into the plicatic acid-lignan pathway is considered to (largely, if not exclusively) occur via stereoselective coupling of *E*-coniferyl alcohol (**2**) to initially yield (+)-pinoresinol (**3**), which is then metabolized further to afford plicatic acid (**1**) (Fig. 1). This coupling step apparently requires an oxidase (one-electron oxidant) and a dirigent protein (Latin: *dirigere*, to guide or align), with the latter enabling stereoselective coupling to occur (Davin et al., 1997; Burlat et al., 2001; Halls and Lewis, 2002; Halls et al., 2004; Davin and Lewis, 2005). In western red cedar, there are at least nine dirigent protein isovariants/homologues, several of which were demonstrated *in vitro* as able to help effectuate this stereoselective coupling step (Kim et al., 2002b). There are also 35 putative homologues in species of spruce (*Picea* spp.) (Ralph et al., 2006, 2007).

In this study, it was instructive to develop riboprobes to investigate patterns of gene expression of the dirigent (multigene) family directly (*in situ*) in developing bark (cork cambium), sapwood and heartwood-forming tissues. This was because several of these genes were reportedly involved in plicatic acid (**1**) formation and possibly to other (related) metabolites as well. In this context, earlier studies in other woody plant species had suggested, based on chemical analyses, that various classes of extractives were ultimately formed in regions proximal to the sapwood/sapwood–heartwood transition zone (Hillis, 1987; Magel et al., 1994). Very little is known, however, regarding transcriptional (or post-transcriptional) activity of extractive (lignan)-related genes in the

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doi:10.1016/j.phytochem.2008.06.020

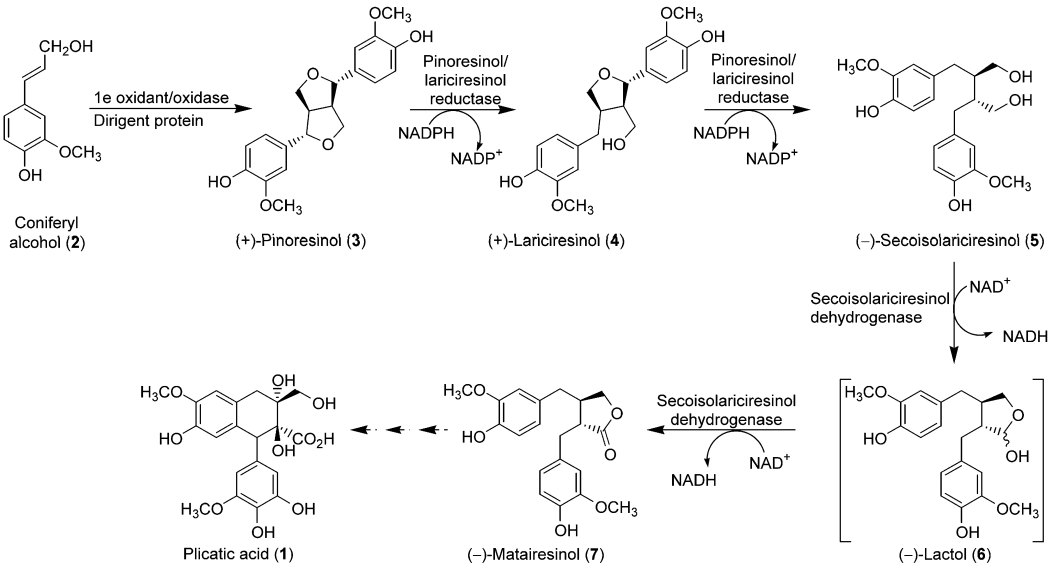

Fig. 1. Proposed biosynthetic pathway to plicatic acid (**1**) from coniferyl alcohol (**2**). Known biochemical steps show conversions and proteins/enzymes involved. Broken arrows indicate unknown conversions.

sapwood, the sapwood–heartwood transition zone and the heartwood tissues. Transcription of a presumed metabolic marker for heartwood formation (sucrose synthase) was, however, demonstrated using random amplification of polymorphic DNA (RAPD) in excised heartwood-forming tissues from *Robinia pseudoacacia* (Magel et al., 2002), indicating the potential to study such tissues, despite the technical challenges presented by heartwood. To our knowledge, the detection of lignan-related transcripts has not been reported for *in situ* analyses of either nascent or mature heartwood-forming tissues.

