PERGAMON

Phytochemistry 61 (2002) 311–322

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Monolignol radical–radical coupling networks in western red cedar and *Arabidopsis* and their evolutionary implications

Myoung K. Kim, Jae-Heung Jeon, Laurence B. Davin, Norman G. Lewis*

Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA

Received 22 April 2002; received in revised form 24 June 2002

Abstract

The discovery of a nine-member multigene dirigent family involved in control of monolignol radical–radical coupling in the ancient gymnosperm, western red cedar, suggested that a complex multidimensional network had evolved to regulate such processes in vascular plants. Accordingly, in this study, the corresponding promoter regions for each dirigent multigene member were obtained by genome-walking, with *Arabidopsis* being subsequently transformed to express each promoter fused to the β-glucuronidase (GUS) reporter gene. It was found that each component gene of the proposed network is apparently differentially expressed in individual tissues, organs and cells at all stages of plant growth and development. The data so obtained thus further support the hypothesis that a sophisticated monolignol radical–radical coupling network exists in plants which has been highly conserved throughout vascular plant evolution.

Keywords: *Thuja plicata*; Cupressaceae; Western red cedar; *Arabidopsis thaliana*; Brassicaceae; Thale cress; GUS reporter gene; Genome walking; Dirigent proteins; Monolignol radical–radical coupling networks

1. Introduction

The successful adaptation to land by vascular plants from their aquatic forerunners was very much facilitated by elaboration of the phenylpropanoid pathway. Two major branches of this specialized metabolism led to the structural biopolymers, the lignins, and to the primarily defense-related substances, the (poly)lignans. Together, they typically account for as much as 30% of all (woody) plant material (van Heerden et al., 1996). Of these, the lignins are deposited in the cell walls of tracheary elements in all major tissues of (woody) plants and, in part, function to help counter the forces of compression acting on the plant body, i.e., thereby enabling the stems to stand upright and the branches to gain specific orientations. The presence of lignins in specialized cell types also provides the means for water and nutrient conduction. By contrast, the (poly)lignans, which are a structurally very diverse class of compounds, primarily provide protection against opportunistic pathogens and other encroachers. Depending upon the specific lignan type being formed in a particular species, these can have various biological properties, e.g. as cytotoxic, antioxidant, antifungal, antiviral and antifeedant substances (for a review see Lewis and Davin, 1999). Like lignins, (poly)lignans are present in all plant tissues (stems, roots, flowers, seed coats, leaves, etc.), albeit in differing amounts.

The lignins and (poly)lignans are derived from monolignol coupling, and their patterns of deposition in various tissues and organs raise the possibility of the existence of distinct monolignol radical–radical coupling networks in planta. Moreover, the discovery of dirigent proteins (Davin et al., 1997) controlling stereoselective 8–8′ coupling of monolignol (radical)s to give lignans, such as (+)-pinoresinol (**1**), established a basis for control of coupling in vivo (see Fig. 1). There is now growing evidence for the existence of a broad class of such monomer binding "coupling" proteins which are responsible for distinctive coupling modes in (poly)lignan biosynthesis, and for arrays of monolignol (radical) binding sites in related proteins for lignin assembly (Gang et al., 1999; Davin and Lewis, 2000; Burlat et al., 2001; Wang et al., 2001). Significantly, the dirigent proteins seem to

* Corresponding author. Tel.: +1-509-335-8382; fax: +1-509-335-8206.

E-mail address: lewisn@wsu.edu (N.G. Lewis).

0031-9422/02/$ - see front matter
PII: S0031-9422(02)00261-3

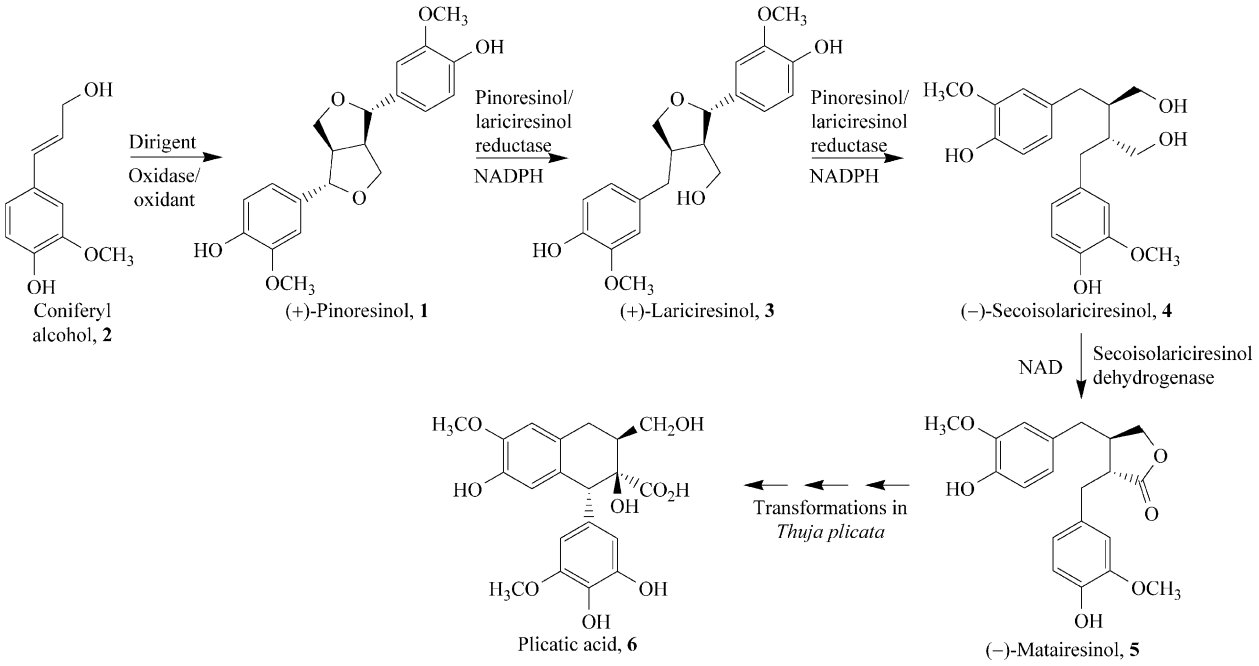

Fig. 1. Proposed biochemical pathway to plicatic acid (**6**) from coniferyl alcohol (**2**) in western red cedar (*Thuja plicata*).

have evolved their functions during adaptation of (vascular) plants to a land base.

Western red cedar *(Thuja plicata)* is a gymnosperm whose life span can range up to and beyond 3,000 years. It is a highly valued commercial species, in large part due to the massive amounts of (poly)lignans derived from plicatic acid (**6**) (Barton and MacDonald, 1971), which are deposited in its heartwood as non-structural infusions. These metabolites typically constitute between 11 and 13% by weight of the heartwood itself and help provide both color and durability; they are derived from (+)-pinoresinol (**1**) and Fig. 1 shows the proposed biochemical pathway to (+)-plicatic acid (**6**). In a previous study (Kim et al., 2002b), we established that there is at least a nine-member dirigent multigene family in western red cedar alone. These genes encode proteins displaying a high degree of homogeneity, i.e. ~73–99.5% identity at the amino acid levels and several were shown to be involved in stereoselective 8–8′ monolignol radical–radical coupling to afford (+)-pinoresinol (**1**) (Kim et al., 2002b).

The purpose of the present study was to establish if a multidimensional monolignol radical–radical coupling network existed throughout the plant body during its life span. To investigate this possibility, we transformed *Arabidopsis* plants with promoters from each of the western red cedar 8–8′ dirigent multigene family fused to the GUS-reporter gene. Western red cedar itself was not used, due to current limitations in gymnosperm transformations and the length of time of the plant to maturity; however, preliminary RT-PCR analyses of various tissues from western red cedar plants suggested the existence of such a network (Kim et al., 2002b). The data obtained in the present study using *Arabidopsis* are consistent with the existence and evolution of sophisticated monolignol radical–radical coupling networks, thereby enabling differential control at the tissue and cellular level. The findings appear to have important ramifications for both the evolution of land-based vascular plants, and for the transcriptional regulation of monolignol radical–radical coupling processes.

