Investigating development of phloem parenchyma transfer cells in *Arabidopsis thaliana*

by

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Thesis submitted for the degree of Doctor of Philosophy in Biological Science

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Declarations

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Statement of Originality
This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

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Acknowledgement of Authorship
I hereby certify that the work embodied in this thesis contains a published book chapter and three published papers, of which I am a joint author. I have included as part of the thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publications.

Thi Thu Suong Nguyen

Assoc/Prof. David McCurdy
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<tbody>
<tr>
<td>ABRC</td>
<td>Arabidopsis Biological Resource Centre</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine di phosphate</td>
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<tr>
<td>AFLP</td>
<td>amplified fragment length polymorphism</td>
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<tr>
<td>AP2</td>
<td>apetala2</td>
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<tr>
<td>ARF</td>
<td>auxin response factor</td>
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<tr>
<td>ARR</td>
<td>Arabidopsis response regulator</td>
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<tr>
<td>At</td>
<td>Arabidopsis thaliana</td>
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>BS</td>
<td>bundle sheath</td>
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<tr>
<td>CaMV-35S</td>
<td>cauliflower mosaic virus 35S</td>
</tr>
<tr>
<td>CC</td>
<td>companion cell</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinases</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
</tr>
<tr>
<td>Cq</td>
<td>quantitative cycle</td>
</tr>
<tr>
<td>DCB</td>
<td>2,6-dichlorobenzonitrile</td>
</tr>
<tr>
<td>DFR</td>
<td>dihydroflavonol reductase</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>FESEM</td>
<td>field emission SEM</td>
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<tr>
<td>FLC</td>
<td>flowering locus C</td>
</tr>
<tr>
<td>FLT</td>
<td>flowering locus T</td>
</tr>
<tr>
<td>FUL</td>
<td>Fruitfull</td>
</tr>
<tr>
<td>GI</td>
<td>Gigantea</td>
</tr>
<tr>
<td>H2O2</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HCL</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>LFY</td>
<td>Leafy</td>
</tr>
<tr>
<td>M</td>
<td>myrosin cell</td>
</tr>
<tr>
<td>mPS-PI</td>
<td>modified pseudo-Schiff propidium iodide</td>
</tr>
<tr>
<td>MQ</td>
<td>Milli Q</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>Na2S2O5</td>
<td>sodium metabisulphite</td>
</tr>
<tr>
<td>NTC</td>
<td>negative control</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PP</td>
<td>phloem parenchyma</td>
</tr>
<tr>
<td>RDR</td>
<td>RNA dependent RNA polymerase</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription/transcriptase</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>real time quantitative RT-PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SE</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SE/CC</td>
<td>sieve element/companion cell</td>
</tr>
<tr>
<td>SE</td>
<td>sieve element</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<tr>
<td>SGS</td>
<td>suppressor of gene silencing</td>
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<tr>
<td>SPL</td>
<td>squamosa promoter binding protein like</td>
</tr>
<tr>
<td>sRNA</td>
<td>small non-coding RNA</td>
</tr>
<tr>
<td>SQN</td>
<td>squint</td>
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<tr>
<td>SUC</td>
<td>sucrose transporter</td>
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<tr>
<td>SWEET</td>
<td>sugar uniporter</td>
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<tr>
<td>TC</td>
<td>transfer cell</td>
</tr>
<tr>
<td>TE</td>
<td>tracheary elements</td>
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<tr>
<td>TED</td>
<td>tracheary element differentiation</td>
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<tr>
<td>TEM</td>
<td>transimission electron microscopy</td>
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<tr>
<td>Tm</td>
<td>melting temperature</td>
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<tr>
<td>TCL1</td>
<td>trichomeless1</td>
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<tr>
<td>TRY</td>
<td>Triptychon</td>
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<tr>
<td>VND</td>
<td>vascular related NAC domain</td>
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<tr>
<td>VPC</td>
<td>vegetative phase change</td>
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<tr>
<td>XE</td>
<td>xylem element</td>
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<tr>
<td>Ze</td>
<td>Zinnia elegans</td>
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<tr>
<td>ZIP</td>
<td>zippy</td>
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<tr>
<td>Zm</td>
<td>Zea mays</td>
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### Units

- °C: degrees Celsius
- bp: base pair
- d: day
- g: gram
- h: hour
- L: litre
- M: molar
- m: metre
- min: minute
- NA: numerical aperture
- mol: mole
- s (sec): second
- v/v: volume for volume
- w/v: weight for volume

### Prefixes

- m: milli (10^{-3})
- µ: micro (10^{-6})
- n: nano (10^{-9})
Abstract

Recent advances in plant membrane transport research have shown that transport proteins embedded within membranes are key targets for improving plant performance, particularly under stresses such as salinity, pathogens or aluminum toxicity. Many transport processes augment plant performance via defined functions in specialized cell types or tissues. Plant transfer cells (TCs) are an example of such cell types. TCs possess increased densities of membrane transporters due to the increased plasma membrane surface area resulting from deposition of wall ingrowths - the unique structure that defines TC identity. These anatomically specialized cells are located at sites specialized for nutrient transport to facilitate apoplastic/symplasmic solute exchange. Therefore, optimizing transport performance by manipulating TC development may provide new avenues for improving plant performance and hence crop yield. Despite such potential, mechanisms underlying the process of TC development and wall ingrowth formation in particular are poorly understood. Therefore, the focus of this study was to elucidate the genetic control of TC development.

Most TCs are formed via a process known as trans-differentiation in which one differentiated cell type irreversibly switches into another cell type with distinct morphological and functional features. Various differentiated plant cell types including epidermal, vascular, gametophyte cells or root hairs, etc., can trans-differentiate into TCs by developing wall ingrowths on the inner surface of their primary cell wall. Among these diverse TC cell types, adaxial epidermal TCs in cultured Vicia faba cotyledons, endosperm TCs in seeds of maize and barley, and phloem parenchyma (PP) TCs in Arabidopsis thaliana (Arabidopsis), represent the three most extensively studied examples of TC development in plants. Due to the readily accessible genetic resources available in Arabidopsis, studying PP TC development provides an experimental system with unique attributes compared to the other systems. These PP TCs, similar to TCs in many other instances, however, are embedded deep within vascular bundles of leaves and leaf-like organs, and hence have mostly been studied by electron microscopy. To accommodate the use of Arabidopsis as a genetic system to study PP TC development,
a rapid way to visualize the extent of wall growth deposition and a simple but efficient method to score this process was imperative. To address this issue, a modified pseudo-Schiff-propidium iodide (mPS-PI) staining procedure was developed in combination with confocal microscopy to visualize wall ingrowth deposition in PP TCs in Arabidopsis. This approach enabled high-resolution three-dimensional imaging of polarized wall ingrowth deposition in PP TCs. Importantly, simplifying the original staining procedure by using bleach in place of lengthy extractions involving organic solvents, SDS/NaOH and enzymes enabled high-throughput assessment of PP TC development at the whole leaf level using a semi-quantitative scoring system to assess the extent of wall ingrowth deposition. A defoliation experiment was performed as an example of using this scoring system to analyze responses of PP TC development to leaf ablation. This staining technique was also employed successfully to image companion TCs in leaf minor veins of pea, implying its general applicability for imaging wall ingrowths in diverse species. The results from this study have been published in *BMC Plant Biology* as: Nguyen and McCurdy *BMC Plant Biology* (2015) 15:109, DOI 10.1186/s12870-015-0483-8.

With the robustness of the simplified mPS-PI staining method, the abundance and distribution of PP TC development in shoots of Arabidopsis ecotype Columbia-0 (Col-0) was surveyed. This analysis unexpectedly discovered that wall ingrowth deposition was highly abundant in early-emerged organs such as cotyledons and juvenile leaves, and remarkably less so in later-emerged adult leaves. This survey was extended to other Arabidopsis ecotypes including Landsberg erecta-0 (Ler-0) and Wassilewskja-2 (Ws-2), leading to the conclusion that wall ingrowth deposition in PP TCs represents a novel trait specific to heteroblasty or vegetative phase change (VPC), a phenomenon where plants progress through juvenile and adult stages of vegetative development. Importantly, mPS-PI staining revealed no major differences in xylem development between juvenile or adult leaves, implying that the responses seen in PP TC development across shoot maturation represent a cell wall deposition event specific to VPC.

