J Plant Growth Regul (2005) 23:301–306
DOI: 10.1007/s00344-004-0024-4

J P G R
Journal of Plant Growth Regulation

The Inflorescence Stem Fibers of *Arabidopsis thaliana Revoluta* (*ifl1*) Mutant

Simcha Lev-Yadun,[1] Sarah E. Wyatt,[2] and Moshe A. Flaishman[3]

[1]*Department of Biology, Faculty of Science and Science Education, University of Haifa-Oranim, Tivon 36006, Israel;* [2]*Department of Environmental and Plant Biology, Ohio University, Porter Hall 317, Athens, Ohio 45701-2979, U.S.A.;* [3]*Department of Fruit Trees, Institute of Horticulture, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan, 50250, Israel*

Abstract

Arabidopsis thaliana is gradually gaining significance as a model for wood and fiber formation. *revolute/ifl1* is an important mutant in this respect. To better characterize the fiber system of the *revolute/ifl1* mutant, we grew plants of two alleles (*rev-9* in Israel and *rev-1* in the USA) and examined the fiber system of the inflorescence stems using both brightfield and polarized light. Microscopic examination of sections of plants belonging to the two different alleles clearly revealed that, contrary to previous views, in 18 (13 in Israel and 5 in Ohio) out of 30 stems (20 in Israel and 10 in Ohio) the mutant produced the primary wavy fiber system of the inflorescence stems. Our findings are further supported by the fact that fibers are seen in the figures published in other studies of the mutant even when it was stated that there were no fibers. The impression of a total lack of the wavy band of fibers is in many cases just a result of poorly lignified secondary walls. This specific gene that reduces lignification in fibers is of great significance for biotechnological developments for the paper industry and thus for the global economy and ecology. We propose that *revoluta,* the first name given to this mutant (Talbert and others 1995), is more appropriate than *ifl1*.

Key words: *Arabidopsis thaliana*; fibers; *INTERFASCICULAR FIBERLESS1 (IFL1)*; Inflorescence stems; Lignification; *REVOLUTA*

Introduction

The developing ability and increasing tendency to use *Arabidopsis thaliana* as a model for wood and fiber formation (Dolan and others 1993; Lev-Yadun 1994, 1997; Dolan and Roberts 1995; Zhong and others 1997, 2001; Zhong and Ye 1999, 2001; Zhao and others 2000; Beers and Zhao 2001; Burk and others 2001; Lev-Yadun and Flaishman 2001; Altamura and others 2001; Chaffey 2002; Chaffey and others 2002; Funk and others 2002; Ye 2002; Ye and others 2002; Little and others 2002; Flaishman and others 2003; Kirst and others 2003; Oh and others 2003; Ko and others 2004; Nieminen and others 2003) offer prospects for great advances in understanding the biology of the formation of these tissues, which are not easy to study in trees. Genes that reduce lignification in fibers are of great sig-

Received: 21 July 2004; accepted: 17 December 2004; Online publication: 7 April 2005
**Corresponding author; e-mail:* levyadun@research.haifa.ac.il