2. Results

2.1. Cloning of genomic DNA of western red cedar DIRIGENT genes

Of the nine *DIRIGENT* isovariants (73.7 – 99.8% identity at the nucleotide level), *DIR5* and *DIR6* as well as *DIR7* and *DIR8,* showed high sequence identities to each other (98.8 and 99.8%, respectively) in their 5′ and 3′ untranslated regions (Kim et al., 2002b). Since it was not possible to design primers specific enough to differentiate *DIR5* from *DIR6* and *DIR7* from *DIR8,* the promoters of only seven isovariants were isolated. To identify the promoters of interest, first genomic DNA was obtained from juvenile (2 years old) western red cedar *(T. plicata)* plants. The genomic sequences of the seven *DIRIGENT* isovariants *(TpDIR1-5, 8* and 9), including their promoter regions, were obtained by chromosome walking (see Section 4 and Table 1). The putative promoter regions of each of their 5′ flanking sequences (2.2 kb for *TpDIR8*gp; 2 kb for *TpDIR1*gp, *TpDIR2*gp, *TpDIR3*gp, and *TpDIR4*gp; 1.6 kb for *TpDIR9*gp, and 0.8 kb for *TpDIR5*gp) were then subjected to a BLAST

Table 1
Oligonucleotides used in PCR for the *DIRIGENT* genomic DNA amplification of 5′ flanking region of putative promoter

Isovariant	Primer name	Primer sequences	Adapter primer
	Adaptor primers (AP1)	5′-GTAATACGACTCACTATAGGGC-3′	
	Adaptor primers (AP2)	5′-ACTCACTATAGGGCACGCGTGGT-3′	
*TpDIR1*g			
1st walking	Cs2p(+)61n	3′-AAGACGAGAGAAGGTGCCACGAGTCTT-5′	AP1
	Cs2p(+)44n	3′-AAACACGAAGTACCCCGAAGACGAGAG-5′	AP2
2nd walking	Cs2p(−)1002n	3′-ACGTAAGGGTGTACGTAGTATTAGGTGAGC-5′	AP1
	Cs2p(−)1017n	3′-CGGTGATGGATCTAACGTAAGGGTGTACG-5′	AP2
	Cs2p(−)1034n	3′-AGAGGGAAGTACCAAACGGTGATGGATC-5′	AP2
3rd walking	Cs2p(−)1254n	3′-CGAACGTATCAATCATAGGAAACTATTTGT-5′	AP1
	Cs2p(−)1223n	3′-TAGCAAGAACTACTTCACGAGATTGATAAA-5′	AP2
	Cs2p(−)1187n	3′-GATAGGTAAGAACATTCTAAGAGTGTAGGT-5′	AP2
*TpDIR2*g			
1st walking	TpS32p(+)134n	3′-AGGTCTCGGTACATTCTTAGACCATAAC-5′	AP1
	TpS32p(+)102n	3′-TAGACGTCTGACAGTATCTACCTTCTTCT-5′	AP2
	TpS32p(+)84n	3′-ACGTGACACAACGAGTTTAGACGTCTGA-5′	AP2
2nd walking	TpS32p(−)379n	3′-GGGTTGAGAAGTGCTAGATGGGTAAAGA-5′	AP1
	TpS32p(−)397n	3′-ACAGTTACTTATAACCACACGGGTTGAG-5′	AP2
	TpS32p(−)435n	3′-ACCTCCATGAGAGTTAGAAATAGTTTGGG-5′	AP2
3rd walking	TpS32p(−)1060n	3′-GTAAAAAGATACAATAGAGGAAAGAACGCA-5′	AP1
	TpS32p(−)1126n	3′-CCCGTAATACACGAAAGTAAATGTGATATA-5′	AP2
	TpS32p(−)1130n	3′-AAAACCCGTAATACACGAAAGTAAATGTGA-5′	AP2
*TpDIR3*g			
1st walking	TpS4p(+)213n	3′-AACAAAGTCGGGGAGTTCCTCGATTAGA-5′	AP1
	TpS4p(+)199n	3′-ACGTTGTAGACGTGAACAAAGTCGGGGA-5′	AP2
	TpS4p(+)188n	3′-TACGTCTCTTACGTTGTAGACGTGAAC-5′	AP2
2nd walking	TpS4p(−)685n	3′-ATACCCTACCCTTGGGAACATACGTAGAC-5′	AP1
	TpS4p(−)704n	3′-CGGGTGTTAGGTATAGTCATACCCTACC-5′	AP2
	TpS4p(−)714n	3′-GGAAAGTTTTTCGGGTGTTAGGTATAGTC-5′	AP2
3rd walking	TpS4p(−)1245n	3′-GGGGGGTTTATTAATGGGTACCGAAGT-5′	AP1
	TpS4p(−)1262n	3′-GTAGTTGGGTGTCTTTAGGGGGGTTTA-5′	AP2
	TpS4p(−)1276n	3′-GAGATATCTTGGAAGTAGTTGGGTGTC-5′	AP2
*TpDIR4*g			
1st walking	TpS10p(+)228n	3′-TCCCTCGGTTAGAGTGGTAATACTGAC-5′	AP1
	TpS10p(+)216n	3′-AACGTCGGGGACTCCCTCGGTTAGAGT-5′	AP2
	TpS10p(+)197n	3′-TTACGTTGTAGACGTGAACAACGTCGG-5′	AP2
2nd walking	TpS10p(+)93n	3′-TTTTGGACGTCTAACGGTACCTTCCTTC-5′	AP1
	TpS10p(+)53n	3′-TAATACGGAAGACACCGAAGACTACAG-5′	AP2
3rd walking	TpS10p(−)1187n	3′-TCACGGTTGAACAGGGTTTAGGTACATT-5′	AP1
	TpS10p(−)1198n	3′-ACGTTCAACATTCACGGTTGAACAGGGT-5′	AP2
	TpS10p(−)1210n	3′-TTCGAGTGTTGTACGTTCAACATTCACG-5′	AP2
*TpDIR5*g			
1st walking	CS1p(+)196n	3′-ACGTTGTAGACGTGAACAACGTCGGGGACT-5′	AP1
	CS1p(+)122n	3′-CGAAGGTTTCGGGACGTTCTTAGAACAC-5′	AP2
	CS1p(+)111n	3′-CGACCTTTTTCTTCGAAGGTTTCGGGACG-5′	AP2
2nd walking	CS1p(−)142n	3′-CAACCCCTACTCCTACTAAGTATGGGTG-5′	AP1
	CS1p(−)182n	3′-GTTTGGTTCTAGTTAAGAACGTGTTTAGGG-5′	AP2
	CS1p(−)220n	3′-GGGCATGATTGAGACTAGATAGGAGTTAT-5′	AP2
3rd walking	CS1p(−)685n	3′-TCACGGTTGAACAGGGTTTAGGTACATT-5′	AP1
	CS1p(−)704n	3′-ACGTTCAACATTCACGGTTGAACAGGGT-5′	AP2
	CS1p(−)714n	3′-TTCGAGTGTTGTACGTTCAACATTCACG-5′	AP2
*TpDIR8*g			
1st walking	TpS11p(+)58n	3′-AACACGTAAGACACCGAAGACCAGAGGT-5′	AP1
	TpS11p(+)46n	3′-TCTCAAGACTTAAACACGTAAGACACCG-5′	AP2
	TpS11p(+)22n	3′-ACGTGTTACCGTTAGACCTTACCTTCTC-5′	AP2
2nd walking	TpS11p(−)511n	3′-CTAAGTCACACACTAAGTGACTTACGAGGC-5′	AP1
	TpS1lp(−)550n	3′-GTGTGATTCTAAGTCACACACTAAGTGAC-5′	AP2
	TpS11p(−)562n	3′-CAGTAACGTCATGTGTGATTCTAAGTCAC-5′	AP2
*TpDIR9*g			
1st walking	RT-CS-C1(+)297n	3′-ATGAGAACTGTTGTTAGAAGTAAGA-5′	AP1
	RT-CS-C1(+)276n	3′-ACGACACAAACTACTAGGATAATGA-5′	AP2
	RT-CS-C1(+)255n	3′-TTGTTGGTAAAACCCTTATAACGA-5′	AP2