In light of this finding, the potential regulatory role(s) for microRNA miR156 and its targets *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) transcription factor
The abundance of miR156, miR172, SPL3, SPL9, SPL10 and SPL15 all correlated with that of wall ingrowth deposition in PP TCs across shoot maturation from juvenile, transition and adult leaves, and across maturation of individual juvenile and adult leaves. In all cases, levels of miR156 accumulation showed a positive correlation with the extent of wall ingrowth deposition, whereas levels of SPL9, SPL10, SPL15, and to a lesser extent SPL3 and miR172, negatively correlated with wall ingrowth abundance. Additionally, altering the onset and/or progression of VPC by either prolonged leaf ablation, growth of plants under short days, or genetic manipulation of components of the miR156/SPL module, resulted in corresponding changes in levels of wall ingrowth deposition. In particular, overexpression of miR156 caused an increase in PP TC development, whereas reducing its accumulation or activity led to reduced wall ingrowth abundance. The spl9-4/spl15-1 double mutant had increased levels of wall ingrowth abundance compared to Col-0. Analysis of plants carrying miR156-resistant forms of SPLs, including rSPL3, rSPL9, rSPL10 and SPL15-1D lines, showed that wall ingrowth deposition was decreased in SPL9- but not SPL3-group genes, indicating that SPL9-group genes may function as negative regulators of wall ingrowth deposition in PP TCs. These findings represent a significant step towards a better understanding of the genetic pathways required for constructing wall ingrowths in PP TCs. A manuscript describing the novel linkage of PP TC development and VPC and the novel role for the miR156/SPL module in regulating PP TC development has been accepted to *Plant Physiology* (published on-line 12/01/2017).

The novel observation of heteroblastic development of PP TCs also contributes to the knowledge of physiological role(s) for wall ingrowth deposition in plant development. In many anatomical instances, the presence of TCs has been shown to optimize nutrient transport across relevant plasma membranes. Published work on PP TCs in Arabidopsis has also suggested a role for these cells in enhancing export of photoassimilates from collection phloem in leaves. However, the striking differences in wall ingrowth abundance reported here between mature juvenile and mature adult leaves call into question this role in Arabidopsis. Analysis of mutants disrupted in ATSWEET11/12 or SUC2, the two well-characterized classes of proteins functioning in
phloem loading in Arabidopsis, did not significantly affect wall ingrowth deposition in PP TCs. These and several other layers of evidence necessitate re-evaluating the general consensus that PP TCs in Arabidopsis, or at least wall ingrowth deposition in these cells, plays an important role in phloem loading. In light of these observations, a potential role for wall ingrowth deposition in PP TCs in acting as a physical barrier to defend access of invading pathogens to sugar-rich sieve elements is also discussed.

In summary, the study detailed in this thesis extends the currently limited understanding of the genetic regulation of TC development. The involvement of the miR156/SPL module as a regulator of heteroblastic development of PP TCs in Arabidopsis has been identified. Moreover, the simplified mPS-PI staining procedure used in combination with semi-quantitative assessment of wall ingrowth deposition in PP TCs provides a convenient, accurate and rapid way to analyze PP TC development for future studies of this process. In particular, this approach offers the prospect of using reverse genetics to identify essential genes controlling TC trans-differentiation.
Publications arising from this thesis

Book chapter:


Journal Articles:


CHAPTER 1

General Introduction
1.1 Introduction

Transfer cells (TCs), as their name suggests, play critical roles in transferring solutes at key anatomical sites for nutrient exchange within plants and between plants and their external environment (Gunning and Pate, 1969; Pate and Gunning, 1972). This transport capacity is conferred by wall ingrowths, which are wall protuberances deposited secondarily on the inner face of the primary cell wall and extending into the cell lumen. These ingrowths amplify the area of plasma membrane that lines them, therefore increasing the cell’s transport surface-to-volume ratio which in turn enhances potential trans-membrane flux of solutes (Gunning et al., 1968; Gunning and Pate, 1974).

TCs are ubiquitous as they occur in all examined plant species, from seedless plants to gymnosperms and flowering plants (Gunning and Pate, 1969; Pate and Gunning, 1972; Gunning, 1977). This ubiquity, which had been overlooked for decades since the first published observation of the cell type in 1884 by Fischer, was then fully recognized in a series of systematic and comprehensive reviews by Brian Gunning and John Pate (Gunning and Pate, 1969, 1974; Pate and Gunning, 1969, 1972; Gunning, 1977), and more recently by Offler et al. (2003). Anatomical sites of TCs fall into four main groups corresponding to four categories of trans-membrane flux: absorption/secretion of solutes from or to the external environment, and absorption/secretion of solutes from or to internal, extra-cytoplasmic compartments (Gunning and Pate, 1969). The existence of intensive nutrient transport, albeit coincides with the occurrence of TCs, does not always ensure wall ingrowth formation. For examples, TCs develop in both maternal and filial interfaces of *Vicia faba*, pea, wheat and barley seeds but are present at just the filial site of corn and sorghum seeds and absent in seeds of *Phaseolus vulgaris* and rice (Offler et al., 2003). Under certain stress situations or when abnormal stimuli are applied, wall ingrowth deposition can also be induced in cells that do not normally develop TCs. For instance, nematode infection induces giant TC-like cells in roots of potato (Jones and Northcote, 1972b) and coleus (Jones and Northcote, 1972a), or wall ingrowth formation can be induced in rhizodermal TCs of tomato upon iron and phosphorous starvation (Schikora and Schmidt, 2002). Collectively, these examples imply that most, if not all plants have the
general competency to make TCs, and that the absence of TCs under normal conditions merely because the plant does not make use of such genetic information (Gunning and Pate, 1974; Gunning, 1977). This observation has raised a long-standing question of why and when a given tissue of a given species develops TCs with wall ingrowths if its genome encodes capacity to do so. This question strongly implies that specific signals are required for TC development and thus response to these signals may vary depending on the developmental context of the tissue or its exposure to specific biotic or abiotic stresses.

A growing number of experimental systems and technologies have been applied to investigate TC biology since attention to these cells was drawn by Gunning and Pate’s reviews. This PhD project aims to explore physiological and genetic regulation of TC development. The TCs studied here are phloem parenchyma (PP) TCs in leaf veins of the model flowering plant *Arabidopsis thaliana* (Arabidopsis), and the particular question being asked is whether PP TC development is a novel trait specific to vegetative phase transition of shoots and also regulated by the genetic program controlling shoot ontogeny.

### 1.2 Transfer cells and the wall-membrane apparatus

TCs are characterized by a distinctive wall-membrane apparatus composed of the elaborate wall ingrowths together with the plasma membrane that lines them. This apparatus represents a unique structural/functional module especially well equipped to facilitate intense solute transport (Gunning and Pate, 1969; Pate and Gunning, 1972).

#### 1.2.1 Wall ingrowth structure and composition - a novel form of localized cell wall deposition

Wall ingrowths of TCs can be readily observed by light microscopy, such as the wall protuberances seen in hand-cut sections of *Tradescantia* stem node stained with Toluidine Blue (Gunning and Pate, 1974). To visualize individual wall ingrowths, however, electron microscopy, which provides much greater resolution, is indispensable. Transmission electron microscopy (TEM), scanning electron microscopy (SEM) and more
recently field emission SEM (FESEM) have been employed to study structural details of TCs (Talbot et al., 2001, 2002, 2007b; Offler et al., 2003).

In the early literature, TEM revealed the various morphologies of wall ingrowths in different species, with these ingrowth morphologies ranging from short, apparently papillate projections, to long, filiform structures, with or without being branched and/or interconnected, to robust flange or trabeculae architecture (Gunning and Pate, 1974; Gunning, 1977). This variation in wall ingrowth morphology occurs even within a given species possessing TCs in different anatomical locations, as strikingly seen in the shoot of Helianthemum, where companion cell (CC) TCs of leaf minor veins produce wall ingrowths with a flange morphology whilst ingrowths of xylem parenchyma TCs of the node have a branched, finger-like profile (Jones and Gunning, 1976). Another example of this variation is seen in the grass Lolium, where wall ingrowths in nodal TCs display flange morphology whereas nematode-induced giant cells in roots develop papillate wall ingrowths (Gunning and Pate, 1974). Superimposed on such differences, either species- or tissue-specific, there are two broad morphological categories of wall ingrowth: flange and reticulate (Pate and Gunning, 1972; Talbot et al., 2002), with reticulate being dominant across taxonomic groups (Offler et al., 2003).

