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Regenerative Xylem in Inflorescence Stems of *Arabidopsis thaliana*

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Abstract

By inserting entomological needles into the lower parts of young inflorescence stems of three-month-old *Arabidopsis thaliana* (L.) Heynh var. Colombia plants, we studied the process of regenerative xylem production. Regenerative xylem was formed only in one- to two-day-old inflorescence stems but not in older ones. The regenerative vessels originated from re-differentiation of cortical parenchyma. To characterize the process of regenerative xylem formation, we conducted a histological study from the time of wounding to day 30 after wounding. In the first day after wounding the tissues showed no structural responses except for the wounding itself. After six days, regenerative vessel members were already differentiating in a basipetal pattern, forming a vascular bypass around the wound. Regenerative vessel member formation reached a maximal level on the twelfth day after wounding. Sixteen days after wounding the pith parenchyma started to become loose as if indicating tissue senescence. Altogether, vascular regeneration following wounding in inflorescence stems of *Arabidopsis thaliana* is similar to that in other dicotyledon plants. These findings provide the basis for the use of *Arabidopsis thaliana* as a model system to study the genetics, physiology and cell biology of wound healing and regenerative vascular tissue formation.

Key words: *Arabidopsis thaliana*; Differentiation; Inflorescence stems; Regenerative xylem; Vascular differentiation; Wound healing; Xylem

Introduction

Considerable progress has been made in understanding the role of auxin, gibberellin and ethylene in regulating the differentiation of vascular and various secondary tissues and cell types from experiments involving wounding. Classic wounding experiments (Sinnott and Bloch 1945; Jacobs 1952; Roberts 1960; Roberts and Fosket 1962; Sachs 1981; Berleth and others 2000; and earlier works cited in these studies) in combination with external hormonal or inhibitor applications provided the fundamental developmental-physiological knowledge of the regulation of vascular differentiation. Earlier findings suggested that polar auxin transport induces polar vascular and fiber differentiation, and inhibits periderm (cork) development and dilatation activity, gibberellins are involved with auxin in fiber differentiation, and ethylene is a major activator in ray, cork and dilatation development (Savidge and Wareing 1981; Sachs 1981; Little and Savidge 1987; Lev-Yadun and Aloni 1990, 1992, 1995;

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Jacobs 1998; Fink 1999). Wounding itself influences cell-type differentiation (inhibiting fiber formation and inducing resin or gum ducts) or quantitative parameters, such as the proportion between cell types or cell size (Lev-Yadun 2000, 2002). However, many of the findings concerning the role of growth regulators have already been supported by studies using genetic and molecular methods or tissues of *Arabidopsis thaliana* (Romano and others 1991; Gälweiler and others 1998; Mattsson and others 1999; Sieburth 1999; Berleth and others 2000; Avsian-Kretchmer and others 2002; Little and others 2002).

Regenerative xylem formation following wounding is characterized by the re-differentiation of cortical parenchyma cells into vessel members without cell divisions. The differentiating regenerative vessel members deposit thick, lignified secondary cell walls, perforate their end walls that connect them to the next vessel member and die as do all vessel members. The stimulus for the process is believed to be the polar transport of auxin (Jacobs 1952; Sachs 1981). Very little has been done to characterize wounding in experimental systems of model plants using modern methods (Reymond and others 2000; Nishitani and others 2002). Reymond and others (2000) studied wounded rosette leaves of *Arabidopsis thaliana* and Nishitani and others (2002) used *Zinnia elegans*.

Even after all this progress in understanding the regulation of differentiation, we know only the basics of the processes. For a deeper and more detailed understanding of the primary and secondary plant body we need better systems for the studies. Establishing experimental systems in *Arabidopsis thaliana* for the study of wood and fiber formation may significantly increase the level of understanding of processes at the cellular and genetic levels (see Lev-Yadun 1994, 1997; Zhao and others 2000; Beers and Zhao 2001; Lev-Yadun and Flaishman 2001; Chaffey 2002; Chaffey and others 2002; Funk and others 2002; Little and others 2002). The objective of the present study was to examine the ability to produce and characterize the process of regenerative xylem production in inflorescence stems of *A. thaliana*.