(*continued*)

Table 1 (*continued*)

Isovariant	Primer name	Primer sequences	Adapter primer
2nd walking	RT-CS-C1(−)1977n	3′-CTTTCATAGTAGGTAAAGTAGTACGAAC-5′	AP1
	RT-CS-C1(−)2060n	3′-CTAGAAATTCTACGAAACCTCATACCG-5′	AP2
	RT-CS-C1(−)2068n	3′-GTGATACTTCTAGAAATTCTACGAAACC-5′	AP2

"s" and "n" stand for sense and antisense oligonucleotides, respectively. "−" and "+" designate upstream and downstream. *TpDIR1*g stands for *Thuja plicata DIRIGENT* gene 1 genomic DNA clone for PSD_Tp1 cDNA; *TpDIR2*g for PSD_Tp2 cDNA; *TpDIR3*g for PSD_Tp3 cDNA; *TpDIR4*g for PSD_Tp4 cDNA; *TpDIR5*g for PSD_Tp5 cDNA; *TpDIR8*g for PSD_Tp8 cDNA; *TpDIR9*g for PSD_Tp9 cDNA.

search analysis (http://www.ncbi.nlm.nih.gov/BLAST/). The Expected (E) values computed from the analyses for the aligned sequences were greater than 4×10^{-4}, and hence were only of random statistical significance, indicating that the 5′ flanking sequences for the seven *DIR* isovariants were unique and did not share any substantial levels of homology to known promoter sequences.

2.2. Expression patterns of the western red cedar 8–8′ DIRIGENT multigene family in transgenic Arabidopsis

The study of gene expression at the organismal, tissue and cellular levels can conveniently be carried out using the promoter-GUS fusion strategy in transgenic plants such as *Arabidopsis* (An et al., 1996; Meagher et al., 1999). For the *DIRIGENT* multigene family, it is noteworthy that all members lack any introns. In this study, sets of more than 30 transgenic *Arabidopsis* plants, each housing an individual GUS-promoter construct, were examined, with each being derived through two consecutive generations (T_2). Although there was some small variability with respect to staining intensity and localization, each transformant displayed a very unique and consistent expression pattern as described below.

In order to obtain the *Arabidopsis* transformants, western red cedar *DIR* promoter-GUS reporter T-DNA constructs were first obtained through transcriptional fusion of the GUS-reporter gene to the 5′ region of each of the western red cedar *DIR*'s. Each was then individually used to transform *Agrobacterium* (strain GV 3101) via electroporation (McCormac et al., 1998), with subsequent transformation of *Arabidopsis* being achieved using the floral dip procedure (Clough and Bent, 1998). Transgenic plant selection employed the kanamycin resistance screening method and GUS-reporter visualization was carried out with T_2 plants.

The expression patterns of the seven promoters obtained from the western red cedar *DIR* family were individually examined at different developmental stages in the various organs of transgenic *Arabidopsis*. This included: 1–3 days (germinating seeds showing undifferentiated cotyledons and roots); 7–12 days (showing differentiated cotyledons and the two first emerging leaves); 3–6 weeks at different stages up to maturation, with the latter possessing completely emerged rosette leaves, inflorescences, flowers, and siliques. Histochemical analyses revealed very diverse and unique patterns of western red cedar *DIR* promoter-induced expression for each transformed *Arabidopsis* at all stages of its growth and development, this being in accordance with the presence of multidimensional networks differentially controlling monolignol radical–radical coupling (see Figs. 2–4). None displayed constitutive expression profiles. Transgenic plants harboring promoter less-GUS constructs, as well as wild type plants, were also assayed. However, none of these controls displayed GUS activity, even following a 2-day incubation with X-glu under the same assay conditions (data not shown).

The patterns of expression noted for each of the constructs at each developmental stage are shown in Figs. 2–4, and are summarized below.

2.2.1. TpDIR1gp::GUS construct

The *TpDIR1*gp::*GUS* construct was mainly expressed in the meristematic tissue of the undifferentiated *Arabidopsis* cotyledons and hypocotyls at 1–3 days growth (Fig. 2A and B). At 7 days growth, expression was predominant (Fig. 2C) in the vascular region of the cotyledon tissue, the upper part of the hypocotyl (meristematic region) and in the root tips, whereas by 12-days (Fig. 2D), it was strongest in the vascular tissue of the emerging leaf, as well as in the lateral roots and the root tip. Following 3–6 weeks of vegetative growth, the expression occurred throughout the entire stem (Fig. 3A) and mainly in the differentiated stem xylem bundle region (Fig. 3B), as well as being faintly detected in the basal region of the leaf vascular tissue (Fig. 3C). The reproductive tissue at plant maturity also displayed a different pattern of expression, this being localized throughout the stamen filament in the floral organs during flower development (from young unopened flowers to the post-anthesis stage, Fig. 4A), and in the vascular tissues of the silique (Fig. 4B).

2.2.2. TpDIR2gp::GUS construct

For 1–3 days growth, *TpDIR2*gp::*GUS* construct (Fig. 2E and F) was initially strongly expressed in the hypocotyl and radicle (including the hairs, but not the tip). Following 7 days growth, expression was detected in the vascular tissues of the hypocotyl, the meristematic region and in the cotyledon tip (Fig. 2G), but by 12 days, it was strongest in the emerging leaf trichomes and