TCs with flange wall ingrowth morphology are commonly associated with vascular tissue in grasses but infrequently are also found in other anatomical situations or in other taxa (Pate and Gunning, 1972; Offler et al., 2003). They are characterized by the presence of unliignified curvilinear bands of wall materials, which are similar morphologically in some instances to the lignified wall thickenings seen in tracheary elements (Gunning and Pate, 1974). These bands can be simple, thin ribs as in basal endosperm TCs in Zea mays kernels, an exception for this category since these cells are not vascular-derived, or more elaborate, thicker and larger bars of wall material as seen in xylem parenchyma TCs in nodes of Zea mays (Figure 1a), Triticum aestivum (Figure 1b), Oryza sativa and Festuca rubra (Talbot et al., 2002). These types of thickenings are generally Y-shaped when seen in transverse section (Zee and O’Brien, 1971).
Figure 1 Examples of different morphologies of wall ingrowths in TCs viewed by SEM. (a and b) Flange wall ingrowths in xylem parenchyma TCs of nodes of *Triticum aestivum* (a) and *Zea mays* (b). Flange ingrowths are roughly parallel, long barlike thickenings (arrows), which are similar in morphology but much thinner than wall thickenings of adjacent xylem elements (xe). tc TC. (c–e) Reticulate wall ingrowths in epidermal TCs of *V. faba* cotyledons. Small papillae (arrows) (c) are initially deposited at discrete but apparently random loci on the cytoplasmic face of the underlying wall and are often branched (arrowheads) (d). A multi-layered fenestrated labyrinth is deposited with layers numbered 1-3 (e). Arrows indicate nacent papillate ingrowths emerging from the new layer. Images taken from McCurdy et al. (2008).
The mechanism for deposition of flange wall ingrowths has been proposed to resemble that of secondary wall thickenings of tracheary elements (Talbot et al., 2007a; McCurdy et al., 2008). By contrast, reticulate wall ingrowths represent a novel example of localized wall deposition in plant cells (McCurdy et al., 2008; Talbot et al., 2007b). In *V. faba* cotyledons, visualization by FESEM revealed that wall ingrowth formation in adaxial epidermal cells is initiated by deposition of a thin, uniform layer of wall material over the underlying outer periclinal parent wall (Talbot et al., 2007b). This uniform wall layer is probably a prerequisite for the initial stage of reticulate morphology formation, where the deposition of papillae projections occurs at discrete but apparently random loci (Figure 1c; Talbot et al., 2007b; McCurdy et al., 2008). These projections branch and fuse laterally (Figure 1d) to initiate formation of a fenestrated sheet of wall material, providing a structural base for a new round of deposition from newly deposited papillae, ultimately constructing a multi-layered, fenestrated wall labyrinth (Figure 1e; Talbot et al., 2001; McCurdy et al., 2008). Wall ingrowth deposition is disrupted by the presence of the cellulose biosynthesis inhibitors 2,6-dichlorobenzonitrile (DCB) or isoxaben, suggesting a novel cellulose synthesis mechanism of the deposition of reticulate wall ingrowth morphology (Talbot et al., 2007b; McCurdy et al., 2008).

In transverse view by TEM, reticulate wall ingrowths consist of two distinct regions; an electron opaque inner region and an electron-translucent outer region (Talbot et al., 2007b). Compositional analyses by affinity probes and immunogold labelling showed that composition of the inner region is similar to that of primary walls, composed of cellulose, hemicelluloses and highly de-esterified pectins. The outer region of mature wall ingrowths is enriched in callose, which may provide a “spreading” influence on the penetration of wall ingrowths into the cytoplasm (Vaughn et al., 2007).

### 1.2.2 Functions of transfer cells

Initial confirmation of the functions for TCs is their widespread occurrences in numerous anatomical locations of central physiological significance in plants across the plant kingdom (Gunning and Pate, 1969). Examples of these sites are in the sporophyte-gametophyte junction of mosses (e.g., *Pinguicula*, *Drosophyllum*), in the epidermis of...
submerged leaves of aquatic plants (e.g., *Hydrilla, Vallisneria*), in gland cells of insectivorous plants (e.g., *Pinguicula, Drosophyllum*), in maternal/filial interface in seeds of monocots and dicots (e.g., faba bean, pea), in the basal endosperm of maize kernels or the nucellar projection of wheat seeds, and particularly in the vascular tissue of many crop species (e.g., soybean, kidney bean, mung bean, rice, wheat, pea) (Gunning and Pate, 1969; Pate and Gunning, 1972; Offler et al., 2003). In all these anatomical situations, TCs are postulated to optimize nutrient transport across the plasma membrane.

In addition to the extension of plasma membrane surface area, other ultrastructural specializations indicate the inferred role of solute transport by TCs. For instance, in cross sections of leaf minor veins of pea (*Pisum arvense*), CC TCs are distinguished from other cell types due to their dense cytoplasm, contributed to by a large population of mitochondria with well-developed cristae (Gunning et al., 1968). These organelles are often located close to the plasma membrane, between wall ingrowths or in the interstices of the invaginated labyrinth, and are also accompanied by organelles of the endomembrane secretory system, such as Golgi bodies, endoplasmic reticulum and secretory vesicles (Gunning and Pate, 1969). This unusual abundance of mitochondria in TCs is reckoned to accommodate energy demands for active transport of assimilates (Gunning and Pate, 1974). Another specialization is that the plasma membrane of TCs, particularly at the portions associated with wall ingrowths, are enriched in H⁺-ATPase and sucrose transporters (Harrington et al., 1997a; Tegeder et al., 1999). Recently, Chen et al. (2012) discovered the SWEET sucrose efflux transporters, the long-sought missing link in sucrose phloem loading, in Arabidopsis. These effluxers secrete sucrose into the apoplasm by a uniport mechanism and are localized to the plasma membrane of PP TCs in leaf veins. Collectively, the wall-membrane apparatus of TCs are likely to be the principal sites of facilitated membrane transport.

An interesting conflicting consideration first raised by Pate and Gunning (1972) is how efficiently water and solute molecules diffuse through the massive fabric of wall ingrowths before or after successfully crossing the plasma membrane barrier. The extensive wall ingrowth labyrinths seen in some TCs might actually deter efficient diffusional movement of solutes, thus it is necessary to consider permeability of the wall-membrane apparatus when considering the function of TCs.
1.3 Development of transfer cells

1.3.1 Trans-differentiation of transfer cells

TCs form from diverse differentiated cells types through a process known as trans-differentiation (Offler et al., 2003). Trans-differentiation is defined as an irreversible conversion of one differentiated cell type into another cell type with distinct functional and morphological features, and occurs in both plant and animal cells (Okada, 1991; Sugiyama and Komamine, 1990). In the animal literature, this process has received substantial attention by biologists due to its therapeutic potential in regenerative medicine (Tosh and Slack, 2002; Burke and Tosh, 2005). In contrast, trans-differentiation of plant cells has been overlooked although this phenomenon was recognized very early in plant research (Jacobs, 1952 and references therein). Recently, Nguyen and McCurdy (2016; see Appendix III) reviewed representative examples of trans-differentiation in plants including employing criteria used in animal literature to evaluate these examples. This review also discussed current understanding of molecular pathways underlying this fascinating biological phenomenon, including the trans-differentiation of TCs (see Appendix III).

1.3.2 Transfer cell development in response to developmental cues and external stresses

The development of wall ingrowths in TCs is strongly correlated with the degree of activity of TCs in terms of transporting solutes (Gunning and Pate, 1969; Offler et al., 2003). A clear demonstration of this relationship can be seen in Pisum arvense leaf minor veins, where CC TCs begin to develop their wall ingrowths at the onset of solute flux, and the extent of wall ingrowths roughly parallels the increased export capacity of the leaf as it matures (Gunning and Pate, 1974; Gunning et al., 1968). Comparatively, Ade-Ademilua and Botha (2008) reported a positive correlation between the level of wall ingrowth protrusion of CC TCs in Pisum sativum leaf minor veins and the transition of the leaf from sink to source stage based on Leaf Plastochron Index (LPI). This transition commences at LPI 0 when the walls of CC TCs appear to be in the process of protruding ingrowths, then continues to an advanced stage at LPI 0.5 when the wall ingrowths are more abundant and pronounced and reaches the source stage at LPI 1-2.
in which the wall ingrowths are even more numerous and form elaborated and anastomosed structures (Ade-Ademilua and Botha, 2008). Similarly, in *V. faba*, wall ingrowths in the secretory cells of the extra-floral nectaries are evident only after sugar secretion starts (Gunning and Pate, 1969), and in developing seeds, the development of maternal and filial TCs is correlated with a dramatic increase in the rate of cotyledon dry weight accumulation (Bonnemain et al., 1991; Offler et al., 1997). The degree of wall ingrowth development is also concomitant with the intensity of lateral exchange of solutes to or from sieve elements (SEs) and the xylem. For instance, in the shoot vascular system of *Pisum arvense*, phloem TCs are most prominent in leaf minor veins but less abundant in the stem, whereas xylem TCs are absent in leaf minor veins, occasionally are seen in petioles but are very abundant in the stem (Gunning and Pate, 1969).