Materials and Methods

Plant Materials and Growth Conditions

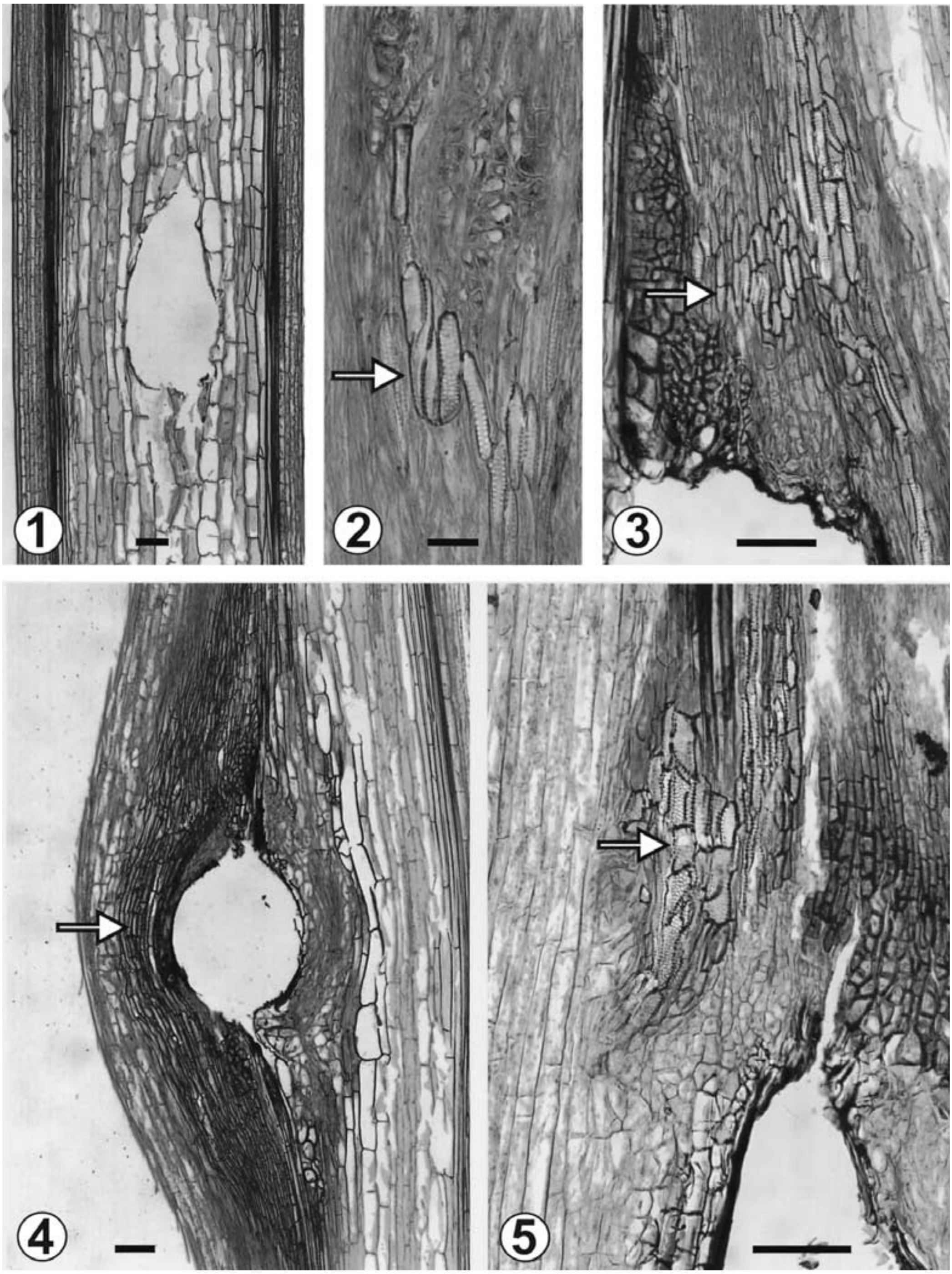
Seeds of *Arabidopsis thaliana* var. Columbia were germinated in the growth chamber under short day conditions (9 h light/15 h dark, 22 ± 1°C, 45 $\mu IS^{-1}m^{-2}$). Several dozens of single, seven-week-old rosettes were transferred to 1.7 liter pots filled with a mixture of peat/tuff/perlite (40%/40%/20% v/v) fertilized once a week with Osmocot (NPK) and irrigated twice a week. The light regime remained short-day (9 h light/15 h dark, 22 ± 1°C) for two months until the rosette leaves filled the pot. Then they were transferred to long-day conditions (12 h light/12 h dark) to stimulate flowering. Development of rosettes of *A. thaliana* larger than usual was induced by repeated excisions of inflorescences (Lev-Yadun 1994). Inflorescence stems that grew from these large rosettes were used for the experiments.