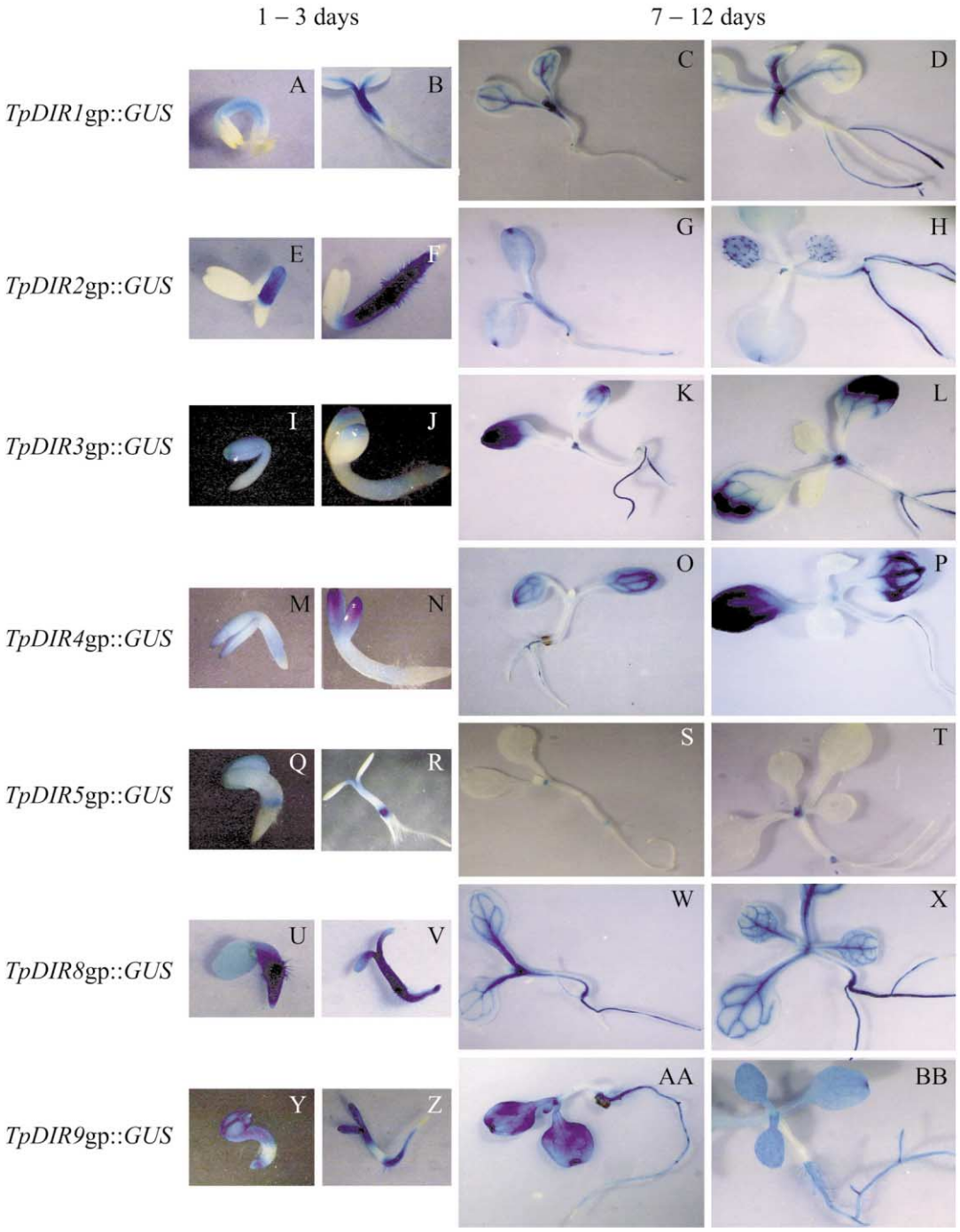

Fig. 2. Histochemical localization of GUS in 1–3 and 7–12 days old seedlings of transgenic *Arabidopsis* plants containing various western red cedar *DIR* promoter::*GUS* fusions. (A–D) *TpDIR1*gp::*GUS*; (E–H) *TpDIR2*gp::*GUS*; (I–L) *TpDIR3*gp::*GUS*; (M–P) *TpDIR4*gp::*GUS*; (Q–T) *TpDIR5*gp::*GUS*; (U–X) *TpDIR8*gp::*GUS*; (Y–BB) *TpDIR9*gp::*GUS*.

in the primary and lateral roots (Fig. 2H), but not in the root tips. By 3–6 weeks growth, however, expression was now restricted essentially to the trichomes in both stems (Fig. 3D and E) and leaves (Fig. 3F). Interestingly, during the early stages of elaboration of the reproductive tissues, expression was strongest in the trichomes of the sepals (Fig. 4C). At full maturity, however, expression was mainly noted in the floral organs (Fig. 4D and E), this including both the flower stalk and the gynoecium: in the latter, expression was strongest in the stigma and the papillar cells, the style and the upper part of the ovary. Overall, expression levels became more intense as flower development progressed, i.e., from the young unopened flowers to the post-anthesis stage (data not shown). Lastly, in the siliques (Fig. 4F) expression was associated mainly with the epidermal layers, and the abscission zone.

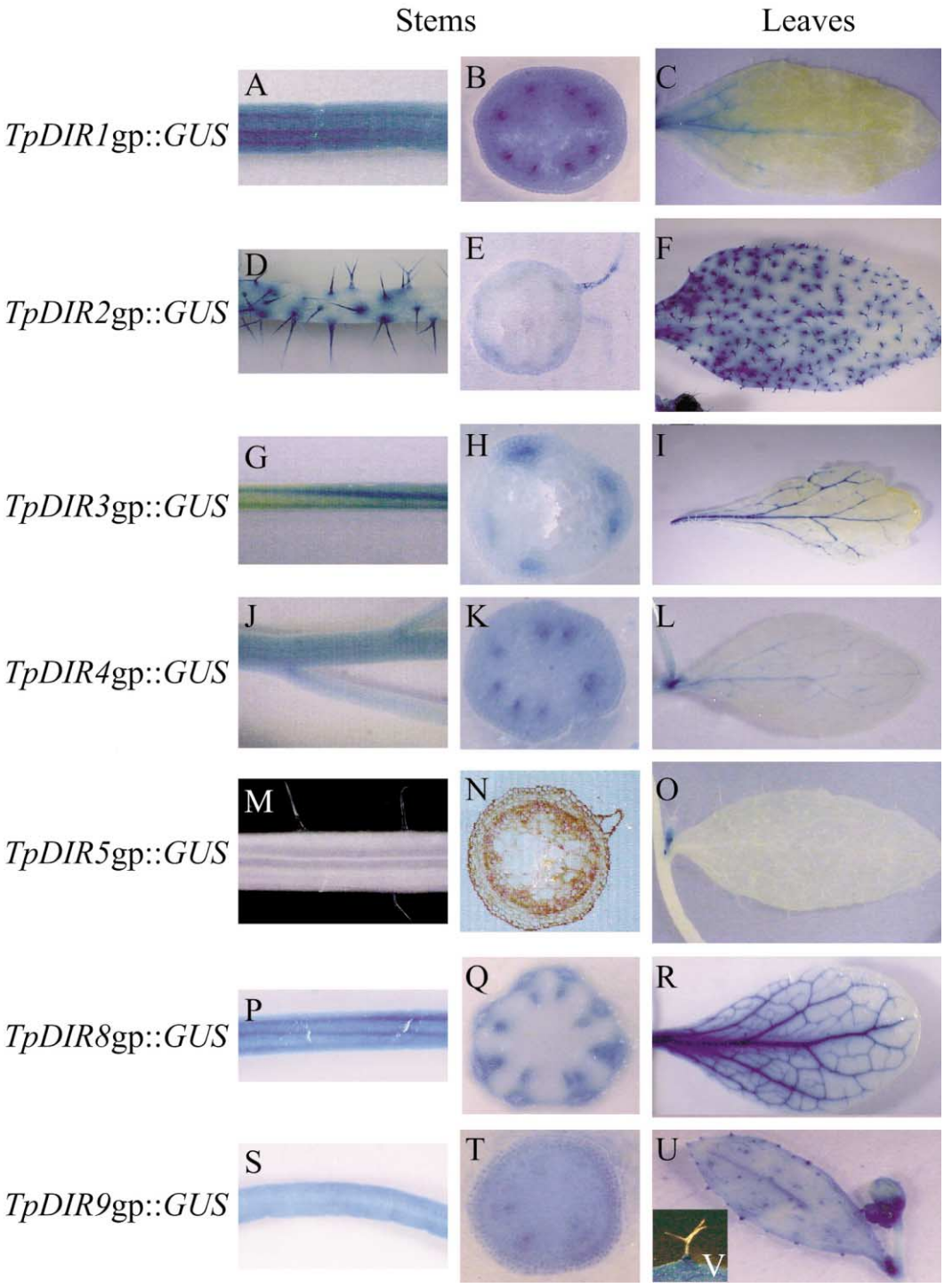

Fig. 3. Histochemical localization of GUS in 3–6 weeks old stems and leaves of transgenic *Arabidopsis* plants containing various western red cedar *DIR* promoter::*GUS* fusions. (A–C) *TpDIR1*gp::*GUS*; (D–F) *TpDIR2*gp::*GUS*; (G–I) *TpDIR3*gp::*GUS*; (J–L) *TpDIR4*gp::*GUS*; (M–O) *TpDIR5*gp::*GUS*; (P–R) *TpDIR8*gp::*GUS*; (S–V) *TpDIR9*gp::*GUS*.