The correlation between the development of TCs and their solute transport activity is given more credence by the observation that the absence or reduction of solutes destined to be exported prevents presumptive TCs from developing or developing completely their wall ingrowths. Conversely, the external stress which abnormally increases demand for solute transport can induce TC differentiation in the cells which are not normally predestined to form TCs. For example, CC TCs in pea leaf minor veins failed to complete wall ingrowth formation when the leaf was kept in darkness (Gunning et al., 1968), but the effect was reversed by a supplementary supply of glucose (Henry and Steer, 1980). Similarly, in cotyledonary nodes of lettuce seedlings, reduction of the upward flow of solutes in xylem caused by manipulated nutritional status of seedlings (grown in either total darkness, with one cotyledon removed, darkened, or in depleted carbon dioxide) stifled the development of xylem-associated TCs. When the seedlings deprived of carbon dioxide were transferred to a normal atmosphere, xylem TCs rapidly initiated regrowth of wall ingrowths (Pate et al., 1970).

Salt stress is a typical example of external stresses inducing the formation of TCs. Kramer et al. (1978) first reported that when the halophyte plant *Atriplex hastata* was grown in high salinity, the epidermal cells and root hairs in the absorbing part of roots *trans*-differentiated to form TCs with extensive wall labyrinths and large numerous
mitochondria. The formation of these wall labyrinths was confined to the outer tangential walls of the cells facing the external medium. This localized formation of wall ingrowths as an adaptation to salt stress was also observed in exodermal cells adjacent to dead epidermal cells which directly exposed them to the nutrient solution. When *Atriplex hastata* plants were grown without salt or at low salt concentrations, no substantial wall labyrinth formation observed (Kramer et al., 1978). Similarly, the complexity of invaginated wall ingrowths, hence the surface area of plasma membrane, in leaf blade epidermal TCs of the seagrass *Zostera capensis* increased with increasing salinity (Iyer and Barnabas, 1993). Therefore, salt stress has been assumed, at least in *Atriplex hastata* and *Zostera capensis*, to have stimulatory effects on the development of wall ingrowths in TCs, however the mechanism causing this response is unknown. In the case of *Zostera capensis*, it may involve the increased production of photosynthate due to the increased number of chloroplasts in leaf epidermal cells exposed to high salinity (Iyer and Barnabas, 1993). Since there is a paucity of plasmodesmata connecting epidermal and mesophyll cells, photosynthate is transferred apoplasmically from the sites of photosynthesis in the epidermis to the interior tissues of the leaf. This movement can be enhanced by enlarged plasma membrane surface area resulting from salt stress-induced wall labyrinths. Such postulation is further supported by an interesting observation that in another seagrass species, *Posidonia australis*, which does not have epidermal TCs, symplasmic connections between epidermal and mesophyll cells are numerous (Iyer and Barnabas, 1993).

### 1.3.3 Signals regulating transfer cell development

Given the important role of sugars and sugar metabolism as signaling elements in plants (Rolland et al., 2006; Ruan, 2014), and the coincidence of TC development with intensive demand for solute transport, whether coordinating with organ development or induced by external stress, it is tempting to postulate that transported sugars are likely inductive signalling molecules for the *trans*-differentiation of TCs. However, since not every transport cell becomes a TC, and TC development is only one of many morphological responses to stress, it appears that sugars are more likely to be an integral component of a suite of coordinated signals that plants employ to specify
whether or not, and in what anatomical locations, to express the genetic competency to make TCs (Gunning, 1977; Offler et al., 2003). The inductive signals and signaling cascades eliciting TC morphology have been extensively investigated in developing seeds of *V. faba*.

Abaxial epidermal cells of *V. faba* cotyledons *trans*-differentiate to form epidermal TCs (Bonnemain et al., 1991; Gunning and Pate, 1974; Offler et al., 1989) immediately prior to seed fill (Offler et al., 1997). In contrast, their adaxial counterparts do not exhibit TC morphology *in planta*, but can be induced to form wall ingrowths when excised cotyledons are cultured with their adaxial surface in contact with an agar-based medium (Offler et al., 1997). This *in vitro* induction of wall ingrowth formation occurs rapidly within three hours after cotyledon excision (Wardini et al., 2007), forming a large population of TCs which are morphologically comparable to abaxial epidermal TCs differentiated *in vivo* (Talbot et al., 2001) and also mimic their enhanced capacity for sucrose transport (Farley et al., 2000). Furthermore, the filial cotyledons can be easily removed from the maternal seed coats without cellular damage by virtue of their cellular isolation (Offler et al., 1997), and the *in vitro* induced adaxial epidermis can be peeled from cotyledons, hence offering a relatively large amount of material readily accessible to experimental approaches (Dibley et al., 2009). Collectively, developing seeds of *V. faba* offer a powerful experimental system to study TC biology.

Based on findings of *in vitro*-induced developmental events of adaxial epidermal TCs of *V. faba* cotyledons, an auxin-ethylene-ROS cascade has been proposed to regulate the development of wall ingrowths in epidermal TCs. The *in planta* formation of wall ingrowths coincides with the onset of storage when the cotyledons enlarge and crush the thin-walled parenchyma cells of the abutting seed coat (Harrington et al., 1997b), consequently leading to a spike in ethylene production (Andriunas et al., 2011). Wound-induced ethylene may in turn drive a concomitant ethylene burst in the juxtaposed abaxial epidermal cells, initiating their *trans*-differentiation to form wall ingrowth morphology (Zhou et al., 2010). Crushing of seed coat cells concurrently brings about the localized down regulation of their extracellular invertase activity (Harrington et al., 1997b), subsequently resulting in a decline of
intracellular glucose concentrations in abaxial epidermal cells (Andriunas et al., 2011). This event counteracts glucose repression of an ethylene signaling cascade, hence activating ethylene response genes and leading to initiation and construction of wall ingrowths (Andriunas et al., 2011). Removing the glucose block also causes a burst in extracellular reactive oxygen species (ROS), in particular H$_2$O$_2$. The extracellular H$_2$O$_2$ has been suggested to induce expression of cell wall biosynthesis genes and act as a polarizing signal that directs the deposition of wall ingrowths (Andriunas et al., 2012; Xia et al., 2012). However, in this *in planta* proposed regulatory pathway, the roles of auxin as part of the suites of coordinated signals is currently unclear (Andriunas et al., 2013), although it was confirmed that auxin indeed induced wall ingrowth formation in adaxial epidermal cells of cultured cotyledons, and likely acted upstream of ethylene (Dibley et al., 2009; Zhou et al., 2010). In addition, it has recently been suggested that a novel cytosolic Ca$^{2+}$ signal spatially directs formation of wall ingrowth papillae at specific loci (Zhang et al., 2014).

1.4 Transfer cells in the leaf vascular system

1.4.1 Examples of transfer cells in the leaf vascular system

A taxonomic and morphological survey of TCs in leaf minor veins of nearly one thousand Angiosperm species revealed that these specialized cells are highly restricted to dicotyledons, and within this group TCs are predominantly associated with herbaceous species, with very few exceptions found in woody plants (Pate and Gunning, 1969). These TCs, along with their counterparts in stem and root vascular tissue, were predicted to function in long-distance transport (Gunning and Pate, 1969, 1974). Four types of TCs in leaf minor veins have been described based on morphological attributes: types A and B are modifications of phloem cells, and far more widespread and conspicuous than the other two types, C and D, which are modified xylem parenchyma and modified bundle sheath cells, respectively (Pate and Gunning, 1969). Phloem TCs, of which one type is the subject of this present study, are described in further detail below.
The Type A TC is regarded as a modified CC by virtue of their anatomical equivalence, hence called CC TCs (Pate and Gunning, 1969). The term “transfer cell” was originally applied to this cell type in an earlier comprehensive study of its structure and functional significance (Gunning et al., 1968), and then used in a wider context (Gunning and Pate, 1969). CC TCs are characterized by highly dense cytoplasm and relatively non-polarized distribution of wall protuberances or ingrowths. These protuberances are observed around the cell periphery but less prolific on the wall regions which are connected to their associated SEs via numerous plasmodesmata. Type B TCs, known as PP TCs, however, differ strikingly from Type A TCs, with less dense cytoplasmic content and more labyrinthine and a highly polarized distribution of wall ingrowths restricted to the wall regions contiguous with SEs and/or CCs. Both these TC types have numerous mitochondria and well developed endoplasmic reticulum (Gunning et al., 1968; Pate and Gunning 1969; see also Amiard et al., 2007; Wimmers and Turgeon, 1991).

CC TCs are specialized to function in the apoplastic step of phloem loading, where their wall ingrowths facilitate uptake of solutes from the apoplast and then pass them symplasmically to the SEs, hence improving the net flux of photoassimilates translocated in the whole plant (Gunning et al., 1968; Wimmers and Turgeon, 1991). The potential role of PP TCs in enhancing the transport of solutes, however, is a matter of conjecture (Haritatos et al., 2000; Pate and Gunning, 1969), and is discussed in the next section.