We therefore devised a single riboprobe to detect and localize all gene family transcripts of the lignan-forming dirigent protein in such recalcitrant tissues. An 18s rRNA riboprobe was also utilized to serve as a control (i.e. for RNA detectability and also to examine the possibility of tissue preparation artifact). Herein, we now report *in situ* detection of dirigent gene transcription in the cork and vascular cambia, as well as in the ray parenchyma of sapwood. Additionally, the detection of 18s rRNA in the ray parenchyma cells of maturing heartwood tissue also demonstrated RNA localization within the heartwood itself; to our knowledge, this is both a novel finding and a technological advance as such tissue has generally been considered hitherto as non-living. Given the technical challenges of working with heartwood, this finding thus also demonstrates the need for analyzing heartwood tissue directly, especially of ray parenchyma. It is, therefore, suggested that future work on heartwood formation should include heartwood tissue itself by either *in situ* hybridization or possibly by using more sensitive methods, i.e. laser microdissection combined with analyses such as RT-PCR and/or mass spectrometry.

2. Results and discussion

Kim et al. (2002b) had previously demonstrated that many of the dirigent gene isovariants of western red cedar were transcribed in diverse tissues such as the shoot tip, young stem, vascular cambium, needles, roots, and female cones, when individual whole tissues were homogenized and probed using real-time PCR. This diverse distribution suggested widespread plant defense roles for dirigent protein-generated lignans in different tissues and organs in this species. Furthermore, using western red cedar-derived dirigent protein gene promoter elements, Kim et al. (2002a) demonstrated that control of transcription of each of the nine dirigent protein isovariants was targeted to specific and mostly unique tissue types when transformed to, and expressed in, the model angiosperm *Arabidopsis*. These studies lent preliminary insight into the complex dirigent protein metabolic networks that apparently exist in western red cedar.

In the current study, we sought to identify the cell types in western red cedar that harbor mRNA transcripts for dirigent proteins. Initially, it seemed feasible to generate specific riboprobes for at least 7 dirigent isovariant types, because Kim et al. (2002b) showed that PCR could be used to distinguish isovariants 1–4, 5/6, 7/8 and 9; indeed, our PCR results were similar to that of Kim et al. (2002b) (data not shown). Conversely, in this study, DNA blot hybridization following riboprobe construction demonstrated consistent cross-reactivity to all dirigent templates, not just each intended target.

Several factors may account for the reduced specificity observed with the riboprobes. For instance, riboprobes depend on a sequence specificity of over 100 + bp, whereas a technique such as PCR requires specificity over only two 18–25 bp primer sequences. Given the >72% sequence identity between the dirigent isoforms (Kim et al., 2002b), we were thus unable to generate riboprobes with isoform-specificity over a 100-bp span. Additionally, the length of a riboprobe necessitates a much longer hybridization/annealing time (several hours to overnight) than the annealing time of PCR primers ($\leqslant$1 min), thereby providing opportunity for the riboprobe to also weakly (or strongly) bind non-specific sites. Moreover, we also noted a very light, non-specific, binding to all dirigent protein templates by the cloning site sequence from the vector. This negative control riboprobe was made using an empty

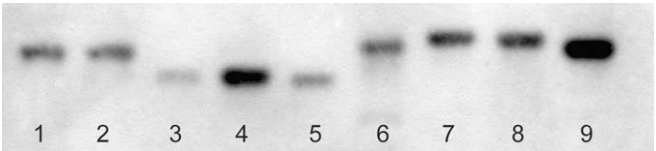

Fig. 2. Cross-reactivity of the generic riboprobe for dirigent protein mRNA to genomic DNA constructs of each of the nine dirigent protein isovariants. The riboprobes reacted to all dirigent protein isoforms, with slightly greater reactivity to isoforms 4 and 9. Numbers on the DNA blot refer to the specific genomic DNA loaded for each dirigent protein isovariant.

vector and tested using the same (previously stripped) DNA blots. Given these findings, a single generic riboprobe was utilized to collectively localize the mRNA transcripts for all dirigent isovariants in western red cedar (Fig. 2) as described in the Experimental.