Wounding Experiments

Several preliminary sets of wounding experiments were done using var. Columbia. Wounding was done either by inserting needles into the inflorescence stems at various positions along their length or by making a longitudinal slit in the middle of the stems or a slit that partially separates a sector of the stem using a sharp razor blade. The wounded stems were sampled one or two weeks after wounding. For the detailed histological examinations we wounded approximately 100 one- to two-day-old (days from bolting) inflorescence stems of var. Columbia by inserting entomological needles that penetrated from side to side. The needles severed either some of the primary vascular strands, the interfascicular zone or both.

Screening for Regenerative Xylem Formation

For quick screening of the formation of regenerative xylem without sectioning, the stems were cleared and examined under the microscope. Clearing started by overnight incubation of the wounded stems in 95% ethanol at room temperature. They were then rinsed in water, boiled briefly in lactic acid, and kept immersed in the acid for 16 h. The cleared tissues were then washed several times in water and left immersed in water for 1 day to wash out the lactic acid. Some cleared stems were then stained with Safranin and fast green and some were not. Both stained and non-stained stems were dehydrated with absolute ethanol, washed and immersed in solvent for histology (Frutarum, Haifa, Israel; Cat. no. 55417), mounted with Entellan new (E. Merck, D-6100 Darmstadt, Germany; Cat. no. 7961) and dried on a hot plate for two days at 50°C. Slides were examined under bright field with a Leitz Dialux 20 microscope equipped with a Nikon F3 camera, at magnifications of ×16 to ×400.



All figures are tangential longitudinal sections through inflorescence stems of *Arabidopsis thaliana* wounded with a needle.
Figure 1. The tissue ruptured by the needle as seen one day after wounding with no changes indicating healing. Bar = 100 μm.
Figure 2. The first regenerating xylem (arrow), formed by re-differentiation of parenchyma, as seen six days after wounding. The newly formed vessel members that bypass the wound are not yet fully lignified. Bar = 40 μm.
Figure 3. An advanced stage in the formation of the regenerative xylem (arrow) as seen nine days after wounding. The upper part of the bypass formed by re-differentiation of parenchyma is many cell-files wide. The white patch at the bottom of the picture is the wound. Bar = 100 μm.
Figure 4. A general view of the wound and bypass 12 days after wounding. The regenerative xylem in the bypass (arrow) is fully lignified as indicated by the red staining. Bar = 100 μm.
Figure 5. A closer view of the wound 12 days after wounding. The regenerative xylem in the bypass (arrow) with its typical pitting is clearly seen. The white patch at the bottom of the picture is the wound. Bar = 100 μm.

Table 1. Regenerative Xylem Formation in Young Inflorescence Stems of *Arabidopsis thaliana* var. Columbia

Days after wounding	Rupture of tissue	Pith parenchyma attached to needle	Regenerative vessels differentiate	Vascular bypass fully developed
1	7			
2		7	2	
4		7	5	
6		7	7	
9		7	7	1
12		7	7	6
15		7	7	7
21		7		7
30		7		7

Seven stems were sectioned at each date; the numbers indicate how many showed the developmental change.