1.4.2 Phloem parenchyma transfer cells in Arabidopsis leaf veins

TCs (type B) in Arabidopsis are known to develop in PP located in the minor vein network of leaves (Haritatos et al., 2000) and sepals (Chen et al., 2012). PP TCs possess bulky wall ingrowths which predominantly abut SEs and to a lesser extent CCs (Figure 2; Amiard et al., 2007; Haritatos et al., 2000). These three cell types together construct the minor vein phloem with proportionate numbers of cells of each type relatively consistent throughout the vein system regardless of vein order. If one observes a typical minor vein in transverse section with five SEs, it is expected that these SEs will be accompanied by seven PP cells and ten CCs (Haritatos et al., 2000). SEs are smaller
than CCs, as in the common collection phloem described by van Bel (1996), and PP cells are larger than CCs (Haritatos et al., 2000). Distinct from other dicotyledonous species which normally have six or seven leaf vein orders, Arabidopsis leaves display typically three or four vein orders (Haritatos et al., 2000) and sometimes up to five (Kang et al., 2007). Almost all veins in Arabidopsis leaves are considered as minor veins (Haritatos et al., 2000), whereas in most dicotyledons they are the fourth or higher orders (Gamalei, 1989). This conclusion results from the observation that a majority of veins in Arabidopsis leaves are found to be intimately embedded in the mesophyll, lacking specialized mechanical supporting cells, therefore, offering sites for phloem loading of photoassimilates (Haritatos et al., 2000). Correspondingly, Chen et al. (2012) discovered that two sucrose effluxers, AtSWEET11 and 12, are present in the phloem tissue, in all probability in PP cells, including both classically defined major and minor veins, which in Arabidopsis are collectively predicted to function physiologically as minor veins (Haritatos et al., 2000).

Pate and Gunning (1969) have speculated that all four types of the leaf minor vein TCs, type A, B, C and D, may function in absorbing solutes from the apoplastic compartment of the vein. However, there are suggestions that type B TCs, at least in Arabidopsis leaf minor veins, do not fall into this functional category. Unlike CC TCs which have the function of uptake of apoplastic nutrients, PP TCs have been assumed to facilitate the efflux of photoassimilates into the apoplasm (Haritatos et al., 2000). This functional potential is conferred by a number of structural details, such as abundant plasmodesmata connecting PP TCs and neighboring bundle sheath cells, offering a symplasmic avenue for sucrose to be delivered from the site of photosynthesis in mesophyll cells, and prominent wall ingrowths of PP TCs asymmetrically deposited on the wall adjacent to SEs and CCs, hence the delivered sucrose is likely pumped out to the apoplast across the plasma membrane associated with these ingrowths (Figure 2; Amiard et al., 2007; Haritatos et al., 2000). In accordance with these observations, the discovery of SWEET effluxers in PP cell plasma membrane putatively associated with wall ingrowths (Chen et al., 2012) strongly supports this assumption.
General introduction

Figure 2 TEM views of PP TCs in Arabidopsis minor veins. (a) Cross-section of a minor vein showing the arrangement of sieve elements (SE), companion cells (CC) and PP TCs (PC). In this image the authors have highlighted in white the areas of wall ingrowth deposition in PCs. The black arrow indicates a junction of a SE, CC and PC, equivalent to the area shown in (b). (b) Higher magnification view of a junction of SE, CC and PP TC (PP) showing localized deposition of wall ingrowths adjacent to SE/CC junction (comparable to the area indicated by the black arrow in (a)). Note also the presence of branched plasmodesmata (arrow) between PP TC and CC. Scale bar = 0.4µm in (b). Image (a) taken from Amiard et al. (2007) and (b) from Haritatos et al. (2000).

A function in defense has also been proposed for wall ingrowth deposition in Arabidopsis PP TCs. Amiard et al. (2007) suggested that the bulky and highly localized wall ingrowth deposition in PP TCs adjacent to SEs may act as a potential barrier to impede systemic pathogen spread, given that PP cells are often the primary target of phloem-feeding pathogens (Ding et al., 1998; Heller and Gierth, 2001). This assumption is further supported by the observations that the extent of wall ingrowth formation in PP TCs can be triggered by jasmonic acid (Amiard et al., 2007; Demmig-Adams et al., 2013). Moreover, aphids, the major group of phloem-feeding insects, activate jasmonic-acid-mediated defense pathways (Louis et al., 2012) and stimulate cell wall modifications (Divol et al., 2005) when feeding on a host plant. Chen et al. (2012) also suggested that the highly localized deposition of wall ingrowths in PP TCs restricts sucrose delivery into the apoplasm specifically to the interface adjacent to SE-CCs, thus limiting the availability of this apoplastic sugar potentially accessed by pathogens. CC TCs in pea typically avoid pathogenic attack and their cell wall invaginations were not enhanced by exogenous methyl jasmonate treatment (Amiard et al., 2007). These
observations collectively support a dual function of PP TCs in enhanced phloem loading and in defense against pathogenic attack.

1.4.3 Signals regulating transfer cell development in Arabidopsis leaf veins

In Arabidopsis, the extent of wall ingrowth deposition in PP TCs is responsive to various stresses, such as exposure to high light or methyl jasmonate (Amiard et al., 2007). These authors measured plasma membrane length in PP TCs from TEM images of plants exposed to either low (100 µmol m\(^{-2}\) s\(^{-1}\)) or high (1000 µmol m\(^{-2}\) s\(^{-1}\)) light, or plants grown under low light and sprayed with methyl jasmonate. These treatments caused greater than two fold increases in plasma membrane length (a proxy for the extent of wall ingrowth deposition) compared to controls (Amiard et al., 2007). These enhanced cell wall thickenings were hypothesized to be triggered by oxylipins, namely jasmonic acid, of which biosynthesis is promoted by increased production of ROS resulting from oxidative stress under high light exposure (Amiard et al., 2007). Their hypothesis was supported by the observation that this response to high light or methyl jasmonate did not occur in the fad7-1 fad8-1 double mutant. This mutant lacks two fatty acid desaturases which generate the polyunsaturated fatty acid alpha linoleic acid, hence blocking synthesis of oxylipin (Falcone et al., 2004). In response to transfer from low light to high light, this jasmonate-deficient mutant showed a significantly lower level of wall ingrowths in PP TCs compared to wild-type, supporting a role for jasmonic acid in up-regulating wall ingrowth formation (Amiad et al., 2007). To further examine this role, Demmig-Adams et al. (2013) exposed the npq1-2 lut2-1 double mutant, deficient in both zeaxanthin and its isomer lutein, to high light. Zeaxanthin and/or lutein are required for photoprotective processes including thermal energy dissipation (which pre-emptively prevents the formation of ROS) and detoxification of already-formed ROS (Baroli and Niyogi, 2000). During these processes, the production of ROS is lowered, leading to the suppression of oxylipin formation and consequently blocking increased wall ingrowth deposition caused by exposure to high light. Therefore, due to the absence of zeaxanthin/lutein-dependent photoprotection and consequently augmented production of oxylipin, transfer of the npq1-2 lut2-1 double mutant from 150 to 350 µmol m\(^{-2}\) s\(^{-1}\) for one week resulted in these
plants displaying enhanced levels of wall ingrowths compared to wild-type (Demmig-Adams et al., 2013).

Cold stress has also been shown to enhance wall ingrowth deposition in PP TCs. When 3- to 4-week-old wild-type plants were grown at 7.5°C for 14 days, PP TCs showed a noticeably increased wall ingrowth deposition in the wall regions adjacent to SEs and CCs (Maeda et al., 2006). Given that cold acclimation induces accumulation of ROS in Arabidopsis (Chinnusamy et al., 2010) and that plant responses to cold stress has been suggested to be directly linked to ROS signaling (Huang et al., 2010), the extent of PP TC formation in Arabidopsis leaf veins as an adaptation to low temperature is likely regulated via a ROS-associated signaling pathway.

To investigate regulatory pathways of PP TC development in Arabidopsis, Edwards et al. (2010) performed a hierarchical bioinformatics analysis of public microarray datasets analyzing changes in gene expression in leaf tissue of plants exposed to high light and cold treatment. From this analysis the authors unexpectedly identified GIGANTEA (GI) as a gene involved in regulating wall ingrowth deposition. The occurrence of PP TCs in GI mutants (gi-3 and gi-2) was reduced 15-fold compared to wild-type. When these mutants were exposed to high light or cold conditions, no enhanced wall ingrowth formation was observed. Since gi-2 mutants have an increased capacity to detoxify ROS (Cao et al., 2006), Edwards et al. (2010) proposed that GI may influence wall ingrowth deposition downstream of a ROS-acting signaling pathway. This observation also supports a potential role for ROS in the ongoing development of wall ingrowths (Edwards et al., 2010).