nificance for biotechnological developments in the paper industry and thus for the global economy and ecology. One of the promising mutants in this respect, inducing alterations in fiber differentiation, is the *A. thaliana* mutant of the gene *REVOLUTA* (*REV*) (Talbert and others 1995), which was studied and cloned in parallel as a different gene *INTERFASCICULAR FIBERLESS1* (*IFL1*) (Zhong and others 1997, 2001; Ratcliffe and others 2000; Zhong and Ye 1999, 2001, 2004). However, these genes were found to be identical (Ratcliffe and others 2000). The gene encodes a class III homeodomain-leucine zipper protein (HD-ZIP) and is involved in the regulation of interfascicular fiber differentiation in inflorescence stems of *A. thaliana* (Zhong and Ye 1999; Otsuga and others 2001), as well as in regulating other apical meristem functions (Meyerowitz 1997; McConnell and others 2001; Emery and others 2003; Greb and others 2003; Mattsson and others 2003). The *REV/IFL1* gene was found to be one of the putative target sequences of microRNA 165 that cleave the wild-type *REV/IFL1* mRNA (Floyd and Bowman 2004; Zhong and Ye 2004). The name *INTERFASCICULAR FIBERLESS1* implies that interfascicular fibers do not form. This is not merely a semantic issue but a substantial developmental matter. Ye (2002) also stated that the *ifl1* mutation leads to a block of vascular cambium activity at the basal parts of inflorescence stems of *A. thaliana*, a description based on the assumption that the interfascicular fibers are part of the secondary xylem formed by the cambium. This blockage was shown to be associated with the reduced expression of the auxin efflux carriers PIN3 and PIN4 (Zhong and Ye 2001; Ye 2002). In Figure 5-J, Talbert and others (1995) show, however, a cambium formed in both interfascicular and fascicular zones, probably a regular case of variability concerning expression of cambial development in the *revoluta* mutant. Such variability seems to be common in various genotypes of *A. thaliana* and is probably influenced by growth conditions (M.A. Flaishman and S. Lev-Yadun unpublished).

The examination of the published histological figures of the mutant (Talbert and others 1995; Zhong and others 1997, 2001; Zhong and Ye 1999, 2001) raised the possibility that fibers are formed in the inflorescence stems and that the mutation mainly alters the secondary cell-wall composition rather than causing its absence. To clear this issue, we grew plants of the mutant and characterized the interfascicular fiber band of their inflorescence stems.

Materials and Methods

Plant Materials and Growth Conditions

Seeds of the mutant *revoluta* of *Arabidopsis thaliana* were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University). In Israel, the *rev-9* allele was used and they were germinated in a growth chamber under short day conditions (9 h light/15 h dark, 22 ± 1 °C, 45 $\mu IS^{-1}m^{-2}$). Two sets of single, four-week-old rosettes were transferred to 0.5 liter pots filled with a mixture of peat/tuff/perlite (40%/40%/20% v/v) fertilized once a week with Osmocot (NPK), irrigated twice a week and grown in a greenhouse. In Ohio, the *rev-1* allele was used and they were germinated in a growth room under short day conditions (9 h light/15 h dark, 21-22 °C, 75 $\mu IS^{-1}m^{-2}$). After two weeks, 10 rosettes were moved to long day conditions (9 h light/15 h dark with the same illumination) and were grown until they flowered. Control wild-type plants were grown under the same conditions.

Sampling and Histological Examination

In the plants grown in Israel, in one group of eight plants, the inflorescence stems were sampled when they were flowering. In two other groups of plants, one of seven and one of five, the plants flowered and had green siliques when sampled. In five of the plants we also sampled the main root to determine whether it performs secondary growth. A segment about 1 cm long was cut off the lower part of 20 inflorescence stems or the upper part of the main root and fixed in a mixture of 3:1 ethanol and glacial acetic acid overnight at room temperature. Control wild-type plants were also sampled in the lower part of 10 inflorescence stems. After fixation, samples were washed three times for 15 min each in PBS (pH 7.2), dehydrated in a series of ethanol solutions (25, 50, 75, 96, and 100%), and embedded in paraffin. Serial cross-sections, 10 μm thick, were prepared from each stem segment with a rotary microtome (American Optical model 820, Spencer) from the whole width of each stem segment, stained with Safranin and Fast Green and mounted with Permount (Fisher Scientific, Cat. No. SP15-100). Slides were examined under brightfield and polarized light with a Leitz Dialux 20 microscope equipped with a Nikon F3 camera, at magnifications of X40 to X160.