2.2.3. *TpDIR3gp::GUS construct*

The *TpDIR3*gp::*GUS* construct displayed a third type of expression pattern. For 1–3 days of growth (Fig. 2I and J), moderate expression was observed in the terminal region of the undifferentiated cotyledons, the hypocotyl and the radicle (except the root hairs and tip). Following 7–12 days growth (Fig. 2K and L), however, intense GUS expression was evident in the vascular region of the cotyledons, the meristematic region and the root (but not the root tip). GUS expression patterns were similar at days 7 and 12, although it was noteworthy that the two emerging leaves at day 12 lacked

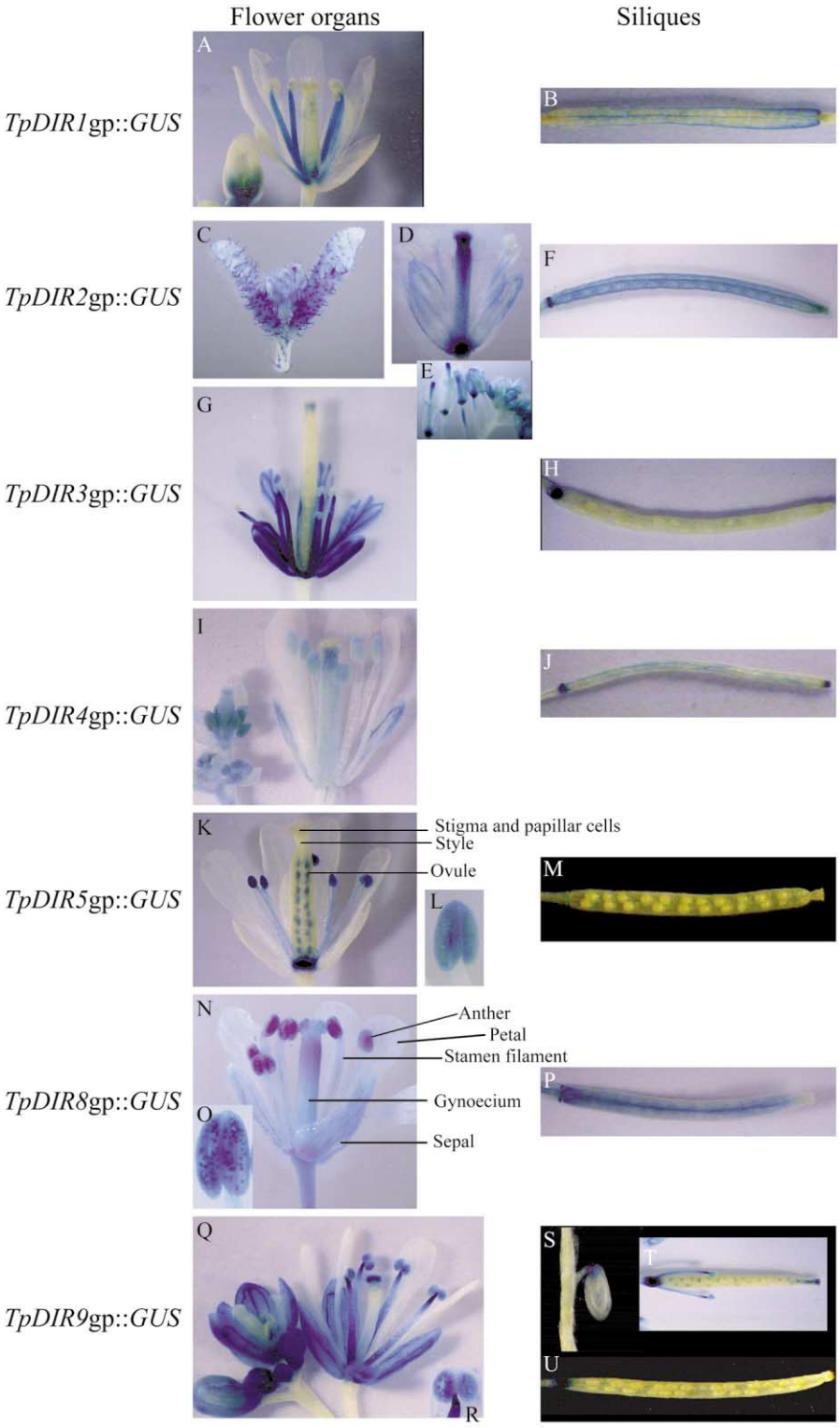

Fig. 4. Histochemical localization of GUS in flowers and siliques of transgenic *Arabidopsis* plants containing various western red cedar *DIR* promoter::*GUS* fusions. (A and B) *TpDIR1*gp::*GUS*; (C–F) *TpDIR2*gp::*GUS*; (G and H) *TpDIR3*gp::*GUS*; (I and J) *TpDIR4*gp::*GUS*; (K–M) *TpDIR5*gp::*GUS*; (N–P) *TpDIR8*gp::*GUS*; (Q–U) *TpDIR9*gp::*GUS*.

observable GUS staining (Fig. 2L). From 3 to 6 weeks, GUS expression was mainly observed throughout the differentiated phloem bundle region of the stem sections (Fig. 3G and H), as well as being restricted to the (primary) veins of the leaf tissue (Fig. 3I). At plant maturity, expression was noted in the floral organs (Fig. 4G), where moderate to high levels of GUS staining were observed in each of the four whorls with different patterns

of expression being noted in the sepals, petals, stamens and gynoecium. Throughout the sepals, GUS expression was at a significant level including in its vascularization. A similar trend was observed with the petal vascular tissue and the stamen filament (not the anther), whereas in the gynoecium, it was apparently restricted to the style region. On the other hand, in the silique expression was restricted to the receptacle (Fig. 4H).

2.2.4. *TpDIR4gp::GUS construct*

Moderate expression levels were initially restricted to the terminal region of the undifferentiated cotyledons at 1–3 days growth, (Fig. 2M and N). By 7–12 days growth, expression was now mainly present in the vascular region of the cotyledon and weaker in the root (but again not the root tip) (Fig. 2O and P). From 3 to 6 weeks, however, only faint expression levels were observed throughout the entire stem (Fig. 3J), this occurring mainly in the differentiated stem xylem bundle region (Fig. 3K). Very low levels of expression were observed in the vein of the leaf; by contrast, it was stronger in the corresponding meristematic region (Fig. 3L). At plant maturity, *TpDIR4*gp::*GUS* expression was again noted in the floral organs (Fig. 4I), albeit only as faint expression in the sepals, anthers and style. Note, however, that while different patterns were observed in these organs, the staining intensity decreased with flower maturation. Lastly, in the silique (Fig. 4J), expression was restricted to the abscission and stigmatic regions with a very faint staining in the epidermis.

2.2.5. *TpDIR5gp::GUS construct*

*TpDIR5*gp::*GUS* expression was very precisely localized to the hypocotyl-root transition zone and to the shoot meristems from 1–12 days growth (Fig. 2Q–T). From 3 to 6 weeks, only near background levels were noted in the stem sections (Fig. 3M and N), with a higher level of detectable expression being restricted to the corresponding leaf meristematic region (Fig. 3O). At full maturity, in the floral organs (Fig. 4K), a striking pattern of expression was observed in the flower stalk regions, the stamens (filament and anther tissue, but not the pollen, Fig. 4L), and the ovules. On the other hand, the silique was essentially devoid of any GUS expression except for perhaps a very faint level in the abscission zone (Fig. 4M).

