# A RING Domain Gene Is Expressed in Different Cell Types of Leaf Trace, Stem, and Juvenile Bundles in the Stem Vascular System of Zinnia[1]

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The in vitro zinnia (*Zinnia elegans*) mesophyll cell system, in which leaf mesophyll cells are induced to transdifferentiate into tracheary elements with high synchrony, has become an established model for studying xylogenesis. The architecture of the stem vascular system of zinnia cv Envy contains three anatomically distinct vascular bundles at different stages of development. Juvenile vascular strands of the subapical region develop into mature vascular strands with leaf trace segments and stem segments. Characteristic patterns of gene expression in juvenile, leaf trace, and stem bundles are revealed by a molecular marker, a RING domain-encoding gene, *ZeRH2.1*, originally isolated from a zinnia cDNA library derived from differentiating in vitro cultures. Using RNA in situ hybridization, we show that *ZeRH2.1* is expressed preferentially in two specific cell types in mature zinnia stems. In leaf trace bundles, *ZeRH2.1* transcript is abundant in xylem parenchyma cells, while in stem bundles it is abundant in phloem companion cells. Both of these cell types show wall ingrowths characteristic of transfer cells. In addition, *ZeRH2.1* transcript is abundant in some phloem cells of juvenile bundles and in leaf palisade parenchyma. The complex and developmentally regulated expression pattern of *ZeRH2.1* reveals heterogeneity in the vascular anatomy of the zinnia stem. We discuss a potential function for this gene in intercellular transport processes.

Modern dicot angiosperms possess a highly evolved vascular system that is periodically renewed and augmented by the activity of cambial cells (Foster and Gifford, 1959). The vascular system is patterned early in embryo development as a central cylinder, with a central core of cambium, surrounded by phloem and xylem. After germination, roots maintain the vasculature as a central strand, but, in the stem, the central vascular cylinder differentiates as a fixed number of vascular strands. This species-specific base number of vascular strands is then maintained by the plant as the minimum number of vascular strands (Esau, 1962). The arrangement of vascular strands is further elaborated by branching and anastomosis to supply lateral organs, like leaves and axillary branches.

The postembryonic development of the stem vascular system results in an anatomically complex structure. Procambial strands at the apical meristem form protoxylem and protophloem elements of young bundles, which are gradually displaced by metaxylem and metaphloem as each stem segment matures. The anatomy of a mature bundle is, therefore, different from that of a young bundle (Esau, 1962). Partially occluded tracheids of protoxylem are displaced by the larger open vessels of metaxylem in the main stem, except in the vein supplies to leaves, in which tracheids are maintained (Eames and MacDaniels, 1925). Metaphloem contains companion cells and sieve elements to improve transport efficiency and fibers to provide strength.

The neighboring parenchyma and companion cells of the conducting elements in the shoot also differentiate. These cells frequently develop wall ingrowths, characteristic of transfer cells. Xylem parenchyma of the leaf traces develop extensive wall ingrowths in the upper internode region. Phloem companion cells develop transfer cells in the nodal complex and are mostly associated with vascular connections to the axillary bud (Gunning et al., 1970).

Although the anatomy of the stem vascular system has been meticulously studied, the molecular genetics underpinning its inherent heterogeneity is largely mysterious. In this article, we show how the dynamic nature of vascular development is revealed by the complex expression pattern of the *ZeRH2.1* gene.

The *ZeRH2.1* gene fragment was isolated during a complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) analysis (Milioni et al., 2002) of the time course of differentiation to tracheary elements in the zinnia (*Zinnia elegans* cv Envy) mesophyll cell system. In the zinnia system, about 60% of freshly isolated leaf mesophyll cells transdifferentiate into tracheary elements upon induction by exogenous

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auxin and cytokinin (Fukuda and Komamine, 1980). We obtained a full-length cDNA sequence for *ZeRH2.1* using a cDNA library derived from the in vitro system. The *ZeRH2.1* gene is a member of the large RING domain-containing family (Kosarev et al., 2002) that in plants comprises nearly 400 genes, very few members of which have been characterized. RING domains are defined by a conserved pattern of Cys and His residues capable of coordinating two atoms of zinc within a characteristic cross-brace structure. Various RING proteins show binding to E2 ubiquitin-conjugating enzyme, are known to mediate protein-protein interactions, and are reported to be involved in a wide range of cellular processes, including development, oncogenesis, apoptosis, and viral replication (Saurin et al., 1996; Borden, 2000). At the molecular level, the domains are involved in a variety of functions, from transcription and RNA processing to organelle transport and peroxisomal biogenesis (Borden, 1998). Only a handful of the plant genes with RING domains have been studied in any depth (Kosarev et al., 2002), *COP1* being probably the best known (Torii et al., 1999).

In this article, we have integrated a detailed anatomical analysis of the zinnia vasculature with gene expression analysis to explain the complex in situ pattern of *ZeRH2.1*. We describe how the development of the stem vascular system results in differential expression patterns of the *ZeRH2.1* gene in anatomically distinct vascular bundles. We propose a role for the *ZeRH2.1* gene in intercellular transport processes and in transfer cell function.

## RESULTS

### Expression Pattern of *ZeRH2.1* during Formation of Tracheary Elements in the Zinnia System

Using a cDNA-AFLP approach, Milioni et al. (2002) isolated more than 600 transcript-derived fragments that showed differential expression during tracheary element formation in vitro. One of these was a 120-bp-long transcript-derived fragment with sequence similarity to RING domain-containing proteins of Arabidopsis (*Arabidopsis thaliana*). The corresponding full-length zinnia cDNA was obtained by RACE-PCR, using an adapter-ligated cDNA library prepared from zinnia cells differentiating in vitro. We obtained a 1,592-bp-long sequence that had an open reading frame of 1,387 bp with a RING domain at the 3′ end (accession no. AJ867840). RING domains have characteristic conserved sequences of eight metal-ligand residues that include either one or two His (Borden, 2000). As the zinnia cDNA sequence contains the latter, we named the gene *ZeRH2.1* (Zinnia gene with RING H2 domain, 1). The C terminus of the putative ZeRH2.1 protein contained a conserved domain of unknown function and a RING domain (Fig. 1A), and showed 65% identity at the amino acid level to the translated protein sequence of an Arabidopsis gene *At4g34040*. According to a classification of RING domain-containing Arabidopsis genes (Kosarev et al., 2002), *At4g34040* belongs to cluster 2.3 containing 11 genes. ClustalW alignment of putative protein sequences for the Arabidopsis genes in cluster 2.3 and *ZeRH2.1* (Fig. 1B) shows sequence conservation is limited to the C terminus only. A phylogenetic tree constructed using putative protein sequences of *ZeRH2.1* and Arabidopsis protein sequences in cluster 2.3 (Fig. 1C) shows *At1g45180* as the closest homolog of *ZeRH2.1*.

We have estimated previously that 80% of the expression patterns observed in cDNA-AFLP gels can be confirmed by reverse transcription (RT)-PCR or northern gel-blot analyses (Milioni et al., 2002). The cDNA-AFLP analysis indicated that *ZeRH2.1* was first expressed 4 h after addition of auxin and cytokinin to the culture medium in the zinnia system. However, the expression pattern of *ZeRH2.1* using RT-PCR is not consistent with the cDNA-AFLP pattern and showed that the gene is expressed in freshly isolated mesophyll cells and at all stages of the time course of tracheary element formation in the zinnia system (Fig. 1D) and also in cultures containing either auxin or cytokinin alone (data not shown).

### *ZeRH2.1* Is Highly Expressed in Different Cell Types in Different Vascular Bundles of the Mature Zinnia Stem

The term mature zone is used to describe stem sections obtained from any internode between node two and the hypocotyl, counting from the first fully expanded leaf at the shoot apex, as node one (Fig. 2B). Sections were taken from plants with three or four nodes.