In the plants grown in Ohio, ten inflorescence stems were sampled 1 cm above the soil line. The stem segments were embedded in 5% agar, and sections 100 μm thick were made using a vibratome 1500

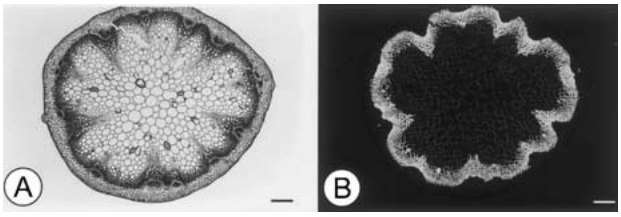

Figure 1. Cross sections through wild-type inflorescence stem that typically formed a wavy interfascicular fiber band. **A** bright field, **B** under polarized light. Bar 200 μm

series. The fresh sections were stained with phloroglucinol solution [5 g phloroglucinol (Sigma Aldrich, St. Louis, MO) in 50 ml 95% ethanol with 50 ml concentrated 12N HCl] for 3-30 min, washed thoroughly with distilled water and mounted in water. The sections were examined under brightfield and polarized light with a Nikon Labphot-2 microscope.

Identification of Secondary Cell Walls

A simple method for examining plant cells with secondary cell walls is to use polarized light. Secondary cell walls usually contain both cellulose and lignin. However, identification of cells with secondary cell walls in unstained material or using common tissue staining may be difficult, especially in the early stages of their formation. The microfibrils of the cell wall contain crystalline cellulose, which is birefringent (it has two refractive indices). The refractive index to light waves parallel to the long axis of the cellulose chains and microfibrils is larger than to light passing at right angles. Light that is polarized by passage through a polarizing filter will not pass through a second polarizer oriented at 90° to the first. When a piece of birefringent material (in this case a secondary cell wall containing crystalline cellulose in the sectioned tissue) is placed between such crossed polarizers so that its axis of major or minor refraction is at 45° to the planes of polarization of the polarizers, then the light passing through it becomes polarized and is able to pass through the second polarizer. The birefringent material between the polarizers appears bright to the viewer (Lyndon 1990). In the case of *A. thaliana*, this is an easy method for distinguishing between parenchyma with primary cell walls and xylem and sclerenchyma (fibers and sclereids) with secondary cell walls (Lev-Yadun 1994, 1997; Lev-Yadun and Flaishman 2001).

Results

Wild-type plants had the typical wavy fiber band in the inflorescence stems (Figure 1). Our histological