Collectively, ROS generated in response to abiotic stress such as high light or low temperature may account for the extent, but not induction, of PP TC development in Arabidopsis. Wall ingrowth deposition in PP TCs is highly polarized to the cell wall region adjacent to SEs and CCs (Amiard et al., 2007; Haritatos et al., 2000), therefore presumably it is induced by a localized ROS signal. Andriunas et al. (2013) suggested that ROS in SEs undergoing partially programmed cell death is the best candidate for generating localized extracellular ROS. Once produced in SEs, ROS could be transported through aquaporins into the apoplasm, which then provides positional information to induce cell wall synthesis and polarized wall ingrowth deposition in abutting PP cells.
(Andriunas et al., 2013). Interestingly, in early literature, it was also assumed that stimuli emanating from neighboring compartments regulate which specific regions of cell wall should be depositing wall ingrowths in TCs (Pate and Gunning, 1972).

Another factor which has been hypothesized to participate in directing the polarization of mature wall ingrowths is polyunsaturated fatty acid composition of endoplasmic reticulum-derived membrane lipids (Maeda et al., 2008). Alteration of this composition, as a result of tocopherol deficiency, may affect properties of Golgi-derived vesicles possibly involved in deposition of polarized wall ingrowth maturation, hence leading to abnormal cell wall morphology of PP TCs in low-temperature-treated vte2 mutants (tocopherol-deficient mutants) (Maeda et al., 2008; Maeda et al., 2006). Unlike wall ingrowth deposition confined to SE and/or CC boundary as seen in PP TCs of low temperature-treated wild-type (Col-0) and untreated Col-0 and vte2, wall ingrowths were present around the entire PP TC periphery in low temperature-treated vte2 (Maeda et al., 2006). Other low temperature-induced vte2 phenotypes include heavy deposition of callose around and in the wall ingrowths coinciding with rapid reduction of photoassimilate export, followed by increased soluble sugar levels in leaves (Maeda et al., 2006).

1.5 Transfer cells and heteroblasty

One of the main hurdles of TC study is the lack of robust means to assess the abundance of wall ingrowths and TCs since these cells are typically buried deep within inner tissues such as vascular bundles, and hence necessitating the use of TEM or SEM for observation. Therefore, the first aim of this thesis is to adapt available fluorescence-based staining approaches to stain PP TCs in Arabidopsis as alternatives to time-consuming electron microscopy techniques. The modified pseudo-Schiff propidium iodide (mPS-PI) staining technique of Truernit et al. (2008) was successfully adapted for this purpose, which enabled high-resolution three dimensional imaging of wall ingrowths in PP TCs by confocal microscopy (Nguyen and McCurdy, 2015; see Chapter 2). This procedure enables PP TC abundance to be assessed at different developmental stages at the whole leaf, and more importantly, at the whole shoot
levels. During the course of mapping the presence of PP TCs across rosette development as a reference for further studies of PP TC development, it was apparent that the abundance of wall ingrowth deposition in PP TCs and the distribution of PP TCs with abundant wall ingrowths were distinctively different at different developmental stages of shoot ontogeny. For example, early-emerged leaves displayed PP TCs with abundant wall ingrowths, and these PP TCs were more-or-less uniformly distributed across the whole leaf vein system (except the basal half of midvein). In contrast, in later-emerged adult leaves, PP TCs contained much less abundant wall ingrowth deposition, these PP TCs were restricted to minor veins, and were distributed across the leaf in a distinctive basipetal gradient (see Chapter 3). These observations suggested that wall ingrowth deposition in PP TCs in Arabidopsis leaves reflects heteroblasty.

Genetic regulation of heteroblasty and phase transitions of shoot growth has been studied extensively over the last twenty years, yielding great progress in understanding of the roles of small non-coding RNAs (sRNAs) in controlling shoot maturation (Poethig, 2013; Fourcacre and Poethig, 2016). This background offers a fertile platform to study heteroblastic development of PP TCs in Arabidopsis, hence providing an opportunity to move forward the understanding of TC biology more generally. Therefore, the main biological question to be addressed in this thesis is whether heteroblastic features of PP TCs in Arabidopsis are controlled by the same genetic mechanism regulating shoot ontogeny (Chapter 3). Consequently, the next sections of this introductory chapter are devoted to reviewing heteroblasty and other related phenomena of plant shoot maturation, and in particular focusing on vegetative phase change.

1.6 Historical review of the concepts of heteroblasty, juvenility and phase change in shoot development

1.6.1 Argumentation about Goebel’s original sense of terms

The term “heteroblasty” is derived from Greek words (“heteros”: different; “blastos”: sprout or shoot) and was originally coined by Karl Goebel to describe ontogenetic
changes in morphological features of shoots with these changes being abrupt and substantial, as opposed to “homoblasty”, which describes subtle and gradual changes in shoot morphology during ontogeny (Goebel, 1900). More or less a century later, usage of the term has changed considerably, in at least two aspects. Firstly, the distinction between heteroblasty and homoblasty has virtually disappeared or has become meaningless as slight changes in leaf size or shape have been referred to as heteroblastic development of leaf morphology (Zotz et al., 2011). This is not surprising, however, because Goebel himself was also aware of an inherent problem of his concept lying in the fact that there is no sharp distinction between these two types of development (Allsopp, 1967), and thus the term “heteroblasty” was meaningful only in a relative sense (Brink, 1962). It is now commonly accepted that heteroblasty also includes gradual transitions in shoot morphology whereby almost all plants display a certain degree of morphological variation along the shoot axis (Bongard-Pierce et al., 1996; Zotz et al., 2011). Nonetheless, some plant biologists, particularly in New Zealand, fully support the use of Goebel’s original sense of heteroblasty because it describes a significant aspect of New Zealand flora (Sooda et al., 2011; Jameson and Clemens, 2015). Secondly, the notion of heteroblastic development has become not only restricted to morphological features of shoots but has also been extended to embrace ontogenetic variation in anatomical, physiological and biochemical traits such as epidermal features, leaf venation patterns, photosynthetic rate, rooting ability of cuttings, disease or insect resistance, production of anthocyanin and chlorophyll, etc. (Kerstetter and Poethig, 1998; Poethig, 2013).

Other terms introduced by Goebel were “juvenile” and “adult” to describe earlier and later “sections of the developmental history” of shoots, respectively (Goebel, 1900). He noted that the appearance of germ-cells or the formation of sexual propagative organs always marks a climax of development and thus called a long series of developmental stages intercalated between germination and such climax “juvenile stages”. The “adult form”, according to Goebel, has the chief duty to produce sexual organs. In subsequent literature, juvenile phase is defined as developmental stages occurring after germination and during which the bud meristem is not competent to respond to floral inductive signals, hence unable to flower even under inductive
conditions; whereas the beginning of adult phase is recognized by the acquisition of flowering competency (Allsopp, 1967; Poethig, 1990; Jones, 1999; Zot et al., 2011; Samach, 2012). The transition from juvenile to adult phase is known as phase change.

Controversially, the terms “juvenile” and “adult” have been widely used to describe heteroblastic traits of vegetative development, in some cases irrespective of, but in other cases also encompassing, the reproductive status of plants. This terminological usage is persistently embedded in the literature, largely attributed to Poethig and co-workers. These workers (e.g., Yang et al., 2011; Poethig, 2013) reasoned that juvenile and adult phases were initially defined by Goebel not on the basis of reproductive competence but vegetative morphology. Indeed, Goebel (1900) also used the terms “juvenile” and “adult” when describing morphological changes during heteroblastic development. He called leaves produced early in shoot development and positioned at lower nodes “primary”, “earlier” or “juvenile” leaves, and leaves formed later and at higher nodes “adult” leaves (Goebel, 1900; Figure 3). He did so for many examples which he used to illustrate the great differences in “the outward changes of configuration” of leaves (leaf morphology) between juvenile and adult phases of heteroblastic species, which in part lead to the misinterpretation that this is the main purpose of employing the terms. However, it is important to repeat that Goebel’s original recognition of an “adult form” was hinged on the appearance of reproductive organs, which he called “a climax of development”, and the juvenile phase was defined as all developmental events occurring between germination and the status in which plants reach that climax (Goebel, 1900). He applied this concept to both categories of development, namely heteroblasty and homoblasty, which is unarguable as all plant species progress through a reproductive stage.
Figure 3 (See legend on next page)
General introduction

Figure 3 Examples of homoblastic and heteroblastic plants. (a) and (b) Casuarina plants show homoblastic development. (a) Seedling plant of Casuarina torolosa; (b) Bonsai plant of Casuarina equisetifolia. The shoot-axes of these plants have rudimentary cyclic leaves concrecent in a sheath. These leaves retain the same morphology along the shoot axes, even until the definite adult form of shoots is reached (Goebel, 1900). (c), (d) and (e) Acacia plants show heteroblastic development, characterized by dramatic differences in morphology between “primary” and “adult” leaves. (c) 1 - 4 primary leaves which are horizontally oriented and possess a bipinnate lamina and a normal leaf-stalk; 7 - 9 phyllodes (adult leaves), attained over the course of morphological changes during which the leaf-stalk gradually broadens while the lamina is reduced; the transition from primary leaves to phyllodes takes place over several nodes, resulting in the production of “transition” leaves, 5 and 6, in which both leaf types are present in a single leaf; n nectaries. Arrows in (d) and (e) point to examples showing transitions to phyllodes. Note that the Acacia pycnantha seedling in (d) has only one “transition” leaf, whereas Acacia implexa in (e) has at least three. (a) and (c) are drawings sourced from Goeble (1900); (b), (d) and (e) are images sourced from the Wee Trees Bonsai Forum website (http://weetrees.co.uk/phpBB3/) and the Florabank website (www.florabank.org.au).