The vascular system in the zinnia stem is characteristic of many dicotyledons and forms a hollow cylinder sandwiched between dermal and ground tissue (Sachs, 1875). Long-running vascular strands form individual units of the stem vascular system, which are separated by interfascicular regions. These vascular strands are composed of collateral vascular bundles, with phloem located outside the xylem (Fig. 2, A and C).

In situ hybridization of the *ZeRH2.1* antisense riboprobe with transverse sections from the mature zone of the zinnia stem showed that almost all cells contain low levels of transcript (Fig. 2C). Control experiments using the sense probe showed no detectable signal (data not shown). However, certain cells in the vascular bundles show a much higher abundance of transcript. The expression pattern of *ZeRH2.1* in vascular bundles 1, 3, 5, 7, 9, and 11, as numbered in the transverse section shown in Figure 2A, is different from that in the other bundles in the stem (Fig. 2C). Clearly, two classes of vascular bundles are represented in a single transverse section taken from the mature zone of the zinnia stem (Fig. 2C).

To understand the underlying differences between these two classes of vascular bundles, we need to describe the morphology of the vasculature. The

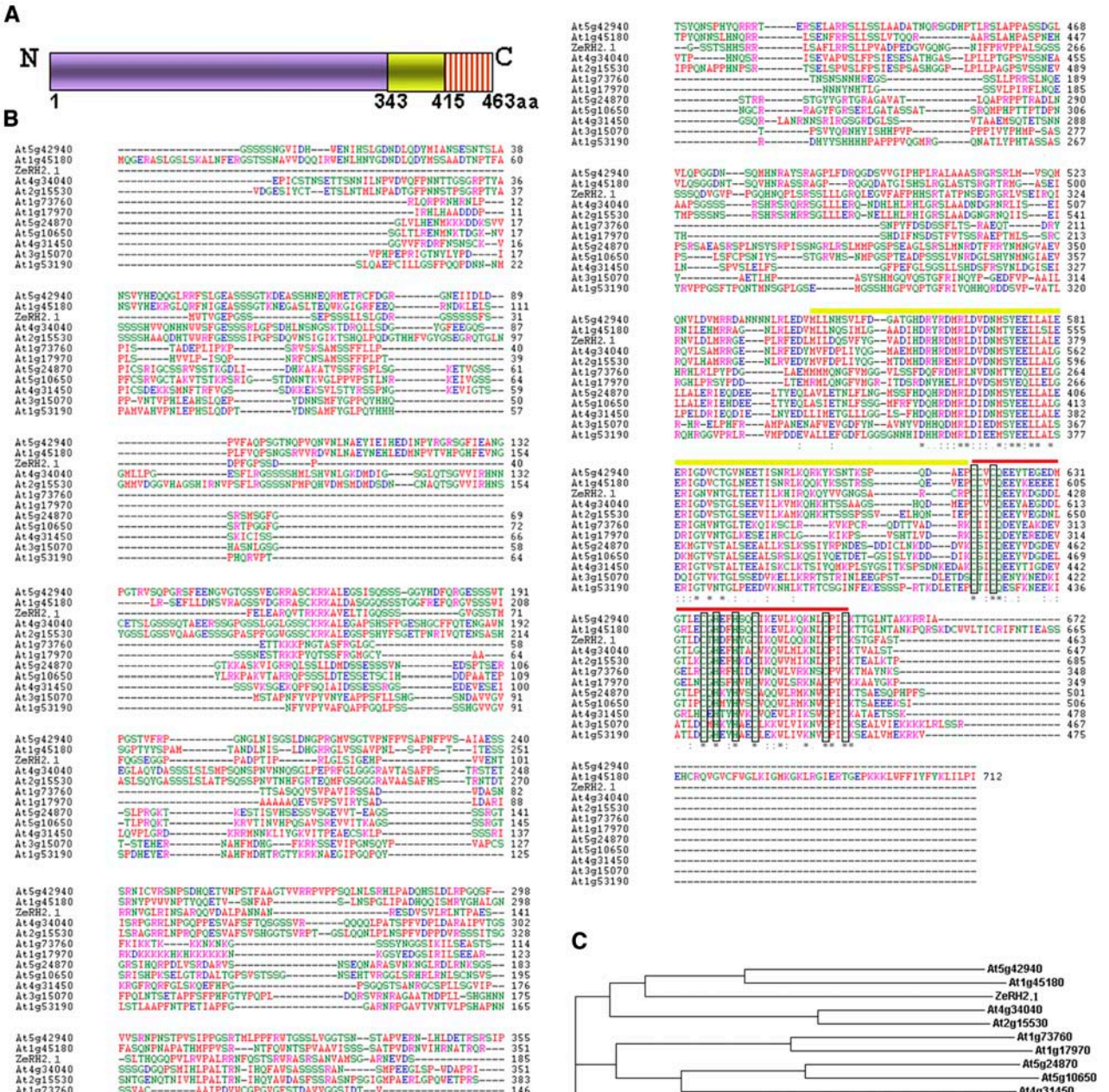


**Figure 1.** A, Schematic representation of the translated protein sequence of ZeRH2.1, showing a divergent region (blue), a conserved domain of unknown function (yellow), and the RING domain (red stripes). B, ClustalW alignment of the predicted ZeRH2.1 protein sequence with sequences in the cluster 2.3 group of Arabidopsis RING domain proteins (Kosarev et al., 2002). Conserved domains at the C terminus, the yellow and red-striped boxes shown in A, are marked by yellow and red lines, respectively. Conserved Cys and His, characteristic of a RING domain, C3H2C3, are marked by rectangles. Red letters represent small and hydrophobic amino acids (AVFPMILW), blue letters represent acidic amino acids (DE), magenta letters represent basic amino acids (RHK), and green letters represent hydroxyl, amine, and basic amino acids (STYHCNGQ). Others are shown in gray. Asterisk (*) indicates identical residues in all alignments, colon (:) indicates conserved substitution, and period (.) indicates

arrangement of leaves along the zinnia stem is decussate, with alternating pairs of leaves at right angles to the pairs of leaves at the nodes above and below (Fig. 2B). Each leaf, which is simple, entire, and obtuse, is connected directly to the stem without a petiole and is trilacunar, that is, supplied by three vascular traces, a midrib and two flanking sideribs (Fig. 2B) that are directly connected to the central stem vasculature. In a stem transverse section, therefore, six such vascular bundles will be present and are referred to here as leaf trace bundles (Eames and MacDaniels, 1925), while the remaining six or more bundles are referred to as stem bundles. In Figure 2A, the six leaf trace bundles are marked with arrows: of these, midrib bundle 1, together with siderib bundles 3 and 11, supply one leaf, while midrib bundle 7, with siderib bundles 9 and 5, supply the opposite leaf.

The *ZeRH2.1* expression pattern provides a molecular marker that distinguishes the leaf trace bundles from the stem bundles. The leaf trace bundles (Fig. 2, C and D) show enhanced expression of *ZeRH2.1* in xylem parenchyma cells, between the rows of xylem conducting elements, but basal levels in phloem cells. However, stem bundles have only basal levels of transcript in their xylem parenchyma cells, but instead have abundant transcript in particular phloem cells (Fig. 2E). At higher magnification (Fig. 2F), the transcript is most abundant in small companion cells next to larger sieve elements. Abundant transcript is also present in some cells in the interfascicular region (Fig. 2, C and G). As shown in Figure 2G, these small cells appear comparable to the companion cells of Figure 2F and may represent young phloem of new developing vascular tissues. Vascular bundles of the hypocotyl do not have abundant transcript in xylem or phloem cells (data not shown).