Histological Examination

After wounding of var. Columbia, seven inflorescence stems were sampled for each sampling date: time zero, 1, 2, 4, 6, 9, 12, 16, 21 and 30 days after wounding. From each specimen, a segment, about 1 cm long with the wounding at its center, was cut off and fixed in a mixture of 3:1 ethanol and glacial acetic acid overnight at room temperature. Several additional samples were taken for cross sectioning at each date but their examination showed that longitudinal sections were better for understanding wound-healing processes. 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 tangential longitudinal sections, 10 µm thick were prepared 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). These slides were examined under the microscope as described above.

Results

The Age of Inflorescence Stems Suitable for Regenerative Xylem Production

To examine the production of regenerative xylem in the inflorescence stems of *A. thaliana*, several preliminary sets of wounding experiments were done using var. Columbia. During the preliminary experiments we defined the best method, the location within the inflorescence stems and the timing for wounding. Many of the slits made with a razor blade resulted in stem breakage. Therefore, insertion of needles was chosen as the method of wounding. The lower part of the stem forms more xylem; therefore this part was used for further experiments. As it became clear that regular regenerative xylem formation following wounding occurred only in young inflorescence stems, only one- to two-day-old inflorescence stems were used for the final experiments. Inflorescence stems that were too thin often broke when wounded, and we used only thicker (approximately 2 mm) ones, some of which became about 2–3 mm thick at the end of the experiment.

The Development of Regenerative Xylem

On the first day after wounding the tissues showed no regeneration-related responses and the wound showed only the rupture of the tissues by the needles (Figure 1). After two days, the pith parenchyma cells were already pressed against the needles that formed the wounds. Four days after wounding, some pith parenchyma cells near the wounds had a somewhat denser cytoplasm and there were more cells with visible nuclei in the vicinity of wounds than in pith regions further away. After six days, the pith parenchyma usually changed cell orientation and the cells became larger. Sometimes, regenerative vessel members that originated from the cortical parenchyma were already formed near the wounding in a basipetal pattern (Figure 2). Nine days after wounding, many regenerative vessel-members in the cortex formed a vascular bypass around the wound (Figure 3). More regenerative vessel-members were seen 12 days after wounding (Figures 4, 5), some of which were spiral. The sections from later dates (16, 21 and 30 days after wounding) did not show more regenerative xylem (Table 1). Sixteen days after wounding, the pith parenchyma started to be loose as if showing tissue senescence.

Discussion

This study describes the formation of regenerative xylem in response to wounding of young inflorescence stems of *A. thaliana* at the histological level and examines basic physiological aspects, such as the influence of the age of tissue. The regenerative vessel members originated in the cortical parenchyma that re-differentiates, probably as a response to the changing polar auxin flow, following the wounding that damaged the original pattern of auxin flow.

The ability to form significant amounts of regenerative xylem declines with the age of the individual inflorescence stems of *Arabidopsis thaliana*. Probably, the physiological changes that cause the whole plant to perform monocarpic senescence, desiccation and death following flowering and seed production start to operate at the level of the inflorescence stem and influence its developmental plasticity. A simpler but related alternative explanation is that the polar auxin flow decreases, thus resulting in a decreasing ability to form regenerative xylem. The third possibility is that the cortical cells lose their ability to re-differentiate for another reason. Our experiments were designed only to show that regenerative xylem differentiation could be induced in *A. thaliana* and cannot answer these questions.

The polar auxin transport is the basic signal for xylem differentiation (Sachs 1981). In fact, the experimental manipulation of auxin transport has already been used to alter xylem differentiation in the inflorescence stem of *A. thaliana*. Application of auxin transport inhibitors resulted in the proliferation of vascular tissues, although vascular discontinuity was noted (Gälweiler and others 1998; Mattsson and others 1999). Recently, Little and others (2002) also showed that experimental modification of polar auxin transport in young inflorescence stems of *A. thaliana* altered secondary xylem production.

We conclude that young inflorescence stems of *Arabidopsis thaliana* have the ability to form regenerative xylem, and thus may serve as a model system for the study of the genetics and physiology of regenerative vascular differentiation. The data shown here help to close the gap in the availability of model systems and enable the use of *Arabidopsis thaliana* to illuminate the biology of the classic wounding experiments with the advantages of modern technical methods and perspectives. The presented findings will probably contribute to spreading the use of *A. thaliana* as a model for wood and fiber development.

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