In the view of this author, although Goebel caused certain confusion by using “juvenile” and “adult” to also illustrate morphological features, his original usage of the terms as depicted above should be retained; however, in cases that necessitate the use of these terms to refer to other aspects of plant development rather than reproductive status of shoots, coherent explanation must be provided to avoid possible terminological confusion or misconception. This view is in part in agreement with Jones’ suggestions (1999), in which she advocated reserving these terms to indicate plant reproductive status to avoid the incorrect synonymizing of the two conceptually distinct processes of heteroblasty and phase change. Additionally, Jones (1999) also pointed out that a premature assumption persists in the literature whereby changes in vegetative attributes signify changes in reproductive competence of shoots, even though little is known about how these two types of changes are related to each other. They can be associated in some cases but decoupled in others (Diggle, 1999; Jones, 1999). Aside from a few workers (e.g., Battey and Tooke, 2002; Jaya et al., 2010; Huijser and Schmid, 2011; Zotz et al., 2011; Poethig, 2013), others paid little attention to Jones’ suggestions. Current literature on heteroblasty and phase change employ these terms to ambiguously describe both (i) the change during shoot ontogeny (both vegetative maturation and reproductive maturation) and (ii) heteroblastic or vegetative characteristics (e.g., juvenile leaves/tissues vs adult leaves/tissues), which has led to an on-going terminological ambiguity and some conceptual issues. Some of these issues are discussed below.
1.6.2 The ongoing terminological confusion in phase change study

In addition to juvenile (non-reproductive) and adult (reproductive) phases traditionally described by Goebel (1900) and others (e.g., Wareing, 1959; Allsopp, 1967; Hackett, 1985), Greenwood (1995) and Poethig (1990) recognized an intercalated phase “adult vegetative phase”, in part owing to the recognition of a period between the acquisition of competency to flower and the actual initiation of reproductive development, a period which can very under natural conditions (Jones, 1999). More or less since Poethig (1990) and Greenwood (1995) (see also Kerstetter and Poethig, 1998), three post-embryonic phases of shoot maturation, namely juvenile vegetative phase, adult vegetative phase and adult reproductive phase, were defined, and at least two post-embryonic phase transitions, vegetative phase change (or vegetative maturation) and reproductive phase change (or reproductive maturation), were delineated. Vegetative phase change is defined as the transition from “juvenile vegetative phase” to “adult vegetative phase” and is normally characterized by morphological changes of vegetative structures along the shoot axis (Poethig, 1990; Huijser and Schmid, 2011). Reproductive phase change is the transition from vegetative to reproductive development and marked by the actual initiation of a completely new structure specialized for gamete production (Poethig, 1990, 2013), a characteristic of “adult reproductive phase” (Huijser and Schmid, 2011). This transition is also known as “floral transition” (Huijser and Schmid, 2011) and is preceded by (i) changes in morphological features of the shoot (namely vegetative phase change) and (ii) a change in the competence of the shoot to respond to signals that evoke floral initiation (Poethig, 2013), with the former being closely associated with the latter in some species (e.g., Hedera helix, maize) but can be uncoupled in others (e.g., Cucurbita argyrosperma subsp. sororia, Eucalyptus occidentalis) (Jones, 1999; Jaya et al., 2010). Particularly, in two closely related species, Eucalyptus tenuiramis and Eucalyptus risdonii, E. risdonii flowers during the juvenile vegetative phase, whereas E. tenuiramis flowers during its adult vegetative phase (Wiltshire et al., 1998). Therefore, vegetative phase change need not necessarily reflect a change in reproductive competence of shoots (Jones, 1999; Huijser and Schmid, 2011). Also, accumulated evidence suggests that vegetative maturation and reproductive maturation are developmentally uncoupled and
regulated by separate genetic mechanisms (Poethig, 1990; Diggle, 1999; Jaya et al., 2010; Zott et al., 2011; Poethig, 2013), although these mechanisms share some common regulatory factors (Huijser and Schmid, 2011; Poethig, 2013). Unfortunately, there persists the assumption that vegetative features (e.g., leaf morphology) bespeak the attainment of reproductive competence, according to Jones (1999) in part attributed to the use of “juvenile” and “adult” to describe heteroblastic traits.

To avoid confusion, Poethig (1990; 2013) has suggested that each process of phase change have its own specific terms, namely juvenile and adult phases of vegetative development, and juvenile and adult phases of reproductive development. However, not much attention has been paid to terminological clarification and thus confusion has occurred, for example, even within the phase change literature of the model species Arabidopsis. Arabidopsis is an annual herbaceous species and hence the interest of phase change study mostly lies in leaf morphology and anatomy, i.e., vegetative phase change (Poethig, 1990). Although the changes in leaf morphology are minor and gradual, Arabidopsis is also considered to display heteroblastic development, but not in a strict sense (Zott et al., 2011). In Arabidopsis, juvenile leaves are characterized by small and round blades without serrations on leaf margins, long petioles and the lack of abaxial trichomes, whereas adult leaves are enlarged and elongated with serrated margins, have short petioles and their abaxial surfaces are covered with trichomes (Telfer and Poethig, 1994; Chien and Sussex, 1996; Telfer et al., 1997; Figure 4a). Other traits have also been recognized and used to distinguish between the juvenile and adult vegetative phases, such as hydathode numbers (Tsukaya et al., 2000), venation patterns (Steynen et al., 2001), blade curvature (Hunter et al., 2006), cell size and cell number (Usami et al., 2009), blade base angle (Li et al., 2012), etc. Unlike other traits which change gradually, the appearance/absence of abaxial trichomes is abrupt and easy to identify, hence being used as a marker for vegetative phase change in Arabidopsis (Telfer et al., 1997). Consequently, it is widely accepted that the appearance of abaxial trichomes signifies the onset of the adult vegetative phase, or equivalently, the end of the juvenile vegetative phase.
Figure 4 Heteroblasty and juvenility in Arabidopsis. (a) Changes in leaf morphology in Arabidopsis. Arrowhead indicates the juvenile-to-adult transition point of vegetative phase change based on the appearance of abaxial trichomes. (b) Estimation of the juvenile reproductive phase length of three Arabidopsis ecotypes Col, Ler and Ws based on the attainment of reproductive competence. The dashed line demarcates the length of juvenile reproductive phase. (c) Estimation of the juvenile vegetative phase of Col, Ler and Ws ecotypes based on the appearance of abaxial trichomes. The arrows indicate the average position of the first leaf bearing abaxial trichomes. The vertical lines denote the estimated time when abaxial trichome development was initiated. Note that the x-axis units are different between the two graphs; see the relevant text for more information. (a), (b) and (c) were adapted from Rodriguez et al. (2014), Matsoukas et al. (2013) and Telfer et al. (1997), respectively.

On the other hand, plant biologists have also been interested in juvenility in Arabidopsis in terms of the attainment of reproductive competence. Arabidopsis is a facultative long-day plant, in which long days promote flowering more rapidly than short days (Gregory and Hussey, 1953). Therefore, reciprocal transfer treatments (Ellis et al., 1992; Adams et al., 2001) can be employed to identify the “juvenile phase” length of Arabidopsis, in this particular context defined as the period in which the shoots are insensitive to respond to the floral inductive cue photoperiod (Matsoukas et al., 2013; Sgamma et al., 2014). In these treatments plants were transferred between conditions that are non-inductive (short-day) and inductive (long-day) at regular intervals following germination, and flowering times of individual plants were
recorded; the end of the “juvenile phase” is defined by the time at which the transitions between photoperiod insensitive phases and the sensitive phase occur (Adams et al., 2001). This “juvenile phase”, if one agrees with Poethig’s suggestion (2013) regarding the separation of vegetative and reproductive phase change terminology, should be clarified as the juvenile phase of reproductive development (hereafter referred to as juvenile reproductive phase). Using the reciprocal transfer approach, Matsoukas et al. (2013) showed that different Arabidopsis ecotypes differ in juvenile reproductive phase length. Columbia (Col) plants have the shortest length, Landsberg erecta (Ler) the intermediate and Wassilewskija (Ws) the longest length (Matsoukas et al., 2013; Figure 4b). Because reproductive maturation can be uncoupled with vegetative maturation (Diggle, 1999; Jones, 1999), the juvenile reproductive phase need not necessarily end at the same time as the juvenile phase of vegetative development. Indeed, Telfer et al. (1997), by using leaf abaxial trichomes as a marker of vegetative phase transition, showed that the three Arabidopsis ecotypes Col, Ler and Ws also differ in length of the juvenile vegetative phase, however in reverse order compared to that of juvenile reproductive phase, namely Ws>Ler>Col (Figure 4c). In particular, the juvenile vegetative phase of Col plants lasts for nine-ten days after planting (Telfer et al., 1997), whereas their juvenile reproductive phase ends around two days from emergence (Matsoukas et al., 2013), or approximately four-five days after planting if the period of seed germination is taken into account (Figure 4b, c). Therefore, at least in Arabidopsis, it is conspicuous that the appearance of adult vegetative traits such as abaxial trichomes is non-concurrent with, and hence is not a sign of, the acquisition of reproductive competence of shoots. Again this observation is in agreement with Jones’ (1999) opposition to the conflation of changes in vegetative attributes (also heteroblastic changes) and changes in shoot reproductive status as discussed above (see Diggle, 1999; Jones, 1999).