### Anatomy of Leaf Trace and Stem Bundles in the Mature Stem

To explain the distinct labeling pattern of the *ZeRH2.1* riboprobe in the two classes of vascular bundles, we analyzed the detailed anatomy of these bundles, using light and electron microscopies. The leaf trace bundle (Fig. 3A) has an arrangement of xylem and phloem cell types very different from that of the stem bundle (Fig. 3D). The phloem of the leaf trace bundle is capped by a larger group of fiber cells and has relatively little active phloem, and the xylem tracheids are arranged in clear radial rows flanked by densely cytoplasmic xylem parenchyma cells (Fig. 3A). At higher magnification, light microscopy (Fig. 3B) and transmission electron microscopy (Fig. 3C) reveal elaborate labyrinthine cell wall invaginations in the xylem parenchyma cells. Cells with this adaptation are commonly known as transfer cells and are characteristic of nodal xylem parenchyma (Pate and Gunning, 1972; Offler et al., 2002).

By contrast, stem bundles (Fig. 3D) have a smaller proportion of phloem fibers and most of the phloem comprises functional companion cells and sieve tube elements (Fig. 3, H and I). The xylem is arranged in an irregular network of vessels and supporting parenchyma cells, which are not so densely cytoplasmic as in leaf trace bundles (Fig. 3D). Micrographs at higher magnifications (Fig. 3, E and F) show that the xylem parenchyma cell walls are secondarily thickened and do not develop transfer cell-like morphology. However, the phloem companion cells of stem bundles have developed peg-like wall ingrowths (Fig. 3I).

Hypocotyl bundles (Fig. 4B) have a morphology characteristic of mature stem bundles. The xylem vessels are irregularly arranged and surrounding parenchyma cells are secondarily thickened. However, unlike young stem bundles, there are few phloem cells and these do not show clear companion cell or sieve element morphology.

In the interfascicular zone, where *ZeRH2.1* transcript is detected in small cells, young vascular bundles develop. Recent divisions of a phloem mother cell into a sieve element and a companion cell can easily be identified (Fig. 3G). The companion cells have a characteristic triangular shape and dense cytoplasm. Enlarged cells destined to be xylem vessel elements are also identifiable.

### *ZeRH2.1* Expression in the Young Zinnia Stem

Stem sections were obtained from just above the first node with fully open leaves. The vascular bundles in this young part of the stem are anatomically similar without distinct stem bundle or leaf trace bundle morphology, and will be referred to as juvenile bundles.

The expression pattern of the *ZeRH2.1* gene in juvenile bundles is distinct from that of either stem or leaf trace bundles of the mature stem. Expression of *ZeRH2.1* is not enhanced in any of the cell types of the xylem (Fig. 4, F–H). Although xylem tracheids of these bundles are radially arranged in a similar fashion to leaf trace bundles (Fig. 4C), the surrounding parenchyma cells are not densely cytoplasmic nor do they show transfer cell-like morphology (Fig. 4D). However, abundant transcript was detected in the phloem (Fig. 4, F–I). As shown in Figure 4G, the expression of the *ZeRH2.1* gene is enhanced in three to four cell

**Figure 1.** (*Continued.*)
semiconserved substitution. C, Phylogenetic tree of the putative protein sequence of ZeRH2.1 with Arabidopsis proteins in the cluster 2.3. D, RT-PCR showing the transcript levels of the *ZeRH2.1* gene at different stages of tracheary element differentiation in vitro: N0, immediately after isolation of mesophyll cells; N24 and N48, 24 and 48 h after isolation; +4, +24, +48, +72 h after induction of tracheary element formation by addition of auxin and cytokinin. The 26S rRNA is used as a standard to show equal loading in each lane.