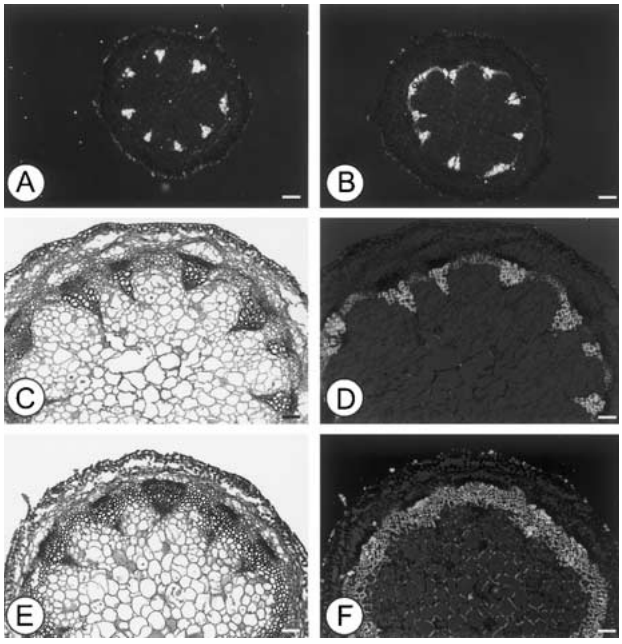

Figure 2. *Revoluta/ifl1* mutant of *Arabidopsis thaliana* (Bar = 100 μm) cross sections through inflorescence stems. **A** A stem that formed no wavy interfascicular fiber band under polarized light. **B** A stem with a thin wavy interfascicular fiber band that occupies only a sector of the circumference is clearly seen under polarized light. **C** A stem seen under brightfield that formed a very thin wavy interfascicular fiber band that looks as if fiber development is impaired. **D** The same stem shown in Figure 2C, but under polarized light. The thin wavy interfascicular fiber band is clearly seen. The secondary cell walls in the primary vascular bundles stain red since they are much more lignified than in the wavy interfascicular fiber band. **E** A stem that formed a thick wavy interfascicular fiber band that looks almost like a wild type under bright field. **F** The same stem shown in Figure 2E, but under polarized light. The thick wavy interfascicular fiber band is clearly seen. The secondary cell walls in the primary vascular bundles stain red because they are much more lignified than the fiber cells in the wavy interfascicular fiber band that stains green.

examination, especially when using polarized light, clearly revealed that the *revoluta* mutant usually produces the primary wavy fiber system of the inflorescence stems (Figure 2 A-F). Eighteen (13 in Israel and 5 in Ohio) out of 30 mutant stems examined (20 in Israel and 10 in Ohio) produced the primary wavy fiber system of the inflorescence stems and only 12 did not (Figure 2A). Indeed, examination of cross-sections of stems under brightfield gave the impression of a considerable reduction in the formation of the band of interfascicular fibers in some of the stems (Figure 2B, C). However, the use of polarized light immediately showed that the fibers were formed (Figure 2 B, D,

F) and that the major apparent difference from the wild type is that their secondary cell walls were poorly lignified. Other stems formed a thick wavy interfascicular fiber band easily seen even under brightfield (Figure 2 E), which looked almost like a wild type. Examination under polarized light better showed the thick wavy interfaseicular fiber band (Figure 2 F). In both types of stems, the secondary cell walls in the primary vascular bundles stained red because they are much more lignified than the wavy interfascicular fiber band that stained green or were stained red only slightly. We could not find a cambial zone or unequivocal evidence for secondary thickening in the inflorescence stems. The main roots that were examined, however, do perform regular secondary growth.

Discussion

A clear understanding of the normal development of the primary wavy band of fibers, cambium and secondary xylem in inflorescence stems of wild type *A. thaliana* (that is, Lev-Yadun and Flaishman 2001; Altamura and others 2001) is needed to understand the nature of the *revolute/ifl1* mutant. The origin, ontogeny and structure of the wavy fiber band that characterizes the inflorescence stems of *A. thaliana* are as follows. In young stems, the wavy fiber band is composed of primary fibers that develop from the ground meristem, forming the outer part of the pith, and they do not develop from the cambium, which does not exist yet at that stage of inflorescence stem ontogeny (Lev-Yadun and Flaishman 2001). In mature, thick stems that preformed secondary growth, the fiber system is comprised of both these primary fibers as well as of secondary ones that belong to the secondary xylem and differentiated from the interfascicular vascular cambium (Lev-Yadun and Flaishman 2001; Altamura and others 2001). Typically, the vascular cambium is not always continuous in the inflorescence stem or may even be lacking altogether when growth conditions (especially low illumination, density and mineral nutrition) do not allow for its formation. Therefore, the secondary xylem and the secondary parts of the wavy band of fibers may sometimes form only in sectors of the circumference.