Hybridization of the 'generic' dirigent riboprobe was successful in all stem sapwood tissues examined. In young stem tissue, hybridization appeared the most intense, occurring in the developing cells of the cork cambium (cc) and radial parenchyma cells (rp) (Fig. 3A and C, respectively), as well as in the vascular cambium (data not shown). It was also detected in the radial parenchyma of mature root sapwood (data not shown). Negative controls (corresponding to the 'sense' strand of the dirigent protein transcript) lacked any detectable hybridization as expected (Fig. 3B and D).

Hybridization was not, however, observed in heartwood tissue (data not shown) under the conditions employed. Indeed, it was not detected, even with application of a three-fold greater concentration of generic dirigent riboprobe, as compared to the control 18s riboprobe (see Experimental). This may suggest that monolignol coupling leading to the plicatic acid-derived lignans are not being formed in the heartwood tissue per se, but are instead generated in the adjacent sapwood parenchyma cells. An additional factor may be the sensitivity of the method; as a linear hybridization reaction, standard *in situ* hybridization detects highly-expressed RNAs well (such as 18s rRNA), but is not as sensitive to mRNAs of relatively lower abundance.

On the other hand, the highly-expressed 18s rRNA gene (control riboprobe) was readily detected in both stem sapwood and heartwood, as well as in the root sapwood (Fig. 4). The 18s rRNA riboprobe was used to control for tissue preparation artifact, the results from which indicated that this was apparently minimized/absent in this experiment. Moreover, the control 18s rRNA riboprobe demonstrated the presence of five RNA-containing cells within the outer heartwood (OHW) and middle heartwood (MHW) zones of stem tissue (locations within the original paraffin-embedded sample are indicated by asterisks, Fig. 4A), the biochemical significance of which is as yet unknown. As an example, the inner-most RNA-positive MHW cell is shown at higher magnification in Fig. 4B (and its tissue location is indicated by an arrowhead in Fig. 4A). This *in situ* finding thus correlates very well with recent reports of successful nucleic acid extraction/amplification from various wood zones, including heartwood (Magel et al., 2002).

Interestingly, heartwood has frequently been defined as having no living cells (International Association of Wood Anatomists (IAWA), 1964; Hillis, 1987; Yang et al., 2004). However, the process of heartwood formation and 'extractive' accumulation has been observed to occur in numerous different possible patterns, with heartwood formation (in *Pinus*) complete only when the nuclei of the parenchyma break down (Taylor et al., 2002, and references therein). Stem sapwood was also used as a positive control for detection of 18s rRNA, with root sapwood as an additional test/sectioning type. Numerous radial parenchyma cells were found to hybridize with the 18s rRNA gene riboprobe (blue coloration, Fig. 4E and H). Again, in this experiment, the negative control in all three cases confirms that probe hybridization is non-random (i.e. lack of blue coloration, Fig. 4C,F and I).

3. Concluding remarks

In conclusion, the localization of the dirigent protein mRNAs to the vascular cambium and radial parenchyma in actively growing stem tissue corresponds well with previous transcript localization of other lignan-related genes, such as that of pinoresinol–lariciresinol reductase in *Forsythia intermedia* and phenylcoumaran benzylic ether reductase in *Pinus taeda* (Kwon et al., 2001), as well as the proposed phenylpropenal double-bond reductase in *P. taeda* (Kasahara et al., 2006). This pattern of expression is again consistent with ray parenchyma cells having an important role in heartwood formation as proposed by Chattaway (1952) and Hillis (1999). It was noteworthy that transcription for the dirigent protein isovariants also occurred in the cork cambium of young tissue