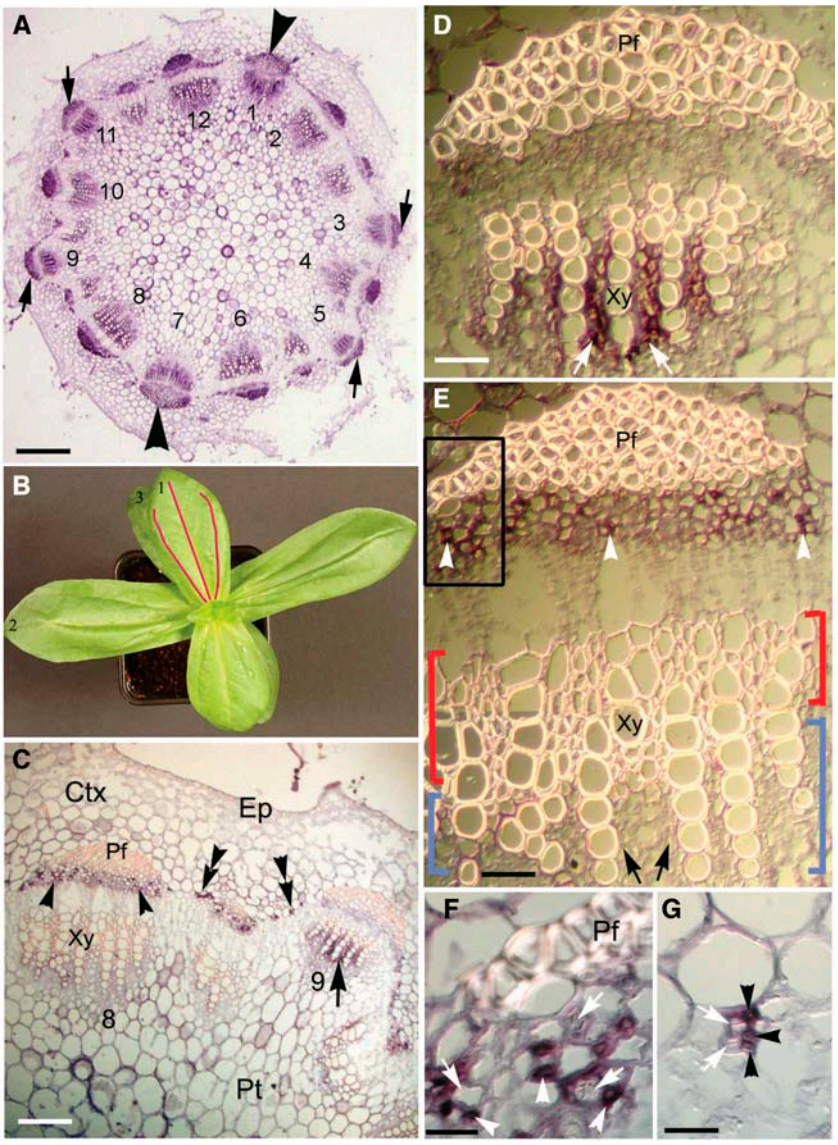


**Figure 2.** In situ hybridization of *ZeRH2.1* antisense riboprobe with transverse sections from the mature zone of zinnia stems. A, Transverse section from the mature zone (below the second node counted from the first fully expanded leaf at the shoot apex) of the zinnia stem as in C, showing the arrangement of leaf trace and stem bundles. Numbered bundles represent the 12 vascular bundles in the zinnia stem. Recently branched bundles are not numbered and can be recognized by comparatively smaller areas of phloem. Bundles 1 and 7 (arrowheads) are leaf midrib bundles supplying opposite leaves at the same node. Arrows indicate each pair of siderib bundles (3 and 11, 5 and 9, respectively) that flank the midribs. The colors in this picture have been enhanced using Adobe Photoshop software to show morphological details. Bar = 380 $\mu$m. B, Top view of a zinnia plant showing leaf arrangement (numbers on the leaves indicate the node number). Each node bears two opposite leaves, each supplied by three leaf traces (indicated by the red lines). C, In situ hybridization of *ZeRH2.1* antisense riboprobe showing distinct labeling patterns in the leaf trace bundles and the stem bundles. Vascular bundle 9, a leaf trace bundle, has abundant *ZeRH2.1* transcript in the xylem parenchyma cells (arrow). Stem bundle 8 has specific cells (arrowheads) of the phloem expressing transcript. Double arrowheads mark a few cells in the interfascicular region with abundant transcript. In addition to these strongly labeled zones, low transcript levels are present in all of the stem cells. Bar = 150 $\mu$m. D, Higher magnification of leaf bundle 9, showing abundant transcript in xylem parenchyma cells (arrows). Bar = 40 $\mu$m. E, Higher magnification of stem bundle 8. Arrows indicate xylem parenchyma cells that express only basal levels of transcript. *ZeRH2.1* transcript is abundant in some cells in the phloem (white arrowheads). Blue brackets mark protoxylem, and red brackets mark new stem bundle metaxylem elements. Bar = 50 $\mu$m. F, Higher magnification of the boxed region marked in E, showing abundant transcript in phloem companion cells (arrowheads). Larger sieve elements (arrows) or parenchyma cells do not show up-regulated gene expression. Bar = 15 $\mu$m. G, Interfascicular region, marked by double arrowheads in C, shows young, developing phloem with abundant transcript in small companion cells (arrowheads); sieve tubes are marked by arrows. Bar = 15 $\mu$m. Ep, Epidermis; Ctx, cortex; Pt, pith; Pf, phloem fibers; Xy, xylem.

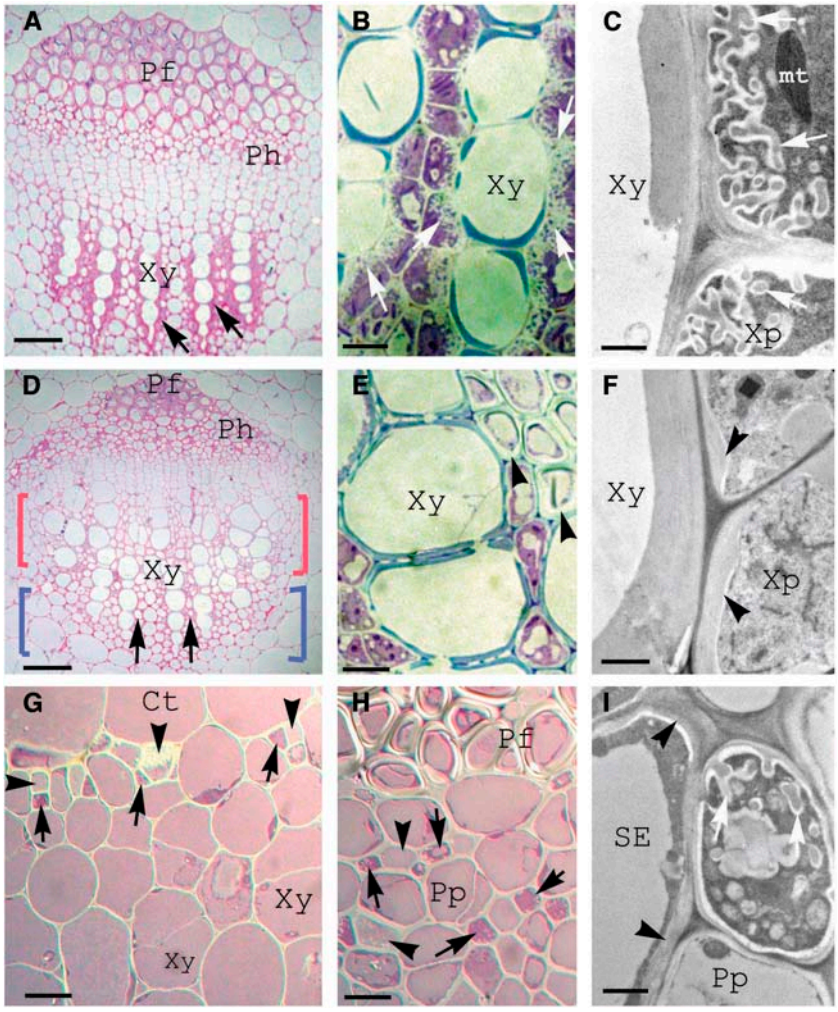


**Figure 3.** Morphology of zinnia leaf trace and stem bundles. A to C, Leaf trace bundles. A, Leaf trace bundle showing characteristic radial arrangement of the xylem tracheids, surrounded by densely cytoplasmic xylem parenchyma cells (arrows). The phloem is largely composed of phloem fibers and a few active phloem cells. Bar = 50 $\mu$m. B, Higher magnification of xylem tissue showing secondary thickenings of the xylem cell wall stained blue. Neighboring parenchyma cells have labyrinthine cell wall ingrowths (arrows), small vacuoles, and dense cytoplasm, characteristic of transfer cells. Bar = 12 $\mu$m. C, Transmission electron micrograph showing wall invaginations (arrows) of the xylem parenchyma cells, surrounded by plasma membrane. Bar = 2 $\mu$m. D to F, H, and I, Stem bundles. D, Stem bundle showing irregular network of metaxylem vessels. Surrounding parenchyma cells are not densely cytoplasmic as in leaf bundles (arrows). The phloem tissue has a higher proportion of active phloem and fewer phloem fibers. Blue brackets mark protoxylem elements, and red brackets mark new stem bundle metaxylem elements. Bar = 50 $\mu$m. E, Higher magnification of xylem elements, marked by red brackets in D, shows parenchyma cells of stem bundles with secondary wall thickenings (arrowheads) and large central vacuole. Bar = 12 $\mu$m. F, Transmission electron micrograph showing secondary wall thickenings (arrowheads) of the xylem parenchyma cells. These cells do not develop transfer cell-like wall ingrowths. Bar = 1.3 $\mu$m. G, A very young vascular bundle in the interfascicular region with enlarged proxylem cells (Xy). Small companion cells (arrows) and sieve elements (arrowheads) have differentiated. Bar = 12 $\mu$m. H, Higher magnification of the phloem of stem bundles showing companion cells (arrows) and sieve elements (arrowheads) embedded between large parenchyma cells. Bar = 12 $\mu$m. I, Transmission electron micrograph of companion cells with transfer cell morphology (arrows indicate wall ingrowths) and sieve elements with secondary wall thickenings (arrowhead) between thin-walled parenchyma cells. Bar = 1.3 $\mu$m. Ct, Cortex; Pf, phloem fibers; Ph, phloem consisting of companion cells and sieve elements; Xy, xylem; Xp, xylem parenchyma; Pp, phloem parenchyma cell; mt, mitochondrion; SE, sieve elements.

layers of phloem. Precursors of phloem fiber cells do not express the gene. Almost all of the phloem cells of a recently divided, very young bundle show accumulation of the transcript (Fig. 4H), as no fiber cells have developed yet. At higher magnification (Fig. 4I), gene expression in the juvenile bundles is confined to some of the larger cells of the phloem. The phloem companion cells of juvenile bundles do not develop wall ingrowths and are roughly the same size as sieve elements (Fig. 4E) and parenchyma cells. It is therefore hard to establish in which phloem cell type the gene is expressed.