Our histological examination, especially when using polarized light, clearly revealed that a majority of the *revoluta/ifl1* plants produce the primary wavy fiber system of the inflorescence stems and that the general impression of a lack of the wavy band of fibers stems from their poorly lignified secondary walls. The flexibility in the differentiation of the wavy fiber band has already been shown at the physiological level. Submergence of the developing inflorescence stem of var. Columbia arrests both the lignification of the wavy band of primary fibers and the development of the cambium in the water-covered sector. When inflorescence stems elongate and emerge from the water, the emerged part resumes the production of the wavy fiber band (Lev-Yadun and Flaishman 2001). Thus, the mechanism for a full or a partial differentiation of the wavy fiber band seems to be quite flexible both in var. Columbia and in the mutant *revoluta/ifl1*. The level of polar auxin flow is known to influence the lignification of fiber cell walls in dicotyledons. Tension wood in dicotyledons is usually formed in the upper side of leaning stems and branches and is induced by lower-than-usual levels of auxin. It is characterized by gelatinous fibers, low in lignin and hemicelluloses and rich in cellulose. These fibers shorten and pull leaning stems and branches upward (Cronshaw and Morey 1965; Timell 1986). Because the *revoluta/if1l* has a dramatically reduced polar auxin flow in inflorescence stems (Zhong and Ye 2001; Zhong and others 2001), it probably induces changes in fiber wall structure, thus resembling those of lignin-poor tension wood fibers. This seems to be the reason for the pendent phenotype of the inflorescence stems of the *ifl1* plants shown in Zhong and others (1997), which have weak, poorly lignified fibers. The polar auxin flow in plants is carried out by a redundant mechanism. There are a number of different auxin efflux carriers or auxin response factors (Taiz and Zeiger 2002; Leyser and Day 2003). This redundancy seems to protect plants from damage if one of the auxin efflux carriers or auxin response factors does not function because of genetic or physiological reasons. It also enables delicate tuning of the system. More specifically, it might result in the developmental variability in the expression of fiber and cambial development in the *revoluta/ifl1* mutant.

An examination of the developmental status of the *revolute/ifl1* mutant as viewed from the published figures (Talbert and others 1995; Zhong and others 1997; Zhong and Ye 1999, 2001) indicates that the conclusions arrived at do not match some of the anatomical facts shown there. Figures 5G and 5J in Talbert and others (1995), Figure 2C in Zhong and others (1997), Figure 2A in Zhong and Ye (1999), Figures 1B, 1C and 10E in Zhong and Ye (2001) and Figure 2D in Zhong and others (2001) clearly show that the interfascicular fibers did form, but were impaired in their lignification, which resulted in poor staining of the secondary cell walls, causing confusion. In Figure 2C of Zhong and oth-

ers (1997) the primary fibers are clearly seen as 4–5 cell files below the arrow that indicates where the authors considered them to be missing. Similarly, the ''ectopic'' fibers in Figure 2A in Zhong and Ye (1999) and in Figure 2B in Zhong and Ye (2001) seem not to be ''ectopic'' but rather part of the natural anatomical variability of inflorescence stems of *A. thaliana*. The use of brightfield illumination alone and the staining with phloroglucinol-HCl (Zhong and others 1997) seems to have contributed to the misunderstanding.

Growth conditions may greatly influence the structure of inflorescence stems of *A. thaliana* (Lev-Yadun and Flaishman 2001). Illumination, for instance, may significantly influence the amount of secondary wood and fibers (Wyatt and others unpublished). Theoretically, the differences between our findings and those of Zhong and others (1997) and Zhong and Ye (1999, 2001) may reflect differences in growth conditions. We grew the plants under long night conditions (9 h light/15 h dark). There are no data in Zhong and others (1997) and Zhong and Ye (1999) concerning the growth conditions, and in Zhong and Ye (2001) only the fact that the plants grew under short night conditions (16 h light/8 h dark) was given. The differences in growth conditions are not the reason for the differences here because there are misidentified fibers in their plates.

We conclude that some of the previous anatomical descriptions of the *revoluta/ifl1* mutant were incomplete and that this problem influences the way we interpret the action of the gene. A regular use of polarized light can better indicate cells with secondary cell walls and help in characterization of the developmental status when regular staining fails. From the published sections and from our microscopic examination it seems that the *revoluta/ifl1* mutation mostly alters fiber lignification rather than blocking their formation altogether. Our proposal is further supported by the fact that a higher level of the *Rev/IFL1* mRNA results in thin-walled interfascicular fiber development (Zhong and Ye 2004). Therefore, the first name *REVOLUTA* given to this gene (Talbert and others 1995) suits it better than *IFL1*. This gene specifically reduces lignification in fibers and is thus of great significance for biotechnological developments within the paper industry and for the global economy and ecology in general.