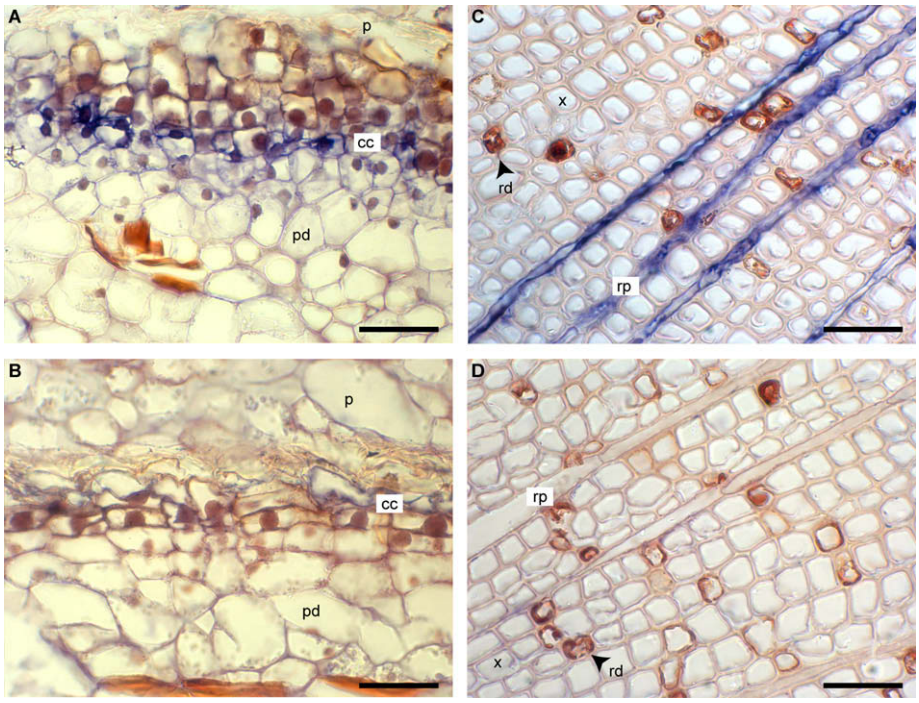

Fig. 3. Detection of dirigent protein gene expression in young stem tissues of western red cedar by *in situ* hybridization. Hybridization of the antisense riboprobe for dirigent protein transcripts was detected in transverse sections by the blue color reaction in cells of the cork cambium (A) and radial parenchyma (C) in young stem tissue (sapwood). A RNA probe of the sense strand of the dirigent transcript was used as negative control (B and D). Abbreviations: cc, cork cambium; p, phellem; pd, phelloderm; rd, resin deposits; rp, radial parenchyma; x, xylem. Scale = 30 μm.