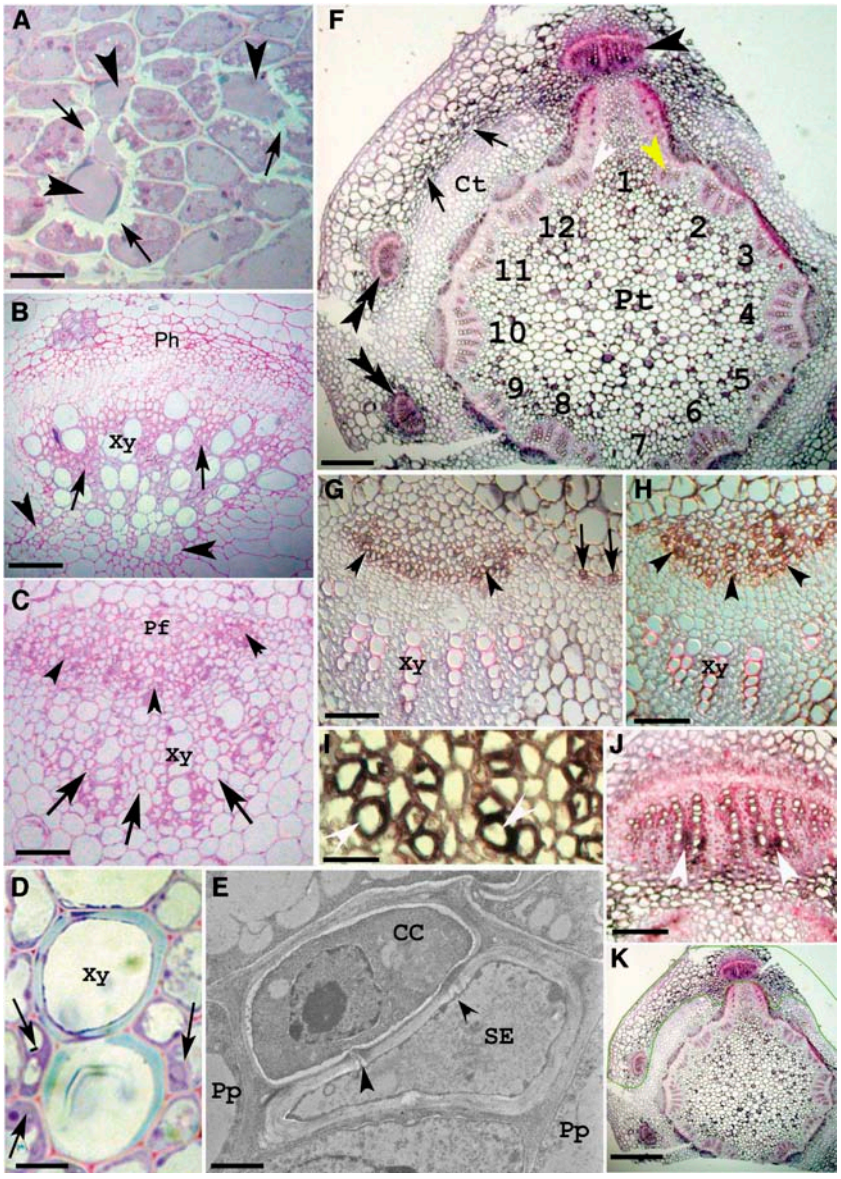


**Figure 4.** A and B, Stem bundle morphology. C to E, Juvenile bundle morphology. F to K, *ZeRH2.1* expression pattern in the young zone of zinnia stem. A, Higher magnification of xylem elements from the internal end of the stem bundle after transition in the long-running internode, showing protoxylem tracheids, marked by blue brackets in Figure 3D. Older xylem vessels forming lacunae are marked by arrowheads. Arrows indicate parenchyma cells with transfer cell morphology. Bar = 20 $\mu$m. B, Hypocotyl bundles with an irregular network of metaxylem vessels (Xy) and secondarily thickened parenchyma cells (arrows), as in stem bundles (Fig. 3, D–F). Arrowheads indicate remaining protoxylem tracheids. Bar = 75 $\mu$m. C, Juvenile bundle showing protoxylem tracheids (Xy) arranged in rows, surrounded by thin-walled parenchyma cells (arrows). The phloem contains three to four cell layers of active phloem (arrowheads) and newly formed phloem fibers (Pf), in which secondary wall thickening is yet to be deposited. Bar = 50 $\mu$m. D, Higher magnification of juvenile bundle xylem, showing secondary thickenings of the xylem cell wall stained blue, surrounded by thin-walled parenchyma cells (arrows), without wall ingrowths. Bar = 12 $\mu$m. E, Transmission electron micrograph showing phloem of a juvenile bundle. The companion cell (CC) is densely cytoplasmic and without wall ingrowths. The neighboring sieve element (SE) is symplastically connected to the companion cell by plasmodesmata (arrowheads). Bar = 0.25 $\mu$m. F, Expression pattern of the *ZeRH2.1* gene in the young zone of the stem, above the first node, with 12 vascular bundles numbered. All of the bundles show juvenile bundle morphology, before developing the morphologies of leaf trace or stem bundles of the mature stem. Black arrowhead and double arrowheads indicate the midrib and the sideribs, respectively, of newly formed leaves. Dark blue staining marks the cells with *ZeRH2.1* gene expression. The section was counterstained pink with basic fuchsin. A number of pith cells (Pt) contain transcript. Transcript levels are enhanced in the future palisade parenchyma (arrow). The transcript is at basal levels in the neighboring stem cortical cells (Ct). Bar = 330 $\mu$m. G, Higher magnification of juvenile bundle 12 shows that gene expression is restricted to the three to four cell layers of the active phloem (arrowheads). Arrows mark cells in the interfascicular region with abundant transcript. Bar = 65 $\mu$m. H, A newly branched bundle indicated by a white arrowhead in F, with transcript expressed in most of the phloem (arrowheads). Bar = 55 $\mu$m. I,

The transverse section in Figure 4, F and K, shows the anatomy of a node subtending a leaf pair above node 1. The overlaid boundary in Figure 4K marks the outline of one of the two future leaves that will arise at this node. The leaf traces destined to supply the future leaf have already diverged from the stem (Fig. 4F, black arrowheads), although the leaf remains physically attached to the main stem. Xylem parenchyma cells of the diverged midrib bundle, which are likely to be in the transition state to form transfer cells, strongly express *ZeRH2.1* (Fig. 4J). A similar pattern of expression was detected in the siderib bundles but was absent from all of the vascular bundles in the central vasculature. In addition to the leaf trace bundles, cells positioned at the top half of the leaf (Fig. 4F, arrows), the future palisade mesophyll cells, also show enhanced gene expression; however, spongy mesophyll cells or the neighboring stem cortical cells do not show up-regulation of the transcript (Fig. 4F).

The expression pattern of the molecular marker *ZeRH2.1* in different types of vascular bundles is summarized in Figure 5.

### Interconnections of Leaf and Stem Vascular Bundles

Two developmental anatomy questions arise from the above observations: (1) how are the leaf trace and the stem bundles connected to each other, and (2) what happens to the stem vasculature after the six leaf trace bundles have supplied the leaves?

To address these questions, we resolved the arrangement of the vasculature in the zinnia stem (Fig. 6). The hypocotyl has four vascular strands, each of which divides once to bring the number of vascular strands to eight. Four of these eight strands divide once again to supply the first pair of leaves, bringing the total number of vascular strands to 12. This represents the base number for zinnia, which the plant will maintain by continued divisions of the vascular strands. At the apical meristem, all of the bundles are identical and show juvenile bundle morphology. Vascular strands that have diverged out from the central vasculature as leaf traces develop leaf trace bundle morphology. As the stem elongates and internodes develop, continuing leaf traces within the central vasculature also develop leaf trace bundle morphology (indicated by green lines in Fig. 6) in the upper region of the internode. As the vascular strand increases in distance from the node, which it supplied as a leaf trace, it undergoes a morphological transition from leaf trace bundle to stem bundle, represented by transition of green lines into black lines, in Figure 6. The peripheral part of the xylem develops irregular metaxylem, characteristic of stem bundles, whereas the internal part of the newly formed bundle contains protoxylem elements with leaf trace bundle characteristics (Fig. 3D) and xylem parenchyma cells with wall ingrowths (Fig. 4A). In mature stem bundles, lacunae form in the protoxylem as the stem elongates and the cells are subject to stretching forces (Fig. 4B). Interestingly, protoxylem tracheids are not obliterated (Fig. 4B), perhaps because of the mechanical support provided by the thick walls of neighboring transfer cells. Older parts of the stem with very long internodes show only stem bundle morphology, illustrated in Figure 6 by black lines, between the hypocotyl and node 4.