2.2.6. *TpDIR8gp::GUS construct*

Of all of the constructs, the *TpDIR8*gp::*GUS* construct was expressed strongest in the vascular apparatus. Following 1–3 days growth (Fig. 2U and V), intense staining was observed in the vascular tissues of the hypocotyl, as well as in the roots and root hairs. By 7–12 days (Fig. 2W and X), an intense level of staining was also observed in the differentiating vascular regions of the cotyledon and the leaf, as well as in the hypocotyl, and in both the main and lateral roots. From 3 to 6 weeks, *TpDIR8*gp::*GUS* was expressed in the stem (Fig. 3P) and most strongly in the differentiated xylem and phloem bundles of the stems (Fig. 3Q). Very high levels of expression were evident throughout the leaf vasculature (Fig. 3R), including both primary and secondary veins. At full maturity, in the floral organs (Fig. 4N), faint expression was localized in the vascular tissue of the sepal and the stamen filament throughout flower development, as well as in the gynoecium (i.e., stigma, style and upper part of the ovary). On the other hand, a significant level of expression was detected in the pollen (Fig. 4O), whereas in the silique staining was evident throughout the lignified partition wall between the 2 valves of the silique (Fig. 4P).

2.2.7. *TpDIR9gp::GUS construct*

At 1–3 days growth, *TpDIR9*gp::*GUS* was expressed in the cotyledon, hypocotyl and radicle but not at the hypocotyl/radicle transition zone (Fig. 2Y and Z). From 7–12 days growth, staining patterns were fairly uniform, albeit decreasing in overall intensity with increasing age, however, staining was absent in the hypocotyl (Fig. 2AA and BB). From 3 to 6 weeks, only faint expression was observed throughout the stem (Fig. 3S and T). In the leaf tissue, GUS activity was mainly associated with the meristematic region (Fig. 3U) and the epidermal cells forming the base of the trichomes (Fig. 3V). At maturity (Fig. 4Q), however, a high level of expression was observed in the flower stalk region, the sepal, the stamen filament, anther (including the pollen, Fig. 4R) and the stigma except in the papillar cells. *TpDIR9*gp::*GUS* was also expressed at the funicle and ovule junction (Fig. 4S) as well as in the style and receptacle (Fig. 4T). However, at later stages of silique maturation, the labeling was restricted to the receptacle (Fig. 4U).

3. Discussion

The expression patterns of the *DIR-GUS* fusions provide evidence for the existence of a multidimensional network involved in control/regulation of monolignol radical–radical coupling in *Arabidopsis,* throughout its entire growth and development. This was particularly interesting given that the corresponding promoters had been obtained from the much more ancient gymnosperm, western red cedar, thereby suggesting that such networks have long been in existence. This, in turn, implies that these control systems are very highly conserved throughout the vascular plant kingdom. At the 1–3 days growth stage of *Arabidopsis* (see Fig. 2, 1st two columns), while there was clearly some overlap in staining (e.g. in the meristematic region) between the various *DIR-GUS* constructs such as *TpDIR1*gp::*GUS*, *TpDIR*gp::*GUS* and *TpDIR8*gp::*GUS* the overall patterns were

spatially and temporally distinct. This became even more evident by 7–12 days growth (Fig. 2, columns 3 and 4), for example, *TpDIR2*gp::*GUS* was mainly trichome and root specific, whereas *TpDIR8*gp::*GUS* was strongly expressed throughout the vasculature apparatus and *TpDIR5*gp::*GUS* was only associated with the hypocotyl-root transition zone and the developing shoot meristem.

These unique patterns were also maintained at further maturation stages of the plant (3–6 weeks), with each again showing quite distinct temporal and spatial staining. For example, *TpDIR1*gp::*GUS*, *TpDIR4*gp::*GUS* and *TpDIR8*gp::*GUS* displayed rather distinct patterns of expression within the vasculature apparatus in the stems (Fig. 3B, K and Q), and this was further underscored in the leaf tissue (Fig. 3C, L, and R). On the other hand, *TpDIR2*gp::*GUS* and *TpDIR9*gp::*GUS* were mainly associated with trichome development, albeit in distinct cellular regions (see Fig. 3F, U and V). *TpDIR3*gp::*GUS* and *TpDIR8*gp::*GUS* also gave staining in the phloem regions (Fig. 3H and Q), but the corresponding expression in the leaf tissue was quite different being restricted to the primary veins (*TpDIR3*gp::*GUS*) and primary and secondary veins (*TpDIR8*gp::*GUS*).

The corresponding different patterns of expression were even more dramatic in the reproductive tissues (floral organs and siliques). For instance, *TpDIR1*gp::*GUS* and *TpDIR8*gp::*GUS* gave opposite staining profiles in the flowers: intense in the stamen filament, none in the anther (*TpDIR1*gp::*GUS*, Fig. 4A), whereas *TpDIR8*gp::*GUS* (Fig. 4N) expression was weak in the stamen filament and strong in the pollen grain; this is most interesting in regard to male sterility. Furthermore, in the silique (Fig. 4P) labeling of *TpDIR8*gp::*GUS* was associated with the wall separating the 2 valves, an area considered to be lignified. *TpDIR2*gp::*GUS* (Fig. 4D) was strongly associated with the gynoecium whereas no staining was observed in this organ with *TpDIR3*gp::*GUS* (Fig. 4G) where, in contrast, intense staining was detected in the sepals, petals, and stamen filaments. *TpDIR5*gp::*GUS* (Fig. 4K) was found both in the male (anthers) and female (ovaries) reproductive organs.

Thus, taken together, these data clearly support the existence of sophisticated multidimensional networks involved in monolignol radical–radical coupling. This in turn raises important questions about the nature of the corresponding transcription factor binding elements. Unfortunately, at this point, nothing is yet known about specific transcription factor binding elements that are involved in regulation and control of monolignol radical–radical coupling. This will, however, be the subject of future study.

Clearly there remains much to be done in unraveling how the proposed monolignol radical–radical coupling networks operate and are controlled, particularly since angiosperm morphology, growth and development differs substantially from that of gymnosperms. Yet such networks can be expected to be substantially highly conserved throughout evolution: indeed, when the promoters of ribulose-1,5-bisphosphate carboxylase from larch (*Larix laricina*) (Campbell et al., 1994), and an caffeoyl CoA *O*-methyltransferase (CCOMT) from loblolly pine (*Pinus taeda*) (Li et al., 1999) were individually linked to the GUS gene and transformed into tobacco, the patterns of expression observed in both cases were as for that in larch and loblolly pine, respectively, i.e. indicating that both promoters displayed conservation of expression patterns in both angiosperms and gymnosperms. Furthermore, since the *Arabidopsis thaliana* genome sequencing (The Arabidopsis Genome Initiative, 2000) has recently revealed the presence of some 16 dirigent protein homologues, experiments can now be designed to establish their individual expression patterns and to correlate these with the radical–radical coupling networks observed with the western red cedar dirigent multigene family. This is presumed to have important evolutionary consequences: the corresponding dirigent proteins appear to have only evolved a function with the adaptation to land by plants. Accordingly, since monolignol coupling to the lignans and lignins is highly conserved throughout the vascular plant kingdom, defining how these networks are differentially controlled should be most instructive.

4. Experimental

4.1. Plant materials

Western red cedar (*T. plicata*) juvenile (~2 years old) plants were maintained in Washington State University greenhouses. For *Arabidopsis* transformations, ecotype Columbia plants were grown to the flowering stage in a growth chamber at 24 °C day/22 °C night under a 16-h photoperiod.