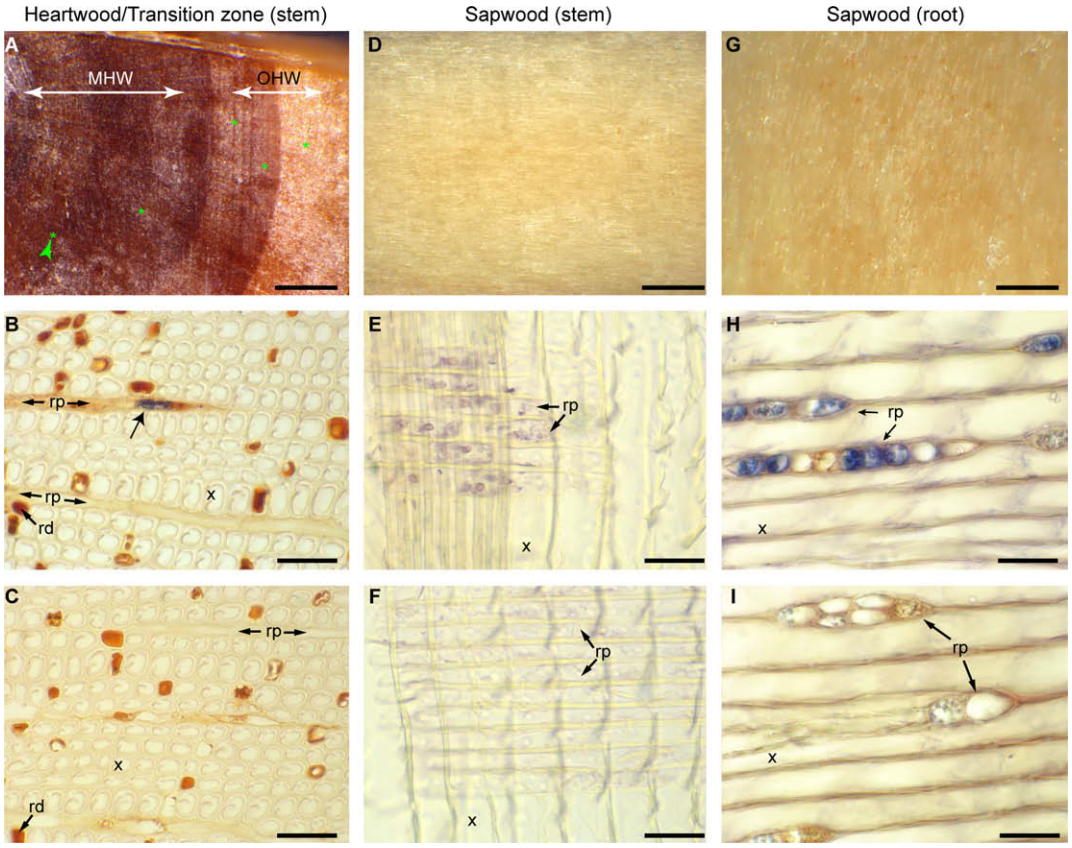

Fig. 4. Examples of cells with active gene transcription in western red cedar stem heartwood (A–C), sapwood (D–F) and root sapwood (G–I) as shown by use of riboprobes specific to 18s rRNA. Stereomicroscope imaging of paraffin-embedded heartwood (A) shows increasing amounts of resin deposits as a light brown (outer heartwood zone, OHW) to dark brown coloration (middle heartwood, MHW) with the location of cells positive for 18s rRNA indicated by asterisks (*). The approximate tissue-level location of the innermost 18s rRNA-positive cell is indicated by an arrowhead (A), and shown to be a radial parenchyma (rp) cell at higher magnification (B). The sense riboprobe (negative control) did not bind to the heartwood tissue (C). For comparison, paraffin-embedded stem sapwood (D) and root sapwood (G) are shown, wherein tissue coloration is very light due to the low level of resin deposition. Antisense riboprobes targeted to 18s rRNA were also applied to tangential stem sapwood (E) and radial longitudinal root sapwood (H) sections, with sapwood being used as a positive control tissue. The presence of active (18s rRNA) transcription (at the time of fixation) in radial parenchyma cells (rp) is confirmed by DIG-detection (blue coloration) by light microscopy (E and H). Specificity of the antisense 18s rRNA probe was again confirmed by use of the sense probe as a negative control (F and I). Abbreviations: MHW, middle heartwood; OHW, outer heartwood; rd, resin deposit; rp, ray parenchyma; x, xylem. Scales: A, D, G = 500 μm; B, C, E, F, H, I = 40 μm.

as well, this being a lignan-rich tissue of western red cedar. This observation thus apparently further supports a role of the dirigent protein multigene family in plant defense functions. Finally, (using the 18s rRNA control probe), the presence of a limited number of RNA-positive cells within the ray parenchyma cells of heartwood suggests that this tissue should also be routinely included in heartwood-forming analyses.

4. Experimental

4.1. Plant materials

Stem and root tissues were collected from a mature (approximately 52 years of age) western red cedar tree, within 30 min after it was felled. The tree was harvested with the assistance of the University of Idaho student logging crew at the University of Idaho Experimental Forest Unit near Troy, Idaho, during a planned thinning on July 7, 2001. Tissues were harvested using a saw for gross cutting and a core-borer to generate core samples 5 mm diameter from 3 locations: approximately 2 in. (sapwood) and 3 feet (sapwood, all heartwood tissues) below the apical meristem of the trunk and from a large surface root (sapwood). Core samples were quickly trimmed to small (2–5 mm^2) blocks, immersed in liquid N_2, and transferred to cold (−20 °C) MeOH-based fixative in the field after the method of Regan et al. (1999). Micrographs in Fig. 3 show transverse sapwood sections made from the stem sample located approximately 2 in. below the apical meristem. Micrographs in Fig. 4 represent transverse sections made from stem heartwood and tangential sections from stem sapwood taken 3 feet below the apical meristem, as well as a radial long section of root sapwood from a large surface root.

4.2. Generation of riboprobes

Following a preliminary experiment, it was found that RNA probes generated from DNA of each dirigent probe isoform were not specific in DNA blot hybridization tests and therefore not useful as individual probes for *in situ* mRNA hybridization (data not shown). For this reason, a single 'generic' probe was constructed for the purpose of comprehensively detecting all mRNAs of the nine dirigent protein isoforms. The initial insert for this generic probe was generated using PCR primers targeted to a 227-bp partial DNA sequence adjacent to the stop codon of the dirigent isoform 4 (nucleotides # 584 to 810 of GenBank accession number: AF210066). These were: CS4-RT1: 5′-GAATTTCCTCTGTATTACTAG-3′ and CS4-RT2: 5′-AATTATTCCAAATGGTACTCC-3′. A control RNA probe was also constructed using a 127-bp PCR product generated by primers based on the 18s rRNA sequence from *P. taeda*