The central stem vasculature only contains six leaf trace bundles at one node, as the vascular bundles supplying the leaf do not continue above that node, shown within the blue rectangle of Figure 6. For example, the vascular supply to leaves of node 2 terminates at that node, and node 1 does not contain any leaf trace bundles that have supplied node 2. Similarly, below the supply node as well, leaf trace bundle morphology remains confined to a single internode. As shown in Figure 6, the siderib bundles branch out just below the supply node. This is not the case for midribs, which originate two nodes below the supply node. The midrib bundle for node 2, for example, originates by the merging of two bundles from node 4. The midrib bundles acquire stem bundle morphology before reaching the node below the supply node; node 2 midrib, for example, will show stem bundle morphology when observed at node 3.

## DISCUSSION

The development of the postembryonic stem vascular system involves three processes: (1) the differentiation of juvenile bundles into leaf trace bundles and stem bundles, (2) the specialization of vascular cells for specific function, and (3) the differentiation of the vascular network as a coherent unit. We have used the expression pattern of a zinnia gene, *ZeRH2.1*, as a molecular marker to help track the differentiation of the zinnia stem vascular system as the plant matures (Fig. 5).

### Vascular Anatomy of the Zinnia Stem

A number of in situ hybridization studies have reported zinnia gene expression patterns in vascular

**Figure 4.** (*Continued.*)
Higher magnification of phloem, showing gene expression in large phloem cells, likely to be companion cells (arrowheads). Bar = 2 $\mu$m. J, Higher magnification of the midrib bundle of the future leaf, marked by a black arrowhead in F. Transcript is abundant in xylem parenchyma cells (arrows), indicating the transition of the xylem parenchyma to transfer cell fate, characteristic of leaf trace bundles. Bar = 122 $\mu$m. K, The boundary of one of the two future leaves is overlaid on the transverse section shown in F. Bar = 700 $\mu$m. Xy, Xylem vessels/tracheids; Xp, xylem parenchyma; Ph, phloem; Pp, phloem parenchyma cell; Pf, phloem fibers; Pt, pith.

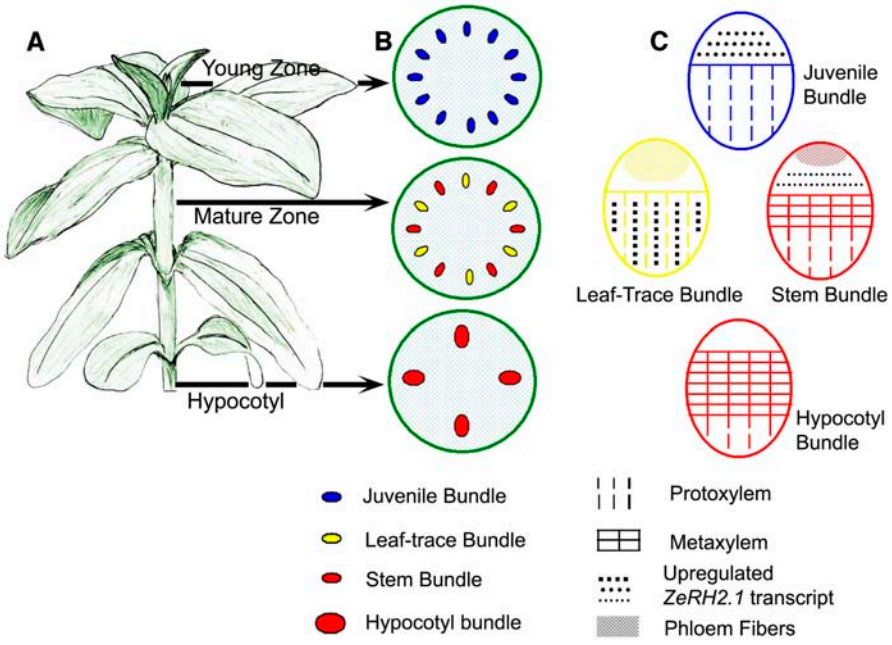


**Figure 5.** Schematic overview of the bundle morphology and the expression pattern of *ZeRH2.1* gene in the zinnia stem vascular system. A, Zinnia plant with young, mature, and hypocotyl zones demarcated. B, Transverse sections showing the vascular bundle arrangements in different zones. The young zone contains 12 juvenile bundles, the mature zone contains alternating stem and leaf trace bundles, and the hypocotyl contains four mature stem bundles. C, Expression pattern of the *ZeRH2.1* gene in different types of vascular bundles, sites of abundant transcript indicated by black stipples. Juvenile bundles show expression in large phloem cells. In the mature zone, there is abundant transcript in xylem parenchyma cells of leaf trace bundles and small phloem companion cells of neighboring stem bundles. Only basal levels of transcript are present in hypocotyl bundles. In juvenile bundles, protoxylem tracheids are arranged in rows, represented by straight broken lines, and the phloem contains large cells. In leaf trace bundles, protoxylem tracheids are arranged as in juvenile bundles but the xylem parenchyma is differentiated to form transfer cells. The phloem consists mostly of fibers. In stem bundles, metaxylem vessels at the peripheral end are represented by rectangles, while at the internal end, protoxylem is represented by lines. The phloem consists of small companion cells and large sieve elements and a few fiber cells at the top. In hypocotyls, the xylem consists of metaxylem vessels.

bundles of the zinnia stem. However, these studies were either limited to the young zone (Demura and Fukuda, 1994; Liu et al., 1999; Im et al., 2000; Endo et al., 2001; Funk et al., 2002; Ohashi-Ito et al., 2002, 2003; Groover et al., 2003; Ohashi-Ito and Fukuda, 2003; Motose et al., 2004) or, when studied in different parts of the plant, the genes of interest were uniformly expressed in all vascular bundles (Ye and Varner, 1994, 1995; Ye, 1996). The complex and dynamic expression pattern of *ZeRH2.1* underscores the importance of assaying expression at different developmental stages.

Understanding the differential expression pattern of *ZeRH2.1* in different developmental zones of the zinnia stem required careful analysis of the anatomy. As zinnia is an established model system for studying xylogenesis, detailed characterization of the stem vascular network is important for functional analysis of the genes in vivo. We define two zones in the stem, a young zone, above the first fully expanded leaf, and a mature zone, between the second open leaf and the hypocotyl (Fig. 5). Juvenile bundles in the young zone have a uniform anatomy, consisting of protophloem and radially arranged tracheids of protoxylem. Juvenile bundles then develop into leaf trace bundles in which xylem parenchyma cells have differentiated into transfer cells, supporting radially arranged protoxylem tracheids. The phloem of these bundles is characterized by metaphloem and is largely composed of companion cells and sieve elements. As the plant grows, the internodes elongate and the long-running vascular strands develop irregularly arranged metaxylem vessels, characteristic of stem bundle morphology (Figs. 3, D–F, and 6). The internal cells of newly formed stem bundles are protoxylem cells (Figs. 2E and 3D), which are later stretched and displaced as metaxylem is formed (Fig. 4A).