4.2. Isolation of western red cedar DIRIGENT cDNA and genomic clones

For isolation of *T. plicata DIRIGENT* genomic DNA, genomic DNA was first purified from emerging shoots of 2 years old western red cedar trees using the procedure of Lassner et al. (1989). Genomic DNA clones (*TpDIR 1, 2, 3, 4, 5, 8*, and *9*g) from the corresponding dirigent cDNAs (PSD_Tp1, 2, 3, 4, 5, 8, and 9) (Gang et al., 1999; Kim et al., 2002b) were isolated by deployment of the Universal GenomeWalker™ Kit (Clonetech, Palo Alto, CA): *TpDIR1*g stands for *Thuja plicata DIRIGENT* gene *1* genomic DNA clone for PSD_Tp1 cDNA (GenBank accession no. AF210063); *TpDIR2*g for

PSD_Tp2 cDNA (GenBank accession no. AF210064); *TpDIR3*g for PSD_Tp3 cDNA (GenBank accession no. AF210065); *TpDIR4*g for PSD_Tp4 cDNA (GenBank accession no. AF210066); *TpDIR5*g for PSD_Tp5 cDNA (GenBank accession no. AF210067); *TpDIR8*g for PSD_Tp8 cDNA (GenBank accession no. AF210068) and *TpDIR9*g for PSD_Tp9 cDNA (GenBank accession no. AF487405). The genomic DNA was digested separately with either *Dra*I, *Eco*RV, *Pvu*II, *Sca*I, or *Stu*I, and ligated to a GenomeWalker Adaptor (5′-GTAATACGACTCACTATAGGGCACGCGTGG TCGACGGCCCGGGCTGGT-3′). Anchor ligated genomic DNA fragments were amplified using an Expand™ High Fidelity PCR system (Boehringer Mannheim, Indianapolis, IN) with adaptor primers (AP1 and AP2; Clonetech, Palo Alto, CA) and gene specific primers (see Table 1) designed from the corresponding cDNA sequences. PCR was consecutively performed to the 2nd or 3rd round using adaptor primers and a set of three nested primers (see Table 1) under the following conditions: 1st cycle at 94 °C (1 min); 2nd–35th cycles at 94 °C (30 s), 60 °C (45 s), 72 °C (3 min), and finally a 36th cycle at 72 °C (7 min). The amplified fragments were individually gel purified, cloned into a TA cloning vector (Invitrogen, Carlsbad, CA) and sequenced. In order to confirm that the aligned sequences from the partial genome walker sequences were correct, the entire aligned sequences were amplified with gene specific primers (Table 2) via application of an Expand™ High Fidelity PCR system (Boehringer Mannheim, Indianapolis, IN) using undigested *T. plicata* genomic DNA, and further sequenced.

4.3. Western red cedar DIRIGENT promoter-GUS reporter constructs

The putative promoter regions of 5′ flanking sequences of seven different isovanants (~2.2 kb for *TpDIR8*gp; 2 kb for *TpDIR1*gp, *TpDIR2*gp, *TpDIR3*gp, and *TpDIR4*gp; ~1.6 kb for *TpDIR9*gp, and ~0.8 kb for *TpDIR5*gp) were individually amplified using primers (see Table 3) containing the requisite restriction enzyme sites. [*TpDIR1*gp stands for the promoter region of the *Thuja plicata DIRIGENT 1* genomic DNA and so on.] The amplified PCR products so obtained for each isovariant promoter region were digested and ligated into appropriate restriction sites of digested pBI 101.1 and/or pBI 101.3 (Jefferson et al., 1987) for transcriptional fusion to the GUS reporter, thereby generating the corresponding western red cedar *DIR* promoter::*GUS*

Table 2
Oligonucleotides used in PCR for the amplification of *DIRIGENT* genomic DNA

Isovariant	Primer name	Primer sequences	Comments
*TpDIR1*g	CS2(−)491s	5′-TCCGGGGAATCTATTGCTCATATGAGGCCA-3′	Amplification of dirigent gene coding region from genomic DNA
	CS2(+)746n	5′-ACTCGTAGAGTGTGATGTTAACCCTCACGA-3′	
*TpDIR2*g	TpS32(−)273s	5′-CATGTGCACCAACAGTCCCATGCAATAT-3′	
	TpS32(+)701n	5′-CTCATCCCTTACCTCTTATTTTCCTGCACCT-3′	
*TpDIR3*g	TpS4(−)373s	5′-TCATCTTCCACCATGTTGAGCTGACAGA-3′	
	TpS4(+)637n	5′-GGTCCCAAAAACTAGAAATACAGAGCAAGG-3′	
*TpDIR4*g	TpS10(−)316s	5′-TGCCTATGAAAGCCGTGAGGAAATCTCCA-3′	
	TpS10(+)653n	5′-GCCCTAGACATTGAACCAGGGAATGACT-3′	
*TpDIR5*g	CS1(−)384s	5′-TGGATTGGGAAATCCGATCGATTTAACA-3′	
	CS1(+)657n	5′-AAGCCAAGAACTATTCCTCAAAGGCACCCA-3′	
*TpDIR8*g	TpS11(−)486s	5′-CCATCTGTCATACTCATTGGAGGGCCAT-3′	
	TpS11(+)720n	5′-CTGTGCATGAGCACCCAAGTTTTCAATG-3′	
*TpDIR9*g	RT(−)268s	5′-TCAAACCCTTGGCTCTATGATAGATCCAA-3′	
	RT(+)666n	5′-ATGCCCTCAAAAGTCAGAGCTAGTCCCA-3′	
*TpDIR1*g	Cs2p(+)61n	5′-TTCTGAGCACCGTGGAAGAGAGCAGAA-3′	Amplification of 5′ flanking region of putative promoter region from genomic DNA
	Cs2p2ks	5′-GTTGAGGGTGTGGGATTGCATTATTTCC-3′	
*TpDIR2*g	TpS32p2.5ks	5′-TCTTGGTGGCACCCTATAGACACTGGA-3′	
	TpS32(+)83n	5′-CAGTCTGCAGATTTGAGCAACACAGTG-3′	
*TpDIR3*g	TpS4p2ks	5′-CATAAGTGCAGGACTGACCCAGATTCACA-3′	
	TpS4p(+)97n	5′-CCAGCTACGGCAATCTGCAGATTTTATG-3′	
*TpDIR4*g	TpS10p3ks	5′-TGCGCCCTGTGAAATAACCTCTGAGAT-3′	
	TpS10(+)93n	5′-CTTCCTTCCATGGCAATCTGCAGGTTT-3′	
*TpDIR5*g	Cs1p1ks	5′-GGGATCACAACTATTAGAATGTGCCCAA-3′	
	Cs1p(+)61n	5′-GCGAGATTGCAGACACTAGAAGACATAGA-3′	
*TpDIR8*g	TpS11p3ks	5′-ACTAGTAACGGCCGCCAGTGTGCTGGAA-3′	
	TpS11(+)57n	5′-GGAGACCAGAAGCCACAGAATGCACAA-3′	
*TpDIR9*g	RTp2ks	5′-GTGGTCGATGGCCCGGGCTGGTAAAAT-3′	
	RTp(+)54n	5′-GGAGACCAGAAGCCACAGAATGCACAA-3′	

For legend see Table 1.