(GenBank accession number: M82463; a complete 18s rRNA sequence from *Thuja plicata* is not yet available): pt18s-rtF5: 5′-CCAGGTCCAGACATAGTAAGGATTG-3′ and pt18s-rtR5: 5′-GAGGTCTCGTTCGTTATCGGAATT-3′. In both cases, PCR was carried out using Invitrogen™ Taq DNA Polymerase according to the manufacturer's recommendations in 2001. A PTC-0220 DNA Engine Dyad Peltier Thermal Cycler (MJ Research, Waltham, Massachusetts, USA) was used with annealing temperatures of 62 °C and 58 °C for the generic dirigent and 18s rRNA probes, respectively.

The PCR products were individually subcloned into the vector pCRII-TOPO® (Invitrogen Co., Carlsbad, California, USA) according to the manufacturer's instructions for the purpose of generating riboprobes. The DNA sequence of the generic probe insert was verified by sequencing from both the 5′- and 3′-ends and checking against the known dirigent protein gene sequence (GenBank accession number: AF210066). The control 18s rRNA sequence was confirmed using the NCBI Blast program (Altschul et al., 1990) with the five highest E values (all 2e-61) found for *Pinus* species.

From each construct, both anti-sense and sense (control) riboprobes were generated using a digoxigenin (DIG) RNA labeling kit according to the manufacturer's instructions (Roche Boehringer-Mannheim, Indianapolis, IN, USA).

4.3. Generic riboprobe specificity

Cross-reactivity of the antisense generic dirigent riboprobe was tested using a DNA blot of individual constructs (100 ng each) for each of the nine full-length (or near full-length) western red cedar dirigent genomic sequences (see Kim et al., 2002b, for all GenBank accession numbers). Hybridization conditions were based on the method of Kwon et al. (2001), but with the generic probe at a concentration of 100 μg/ml, a hybridization temperature of 42 °C, and detection of the DIG-labeled probes by chemiluminescence and a 4 min film exposure. The generic dirigent protein RNA probe was found to bind all 9 isoform genes at nearly equivalent levels, with more reactivity noted for isoforms 4 (as expected) and 9 (Fig. 2).

4.4. In situ hybridization and imaging

Fixed tissues were dehydrated and paraffin-embedded as described by Kwon et al. (2001). Samples were then sectioned to 20 μm, mounted on RNAse-free slides and the paraffin was removed.

Hybridization was carried out as described previously by Kwon et al. (2001) with the sections pre-incubated in hybridization solution (lacking RNA probes) as a pretreatment to *in situ* hybridization. Concentrations of both antisense and sense riboprobes were quantitated and compared to ensure application of 3.0 μg/ml for the generic dirigent probes and 1.0 μg/ml for the 18s rRNA probes on all respective sections. Individual sections were imaged using a ProgRes C12plus digital camera (JENOPTIK, Jena, DE) mounted to an Olympus BH-2 microscope (Tokyo, JP). Additionally, whole block paraffin samples (from which sections had been made) (Fig. 3A,D and G) were imaged to demonstrate coloration of the respective wood samples using a Wild Heerbrugg M5A stereomicroscope (Gais, CH) equipped with the camera described above. For these, ten images were recorded at progressive depths of focus and compiled into a single in-focus three-dimensional image using Helicon Focus software (Helicon Soft Ltd., Kharkov, Ukraine).

Acknowledgements

The authors thank Drs. Myoung K. Kim, Mi Kwon, and Aldwin M. Anterola for design of the PCR primers described in this paper. Thanks are also extended to Drs. Vincent R. Franceschi (deceased), Christine Davitt and Valerie Lynch-Holm of the Franceschi Microscopy and Imaging Center at Washington State University for technical advice and access to facilities. Thanks also to Harold Osborne for permission to collect western red cedar wood samples from the University of Idaho Experimental Forest and for assistance in collection by the U. of I. student logging crew in 2001. Generous support was provided by the National Science Foundation (MCB-0417291), the US Department of Energy (DE FG03-97ER20259), the USDA Forest Service McIntyre-Stennis Program (WNP00202), and the G. Thomas and Anita Hargrove Center for Plant Genomics.

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