### Expression of the *ZeRH2.1* Gene in Different Types of Vascular Bundle Is Enhanced in Transfer Cells

In the mature stem, leaf trace bundles show strong up-regulation of the *ZeRH2.1* transcript in the xylem parenchyma (Figs. 2, C and D , and 5), whereas stem bundles have abundant transcript in phloem companion cells (Figs. 2, C, E, and F, and 5). Both xylem parenchyma of the leaf trace bundles and companion cells of the stem bundles have been modified into transfer cells (Fig. 3, C and I). Xylem and phloem

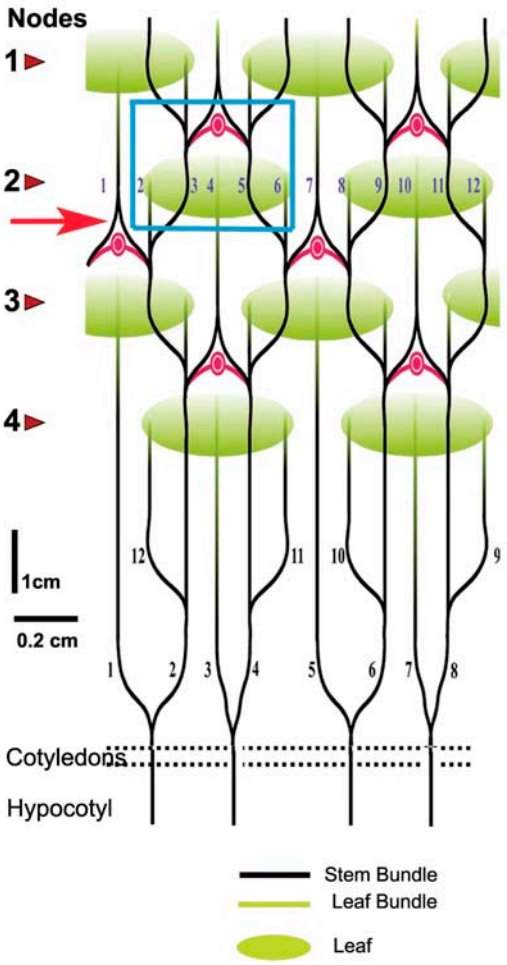


**Figure 6.** Zinnia stem vascular network, showing arrangement and interconnection of the leaf and stem bundles. The hypocotyl contains four bundles, which divide to produce 12 vascular bundles. Green lines indicate leaf trace bundles and black lines indicate stem bundles. Red lines represent vascular supply to lateral branches. The blue rectangle highlights vascular supplies to one leaf showing that the sideribs originate as a branch that leaves the stem bundle immediately after it passes the node below (node 3 in this case), whereas the midrib originates at two nodes below the node it is supplying (node 4 in this case). In the mature zone of the stem, with long internodes, the midrib bundle acquires stem bundle morphology, indicated by the transition of green to black. The leaf trace bundles diverge from the stem vascular cylinder at the supply node and do not form part of the stem vasculature above that node. The red arrow marks the position from which the transverse section shown in Figure 4F is obtained and vascular bundles are numbered as in Figure 4F. Black arrowheads in Figure 4F indicate the leaf trace bundles supplying the leaves on node 3 and are clearly no longer part of the central vascular cylinder of the stem. The newly branched bundles, marked with white and yellow arrowheads in Figure 4F, will merge to form the midrib bundle 1, and the ones on the opposite side will form midrib bundle 7, to supply the leaves on node 1. Vascular bundles 4 and 10 will branch to supply the midribs of the leaves on node 2, flanked by siderib bundles 2, 6 and 8, 12, respectively. Double dotted lines indicate zone of vascular supply to cotyledons.

transfer cells belong to two different classes (Pate and Gunning, 1972). Companion cells are A-type with dense cytoplasmic content and ingrowths distributed around the cell periphery (Fig. 3, H and I), whereas xylem parenchyma are C-type with highly polarized wall ingrowths restricted to the walls in contact with xylem tracheids and their cytoplasm is peripheral (Fig. 3, B and C).

Transfer cell differentiation is coordinated with organ development and occurs at specific locations across particular developmental windows (Pate and Gunning, 1972; Harrington et al., 1997). Wall ingrowths of transfer cells form just as intensive transport starts, and provide efficient and rapid short-distance transport of solutes. Xylem parenchyma cells are best displayed within leaf trace bundles in the upper region of an internode and in the traces leaving the main body of the stem (Gunning et al., 1970). Recently diverged vascular traces supplying young leaves show up-regulation of *ZeRH2.1* transcript in xylem parenchyma cells (Fig. 4, F and J), where wall ingrowths are just beginning to form. In the younger part of the stem, near the apical meristem, internodes are not well developed, and xylem parenchyma cells of juvenile bundles do not develop transfer cells and have basal levels of *ZeRH2.1* transcript (Fig. 4, F–H). The expression of the *ZeRH2.1* gene is, therefore, linked with newly formed transfer cells in leaf traces of the young zone and with functional transfer cells in leaf trace bundles.

The xylem parenchyma cells of the stem bundles have only basal levels of the *ZeRH2.1* transcript (Fig. 2E). As shown in Figure 3, D to F, the peripheral xylem cells of stem bundles that are formed in the long-running internodes have thickened xylem parenchyma without transfer cell-like wall ingrowths. However, the internal xylem cells of these bundles consist of protoxylem with characteristic wall ingrowths in xylem parenchyma cells (Fig. 4A). It is possible that older transfer cells associated with the protoxylem at the internal end of the vascular bundle are no longer functional in stem bundles. As stem bundles age, protoxylem is stretched and then becomes displaced (Fig. 4B). Our results show that only the leaf trace bundle transfer cells, which are in close proximity to the node, contain abundant *ZeRH2.1* transcript. In hypocotyl bundles, no up-regulated expression of the *ZeRH2.1* gene is observed (data not shown) and no transfer cells remain (Fig. 4B).

In the in vitro zinnia system, at least two different cell fates are represented: cells that form tracheary elements and the cells that do not. Several lines of evidence, together with the fact that dilution of cell number in the culture results in suppression of tracheary element formation, suggest that the population of living mesophyll cells that do not differentiate are required to support those differentiating to become tracheary elements (Matsubayashi et al., 1999; McCann et al., 2001; Motose et al., 2001). Most of the riboprobes that we have derived from differentially expressed

sequences identified in our cDNA-AFLP screen hybridize to cells within vascular bundles of stem (Milioni et al., 2002). However, we find a variety of cell types are labeled in addition to young xylem tissue, including cambium, phloem, sieve elements, phloem fibers, and xylem parenchyma (Milioni et al., 2002; P. Dahiya and N. Stacey, unpublished data), and some genes are expressed only in subpopulations in the in vitro system (McCann et al., 2001). This raises the possibility that other vascular cell fates are being explored by isolated cells in the in vitro system and that the cells in culture that do not differentiate as tracheids may be required by those that do and may have some of the properties of other vascular cell types.

### A Potential Function of *ZeRH2.1* in Active Transport

In the angiosperm stem, the relationship of transfer cells with foliar traces holds regardless of the class of structure that a node may subtend; traces to cotyledons, scale leaves, stipules, and various kinds of floral bracts all carry xylem transfer cells just as does the vascular supply to the true leaf (Gunning et al., 1970). In phloem, transfer cells tend to be concentrated to parts of the vascular network where xylem transfer cells are not well developed, for example, in the bud or branch traces, or in the margin of the leaf gap (Gunning et al., 1970). In addition to the abundant expression of *ZeRH2.1* in the xylem parenchyma of leaf trace bundles, companion cells of the stem bundle are modified into transfer cells and accumulate abundant transcript of *ZeRH2.1* gene (Fig. 2, E and G). The phloem of the leaf trace bundle, on the other hand, does not show transfer cell morphology or expression of *ZeRH2.1* gene (Fig. 2D). Gunning et al. (1970) hypothesized that the transfer cell arrangement of the nodal complex might facilitate exchange of solutes between phloem and the transpiration stream (xylem) so that solutes could reach developing flowers and fruits.