Table 3
Oligonucleotides used in PCR for the amplification of promoter region with synthetic restriction sites for GUS fusion

Isovariant	Primer name	Primer sequences
*TpDIR1*g	Cs2p2k5′HindIII	5′-CTAATAGGGCC*AAGCTT*TTTCATTTTT-3′
	Cs2p3′SalI	5′-CTACTCAT*GTCGAC*TGAAATCCCTCGT-3′
*TpDIR2*g	TpS32p2k5′SalI	5′-CCTTAATTAGGAG*GTCGAC*ATGGAGAA-3′
	TpS32p3′BamHI	5′-TGTGCCAT*GGATCC*AAAAGAAGGAGAAA-3′
*TpDIR3*g	TpS4p2k5′SalI	5′-GAAGCAT*GTCGAC*ATTAATTTCACTTTT-3′
	TpS4p3′SmaI	5′-GTAAGCC*CCCGGG*AAAAAATATATGGAAA-3′
*TpDIR4*g	TpS10p2k5′HindIII	5′-GGATCA*AAGCTT*CCTTGGGATTTGTCA-3′
	TpS10p3′BamHI2	5′-CATTGGG*GGATCC*AACTATGCAAATCTCT-3′
*TpDIR5*g	Cs1p1kSalI5′	5′-GCCCTTGGG*GTCGAC*ACTATTAGAATGTGC-3′
	Cs1p1kSmaI3′	5′-GCTTTCATTGC*CCCGGG*TAAAAAATTATTGG-3′
*TpDIR8*g	TpS11p2.6kSalI5′	5′-GACATA*GTCGAC*CTGCAAGACAAGGGAATATT-3′
	TpS11p2.6kSmaI3′	5′-CCAGATTG*CCCGGG*TGCAAAATATATCAAA-3′
*TpDIR9*g	RTp1.6kSalI5′	5′-GAATTCGCCC*GTCGAC*ACACATGCACAACTTCT-3′
	RTp1.6kSmaI3′	5-CAGATTG*CCCGGG*GTGCAAAATAAATCAGAAA-3′

For legend see Table 1. The italic sequences are synthetic restriction sites for GUS fusion.

constructs: pBI-*TpDIR1*gp::*GUS*; pBI-*TpDIR2*gp::*GUS*; pBI-*TpDIR3*gp::*GUS*; pBI-*TpDIR4*gp::*GUS*; pBI-*TpDIR5*gp::*GUS*; pBI-*TpDIR8*gp::*GUS* and pBI-*TpDIR9*gp::*GUS*.

4.4. Transformation and screening of transgenic plants

Agrobacterium strain GV3 101 was individually transformed by electroporation (McCormac et al., 1998) with each T-DNA construct containing a western red cedar *DIR* promoter-GUS reporter. Ecotype Columbia plants, grown to the flowering stage, were next used for transformations using the floral dip procedure described by Clough and Bent (1998). Seeds from each of the resulting T_0 plants were harvested and germinated on medium containing kanamycin (100 mg/l) and carbenicillin (100 mg/l) to identify T_1 transgenic plants. After 2–3 weeks on selection medium, the kanamycin-resistant plants (T_1) were transferred to soil and grown to set seeds (T_2) in a growth chamber at 22 °C under continuous light.

4.5. Histochemical GUS analyses

Histochemical analyses of T_2 plant tissues of transgenic *Arabidopsis* for GUS activity were conducted following the procedures described by Jefferson et al. (1987) with the following modifications: Fresh plant tissues were immediately immersed in GUS staining solution containing 0.25 mM X-glu (GBT, St. Louis, MO) in 50 mM sodium phosphate buffer, pH 7.0, this being vacuum infiltrated for 10 mm and incubated for 5–24 h at 37 °C. The resulting plant tissues were next bleached several times by washing with 70% ethanol in H_2O (v/v). Stained tissues were analyzed under a dissecting microscope (Wild, Heerbrugg, Switzerland) with photographs taken using a Micro Image Video System (Kim et al., 2002a) at Washington State University Electron Microscopy Center.

Acknowledgements

The authors thank Professor Vincent R. Franceschi (Director of the Electron Microscopy Center, Washington State University) for helpful discussions and use of the facilities, as well as Christine M. Davitt and Valerie J. Lynch-Holm (EM Center, Washington State University) for technical assistance. We gratefully acknowledge the United States Department of Energy (DEFG03-97ER20259), the National Science Foundation (MCB-9976684), the National Aeronautics and Space Administration (NAG21198), the United States Department of Agriculture (9603622), and the Lewis B. and Dorothy Cullman and G. Thomas Hargrove Center for Land Plant Adaptation Studies for generous support of this and related studies.

References

An, Y.Q., Huang, S., McDowell, J.M., McKinney, E.C., Meagher, R.B., 1996. Conserved expression of the *Arabidopsis ACT1* and *ACT3* actin subclass in organ primordia and mature pollen. Plant Cell 8, 15–30.

Barton, G.M., MacDonald, B.F., 1971. The chemistry and utilization of western red cedar. Canadian For. Ser. Pub. 1023, 1–31.

Burlat, V., Kwon, M., Davin, L.B., Lewis, N.G., 2001. Dirigent proteins and dirigent sites in lignifying tissues. Phytochemistry 57, 883–897.

Campbell, M.A., Neale, D.B., Harvie, P., Hutchison, K.W., 1994. Tissue-specific and light regulation of a larch ribulose-1,5-bisphosphate carboxylase promoter in transgenic tobacco. Can. J. For. Res. 24, 1689–1693.

Clough, S.J., Bent, A.F., 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16, 735–743.

Davin, L.B., Lewis, N.G., 2000. Dirigent proteins and dirigent sites explain the mystery of specificity of radical precursor coupling in lignan and lignin biosynthesis. Plant Physiol. 123, 453–461.

Davin, L.B., Wang, H.-B., Crowell, A.L., Bedgar, D.L., Martin, D.M., Sarkanen, S., Lewis, N.G., 1997. Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science 275, 362–366.

Gang, D.R., Costa, M.A., Fujita, M., Dinkova-Kostova, A.T., Wang, H.-B., Burlat, V., Martin, W., Sarkanen, S., Davin, L.B., Lewis, N.G., 1999. Regiochemical control of monolignol radical coupling: a new paradigm for lignin and lignan biosynthesis. Chem. Biol. 6, 143–151.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W., 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6, 3901–3907.

Kim, M.K., Choi, J.-W., Jeon, J.-H., Franceschi, V.R., Davin, L.B., Lewis, N.G., 2002a. A simple specimen block staining protocol for localization of GUS expression in transgenic arabidopsis and tobacco. Plant Cell Rep. 21, 35–39.

Kim, M.K., Jeon, J.-H., Fujita, M., Davin, L.B., Lewis, N.G., 2002b. The western red cedar (*Thuja plicata*) 8–8′ *DIRIGENT* family displays diverse expression patterns and conserved monolignol coupling specificity. Plant Mol. Biol. 49, 199–214.

Lassner, M.W., Peterson, P., Yoder, J.I., 1989. Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. Plant Mol. Biol. Rep. 7, 116–128.

Lewis, N.G., Davin, L.B., 1999. Lignans: biosynthesis and function. In: Barton, Sir D.H.R., Nakanishi, K., Meth-Cohn, O. (Eds.), Comprehensive Natural Products Chemistry, Vol. 1. Elsevier, London, pp. 639–712.

Li, L., Osakabe, Y., Joshi, C.P., Chiang, V.L., 1999. Secondary xylem-specific expression of caffeoyl-CoA 3-*O*-methyltransferase plays an important role in the methylation pathway associated with lignin biosynthesis in loblolly pine. Plant Mol. Biol. 40, 555–565.

McCormac, A.C., Elliott, M.C., Chen, D.F., 1998. A simple method for the production of highly competent cells of *Agrobacterium* for transformation via electroporation. Mol. Biotechnol. 9, 155–159.

Meagher, R.B., McKinney, E.C., Kandasamy, M.K., 1999. Isovariant dynamics expand and buffer the responses of complex systems: the diverse plant actin gene family. Plant Cell 11, 995–1005.

The Arabidopsis Genome Initiative, 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408, 796–815.

van Heerden, P.S., Towers, G.H.N., Lewis, N.G., 1996. Nitrogen metabolism in lignifying *Pinus taeda* cell cultures. J. Biol. Chem. 271, 12350–12355.

Wang, C.-Z., Davin, L.B., Lewis, N.G., 2001. Stereoselective phenolic coupling *in Blechnum spicant*: formation of 8-2′ linked (−)-*cis*-blechnic, (−)-*trans*-blechnic and (−)-brainic acids. J. Chem. Soc. Chem. Commun. 113–114.