ACKNOWLEDGMENTS

We thank the *Arabidopsis* Biological Resource Center (Ohio State University) for the seeds of the mutant and Kamelia Loginovsky for her technical assistance. We also thank two anonymous reviewers for their important comments.

REFERENCES

Altamura MM, Possenti M, Matteucci A, Baima S, Ruberti I, Morelli G. 2001. Development of the vascular system in the inflorescence stem of *Arabidopsis* New Phytol 151:381–389.

Beers, EP, Zhao, C (2001) "Arabidopsis as a model for investigating gene activity and function in vascular tissues" In: Morohoshi, N., Komamine, A. (editors), *Molecular breeding of woody plants*, Elsevier Science B.V., Amsterdam, pp 43-52.

Burk DH, Liu B, Zhong R, Morrison WH, Ye Z-H. 2001. A Katanin-like protein regulates normal cell wall biosynthesis and cell elongation Plant Cell 13:807–827.

Chaffey N. 2002. Why is there so little research into the cell biology of the secondary vascular system of trees? New Phytol 153:213–223.

Chaffey N, Cholewa E, Regan S, Sundberg B. 2002. Secondary xylem development in *Arabidopsis*: a model for wood formation Physiol Plant 114:594–600.

Cronshaw J, Morey PR. 1965. Induction of tension wood by 2,3,5-Tri-iodobenzoic acid Nature 205:816–818.

Dolan L, Janmaat K, Willemsen V, et al. 1993. Cellular organisation of the *Arabidopsis thaliana* root Development 119:71–84.

Dolan L, Roberts K. 1995. Secondary thickening in roots of *Arabidopsis thaliana*: anatomy and cell surface changes New Phytol 131:121–128.

Emery JF, Floyd SK, Alvarez J, et al. 2003. Radial patterning of *Arabidopsis* shoots by class III HD-ZIP and KANADI genes Curr Sci 13:1768–1774.

Flaishman MA, Loginovsky K, Lev-Yadun S. 2003. Regenerative xylem in inflorescence stems of *Arabidopsis thaliana* J Plant Gr Regul 22:253–258.

Floyd SK, Bowman JL. 2004. Ancient microRNA target sequences in plants Nature 428:485–486.

Funk V, Kositsup B, Zhao C, Beers EP. 2002. The Arabidopsis xylempeptidase XCP1 is a tracheary element vacuolar protein that may be a papain ortholog Plant Physiol 128:84–94.

Greb T, Clarenz O, Schäfer E, et al. 2003. Molecular analysis of the *LATERAL SUPPRESSOR* gene in *Arabidopsis* reveals a conserved control mechanism for axillary meristem formation Genes Dev 17:1175–1187.

Kirst M, Johnson AF, Baucom C, et al. 2003. Apparent homology of expressed genes from wood-forming tissues of loblolly pine (*Pinus taeda* L.) with *Arabidopsis thaliana* PNAS 100:7383–7388.

Ko J-H, Han K-H, Park S, Yang J. 2004. Plant body weight-induced secondary growth in Arabidopsis and its transcription phenotype revealed by whole-transcriptome profiling Plant Physiol 135:1069–1083.

Lev-Yadun S. 1994. Induction of sclereid differentiation in the pith of *Arabidopsis thaliana* (L.) Heynh J Exp Bot 45:1845–1849.

Lev-Yadun S. 1997. Fibres and fibre-sclereids in wild-type *Arabidopsis thaliana* Ann Bot 80:125–129.