*ZeRH2.1* transcript, however, is also up-regulated in some cells that do not develop transfer cell morphology. In juvenile bundles, the phloem cells accumulate *ZeRH2.1* transcript but do not develop wall ingrowths (Fig. 4, E–I). The leaf palisade parenchyma also accumulates transcript without development of transfer cell morphology (Fig. 4, F and K). This observation is consistent with the constitutive expression pattern of *ZeRH2.1* in the in vitro zinnia system derived from leaf mesophyll and palisade cells (Fig. 1D). The enhanced levels of *ZeRH2.1* transcript in different cell types may be correlated with their involvement in enhanced transportation. Xylem parenchyma cells of leaf trace bundles, companion cells of stem bundles, and phloem cells of juvenile bundles are all placed at crucial junctions of active transportation. Leaf palisade parenchyma cells actively export photosynthates to the rest of the plant. Therefore, we hypothesize that the *ZeRH2.1* gene has a role to play during active transport, with more abundant transcript identifying sites of major intercellular transport.

How the *ZeRH2.1* gene product might facilitate transport remains to be determined. The protein sequence contains a RING domain (Fig. 1). RING domain-containing proteins are components of supramolecular assemblies in cells that act in a variety of unrelated biochemical reactions. Kentsis et al. (2002) showed that purified RINGs from functionally unrelated proteins, including promyelocytic leukaemia protein (Borden, 1998), human KAP-1/TIF1$^{\beta}$ (Peng et al., 2000), lymphocytic choriomeningitis virus Z (Kentsis et al., 2001), breast cancer susceptibility gene product 1, and breast cancer susceptibility gene product 1-associated RING domain protein (Brzovic et al., 2001), self-assemble into supramolecular structures in vitro that resemble those they form in vivo. RING self-assembly creates complexes that act structurally as polyvalent scaffolds, thermodynamically by amplifying activities of partner proteins, and catalytically by spatiotemporal coupling of enzymatic reactions (Kentsis et al., 2002).

In conclusion, our study integrates the complex expression pattern of a molecular marker with the differentiation of the stem vascular system of zinnia. The developmental progression of juvenile bundles to leaf trace bundles, stem bundles, and hypocotyl bundles is marked by the differential expression patterns of the *ZeRH2.1* gene. As all four types of bundles show distinct anatomical characteristics, expression patterns of other vascular genes are likely to follow suit. The most similar sequence to *ZeRH2.1* belongs to cluster 2.3 in the classification of RING domain-containing genes in Arabidopsis (Kosarev et al., 2002). Our study of the expression pattern of *ZeRH2.1* assigns a potential function to genes in this cluster in facilitating active transportation via protein-protein interactions.

## MATERIALS AND METHODS

### Plant Material

Seeds of *Zinnia elegans* cv Envy were obtained from Stokes Seeds, Chiltern, UK. Plants were grown in short-day conditions with 60% humidity at 26°C. Plants with two to three fully expanded leaves were used for in situ hybridization and anatomical studies.

Mesophyll cells were isolated from zinnia leaves and induced to differentiate to tracheary elements in vitro as described previously (Domingo et al., 1998).

### Amplification of cDNA Fragments by 3′- and 5′-RACE PCR

Total RNA and poly(A$^+$) RNA was prepared as described by Milioni et al. (2002). For 3′- and 5′-RACE, adapter-ligated double-stranded cDNA was synthesized using a Clontech Marathon cDNA amplification kit (Clontech, Palo Alto, CA) following the manufacturer's instructions. RACE reactions were performed as per manufacturer's instructions. Amplified cDNAs were subcloned using TOPO TA cloning kit for sequencing (Invitrogen, Groningen, The Netherlands) and sequenced using ABI PRISM dye terminator cycle sequencing ready reaction kit with fluorescent sequencing AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT). Database searches were performed using GCG version 10 software (Genetics Computer Group, Madison, WI) using the BLAST network services (Altschul et al., 1997). Phylogeny and ClustalW alignment (Higgins et al., 1994) were done using the European Bioinformatics Institute Web site (http://www.ebi.ac.uk).

## RT-PCR

Total RNA was extracted from zinnia culture cells as described by Milioni et al. (2002). Endogenous DNA was removed by adding 0.2 $\mu$L DNAseI/$\mu$L (Pharmacia, Uppsala) at 37°C for 10 min. RT-PCR was then performed as described by Wisniewski et al. (1999). One hundred millimolar dNTPs were added to the reaction. For studying the differential expression of *ZeRH2.1*, primer 5′-GGAGGAATACAAGGATGGAGAC-3′ and reverse primer 5′-GCAAAGAATACCATTTGCCAAC-3′ derived from the 3′ end of *ZeRH2.1* cDNA were used. Amplification was performed at 94°C 30 s, 60°C 30 s, and 72°C 30 s. Aliquots (4 $\mu$L) were taken after every third cycle from the 17th to 32nd cycle, loaded on a 1.2% (w/v) agarose gel (Sigma, Poole, UK), and separated by electrophoresis at 80V in Tris-borate/EDTA buffer for 1 h. The gel was stained with ethidium bromide (Sigma) and imaged using Molecular Analyst software (Bio-Rad, Hercules, CA).

The 26S rRNA fragment, isolated by cDNA-AFLP, was used as a control for equal loading of total RNA. Primers for the 26S rRNA (5′-AAAGGATTC-TACCAGTCGCTTGATGGGA-3′ and 5′-ACGCCTCTAAGTCAGAATCCGG-GCTAGA-3′) were mixed with the above reaction.

## Tissue Fixation and Embedding for In Situ Hybridization

Zinnia plants, with two to three fully expanded leaves, were used for fixation and embedding. One-centimeter-long pieces of stem were cut from near to the apical meristem, nodes of open leaves, internode region, and hypocotyl. Stem pieces were placed in fixative immediately after cutting. Tissue fixation was performed as described by Wisniewski et al. (1999). Small petri plates were used as molds, which were heated to 40°C, a little molten wax poured into the bottom, then stem pieces were added and more molten wax was poured on top to submerge the stem pieces. Molds were cooled by floating on cold water. The blocks were stored at 4°C.

## Riboprobe Synthesis and In Situ Hybridization

Gene-specific, digoxygenin-labeled riboprobes were generated from a 357-bp fragment derived from the 3′ end of the *ZeRH2.1* cDNA sequence. T3 and T7 sites of the TOPO sequencing vector were used to make sense and antisense riboprobes. The plasmid was linearized using the *Not*I and *Spe*I sites for antisense and sense probes, respectively. Linearized plasmid was purified by phenol-chloroform extraction and quantified using a Bio-Rad spectrometer. Riboprobe synthesis and in situ hybridization were performed as described by Wisniewski et al. (1999). Photographs were taken on a Nikon Microphot SA microscope, using Nomarski (DCI) optics.

## Stem Vascular Network

Serial hand sections of the zinnia stem were used to determine the vascular strand arrangement.

## Light and Transmission Electron Microscopy

Tissues were fixed and embedded in LR White resin as described previously (Vandenbosch et al., 1989; Dahiya and Brewin, 2000). For light microscopy, 3- to 5-$\mu$m-thick sections were collected on lysine-coated slides and stained with basic fuchsin (Sigma). Photographs were taken using a Nikon Microphot SA microscope using bright-field optics (Nikon, Tokyo). For transmission electron microscopy, 90- to 150-nm-thick sections were collected. Sections were stained with potassium permanganate and uranyl acetate: a few crystals of $KMnO_4$ were added to 10 mL of 0.1 M phosphate buffer pH 7.0 and sections stained for 10 min, washed twice for 2 min in phosphate buffer, and then water for 2 min. Sections were stained with 20% uranyl acetate for 15 min, washed with water, and air-dried. Stem sections were viewed and photographed in a JEOL JEM-1200 EM transmission electron microscope (JEOL U.K., Welwyn Garden City, U.K.) operating at 80 kV.

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requestor.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AJ867840.

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