Lev-Yadun S, Flaishman MA. 2001. The effect of submergence on ontogeny of cambium and secondary xylem and on fiber lignification in inflorescence stems of *Arabidopsis* IAWA J 22:159–169.

Leyser O, Day S. 2003. Mechanisms in plant development Oxford: Blackwell Publishing Company.

Little CHA, MacDonald JE, Olsson O. 2002. Involvement of indole-3-acetic acid in fascicular and interfascicular cambial growth and interfascicular extraxylary fiber differentiation in

Arabidopsis ihaliana inflorescence stems Int J Plant Sci 163:519–529.

Lyndon RF. 1990. Plant development: the cellular basis London: Unwin Hyman..

Mattsson J, Ckurshumova W, Berleth T. 2003. Auxin signalling in Arabidopsis leaf vascular development Plant Physiol 131:1327–1339.

McConnell JR, Emery J, Eshed Y, Bao N, Bowman J, Barton MK. 2001. Role of *PHABULOSA* and *PHAVOLUTA* in determining radial patterning in shoots Nature 411:709–713.

Meyerowitz EM. 1997. Genetic control of cell division patterns in developing plants Cell 88:299–308.

Nieminen KM, Kauppinen L, Helariutta Y. 2004. A weed for wood? Arabidopsis as a genetic model for xylem development Plant Physiol 135:653–659.

Oh S, Park S, Han K-H. 2003. Transcriptional regulation of secondary growth in *Arabidopsis thaliana* J Exp Bot 54:2709–2722.

Otsuga D, DeGuzman B, Prigge MJ, Drews GN, Clark SE. 2001. *REVOLUTA* regulates meristem mitiation at lateral positions Plant J 25:223–236.

Ratcliffe OJ, Riechmann JL, Zhang JZ. 2000. *INTERFASCICULAR FIBERLESS1* is the same gene as *REVOLUTA* Plant Cell 12:315–317.

Taiz L, Zeiger E. 2002. Plant physiology3 Sunderland: Sinauer Associates Inc. Publishers..

Talbert PB, Adler HT, Parks DW, Comai L. 1995. The *REVOLUTA* gene is necessary for apical meristem development and for limiting cell divisions in the leaves and stems of *Arabidopsis thaliana* Development 121:2723–2735.

Timell TE. 1986. Compression wood in gymnosperms Berlin: Springer-Verlag..

Ye Z-H. 2002. Vascular tissue differentiation and pattern formation in plants Annu Rev Plant Biol 53:183–202.

Ye Z-H, Freshour G, Hahn MG, Burk DH, Zhong R. 2002. Vascular development in *Arabidopsis* Crit Rev Cyol 220:225–256.

Zhao C, Johnson BJ, Kositsup B, Beers EP. 2000. Exploiting secondary growth in *Arabidopsis*. Construction of xylem and bark cDNA libraries and cloning of three xylem endopeptidases Plant Physiol 123:1185–1196.

Zhong R, Ye Z-H. 1999. *IFL1*, a gene regulating interfascicular fiber differentiation in Arabidopsis, encodes a homeodomain-leucine zipper protein Plant Cell 11:2139–2152.

Zhong R, Ye Z-H. 2001. Alteration of auxin polar transport in the Arabidopsis *ifl1* mutants Plant Physiol 126:549–563.

Zhong R, Ye Z-H. 2004. *Amphivasal vascular bundle 1*, a gain-of-function mutation of the *IFL1/REV gene*, is associated with alterations in the polarity of leaves, stems and carpels Plant Cell Physiol 45:369–385.

Zhong R, Taylor JJ, Ye Z-H. 1997. Disruption of interfascicular fiber differentiation in an Arabidopsis mutant Plant Cell 9:2159–2170.

Zhong R, Burk DH, Ye Z-H. 2001. Fibers. A model for studying cell differentiation, cell elongation, and cell wall biosynthesis Plant Physiol 126:477–479.