Given all these terminological perplexities, it seems impractical to reconcile or generalize phase-change terms for both the forestry/woody and herbaceous literatures. However, it is important to specify any particular usage of these terms in each circumstance. In this thesis, the term heteroblasty is used to refer to variation in the character of vegetative metamers (repeating units, consisting of one internode, the
node located at its tip, associated leaf/leaf-like organ(s) and lateral buds; Schultz and Haughn, 1991; Barthélémy and Caraglio, 2007) along the axis of the shoot, where the character can be one of or a combination of morphological, anatomical, physiological and biochemical attributes, regardless of the degree of variation. To avoid possible confusion in terminological and conceptual issues as discussed above, and due to current interest of PP TC development overlying with vegetative phase change, leaves produced during early seedling growth are referred to as juvenile leaves and leaves formed later and typically found on fully grown plants are referred to as adult leaves, regardless of reproductive competence of the shoot (see also Jaya et al., 2010). Also, “juvenile shoots” is used to indicate shoots comprising juvenile vegetative organs (e.g., juvenile leaves), and “adult shoots” as shoots having both juvenile and adult vegetative organs, again irrespective of shoot reproductive status.

1.7 Genetic control of heteroblasty and phase change

1.7.1 Overview

Heteroblasty is believed to arise from a number of overlapping processes (Bongard-Pierce et al., 1996; Yang et al., 2011; Poethig, 2013). The first process is known as “shoot maturation” or “phase change” (Wareing, 1959; Brink, 1962) or “ontogenetical aging” (Fortanier and Jonkers, 1976), predicted some time ago to be analogous to “genotypic” changes (Wareing, 1959) and now demonstrated to be clearly under genetic control, resulting in heteroblastic traits changing in regular, predictable and species-specific patterns (Diggle, 2014). The second process is termed “physiological aging” (Ashby, 1948; Wareing, 1959; Fortanier and Jonkers, 1976), which is the loss of shoot vigor or deterioration in general and involves heteroblastic effects easily reversed by changing growth conditions of the shoot (e.g., by grafting, Mencuccini et al., 2007). Heteroblasty has also been considered to encompass the phenomenon of seasonal changes in vegetative metamers, with these changes occurring reproducibly and repeatedly over growing seasons (Green et al., 1979; Godley, 1985) and defined as “seasonal heteroblasty” (Jones, 1999). Another cause behind the phenomenon of heteroblastic development is environmental heterogeneity (e.g., temperature,
photoperiod, soil humidity, carbohydrate balance, etc.; see Ashby, 1948; Cook, 1969; Jones, 1995), which can yield morphological variations very similar to the ontogenetic changes resulting from genetically programmed shoot maturation or phase change (Diggle, 2002), without necessarily being mediated by the same regulatory mechanism (Jones, 1995). Such variation is regarded as “morphological plasticity” (Diggle, 2002).

Many of these various types of heteroblastic traits change simultaneously or overlap with each other during shoot development, therefore, it is difficult to differentiate traits that are potentially controlled by genes involved in the developmental phenomenon of phase change from those regulated by factors related to shoot physiological aging and/or environmentally induced fluctuations (plasticity). To further complicate matters, heteroblastic traits as well as the progression (quickly or gradually) of heteroblasty of individual traits vary from species to species (Hackett, 1985; Poethig, 2013). Additionally, as observed by Bongard-Pierce et al. (1996), unlike woody species, phase-specific traits are much more plastic in herbaceous plants and hence often being mistaken for aging-related traits, which are regulated mainly by quantitative changes in the physiology of the shoot (Allsopp, 1967). This complex nature of heteroblasty has partly impeded our understanding of the functional importance of this phenomenon despite the fact that many quantitative approaches have been employed to study heteroblasty, including the use of single quantitative traits such as leaf index (Tsukaya, 2002; Zotz et al., 2011) or the combination of multiple traits (Steynen et al., 2001; Lièvre et al., 2016).

Heteroblastic traits that are components of the phase change program have been of great interest due to its theoretical importance relative to morphogenetic control, differentiation and determination in plant development and its significant economic implications such as in horticulture (Hackett, 1985). Consequently, the last three decades have witnessed extensive research on this type of heteroblasty, of which the major contributor is primarily but not limited to vegetative phase change (Poethig, 2013). It has emerged that floral transition or reproductive phase change also acts in concert with vegetative phase change to affect a subset of phase-specific heteroblastic traits (also known as “vegetative traits” - see above; Wang et al., 2009; Deng et al., 2011; Willman and Poethig, 2011). Central to this regulatory network is the
microRNA miR156 and its targets *SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL)* transcription factor genes, which have been shown to regulate both vegetative phase change and flowering time (Figures 5, 6; Wu and Poethig, 2006; Wang et al., 2009; Yamaguchi et al., 2009). Therefore, the miR156-SPL module can be used as a molecular marker to determine whether an observed heteroblastic trait is genetically regulated by the same mechanism controlling shoot maturation, sometimes regardless of this trait being a result of vegetative maturation or reproductive maturation or a combination of both (in some cases it is impractical to separate the effects of one process from that of the other).

### 1.7.2 miR156 and its *SPL* targets

MicroRNAs (miRNAs), comprised of 20 to 24 nucleotides, are a class of small endogenous regulatory RNAs that can repress the protein levels of their targets through transcript cleavage or translational inhibition, thereby regulating diverse biological processes in eukaryotic organisms (Bartel, 2004; Rogers and Chen, 2013). In plants, these processes include leaf development, developmental phase transition and floral development, shoot and root development, vascular development, signal transduction, pathogen infection responses and environmental stress responses (Zhang et al., 2006). miR156 is amongst the most extensively studied miRNAs in plants, was first identified in Arabidopsis (Reinhart et al., 2002) and later found to be present in all land plants from mosses, lycopods, ferns and gymnosperms to a large number of monocots and dicots (Arazi et al., 2005; Axtell and Bartel, 2005; Sunkar and Jagadeeswaran, 2008). In Arabidopsis, miR156 directly targets 11 of 17 *SPL* genes, which can be categorized into four major clades: the *SPL3* clade (*SPL3, SPL4, SPL5*), the *SPL9* clade (*SPL9, SPL15*), the *SPL10* clade (*SPL2, SPL10, SPL11*) and the *SPL13* clade (*SPL6, SPL13A, SPL13B*) (Figure 5; Riese et al., 2007; Gandikota et al., 2007; Guo et al., 2008). Based on size of proteins which they encode, these *SPL* genes can be broadly divided into two groups, represented by *SPL3* (*SPL3, SPL4, SPL5*) and *SPL9* (the remaining *SPLs*). *SPL3*-group genes encode small proteins (131 - 181 amino acids) which consist mostly of the SBP DNA binding domain (Cardon et al., 1997; Schwab, 2012), whereas *SPL9*-group genes encode much larger proteins which harbor a C-
terminal domain responsible for protein-protein interactions (Yu et al., 2012; Zhang et al., 2015). Another difference between these two groups is that the miR156-binding site is in the non-coding 3’UTR of SPL3—but in the coding region of SPL9-group genes (Figure 5; Schwab, 2012). These structural dissimilarities of the two transcription factor groups probably lead to different mechanisms by which miR156 regulates their expression. SPL9 expression is repressed through transcript cleavage while SPL3 expression is regulated mainly by translational inhibition (Gandikota et al., 2007).

**Figure 5** The SPL transcription factors targeted by miR156 in Arabidopsis. The miR156-binding site (red) is located in the 3’UTR (untranslated region) of SPL3/SPL4/SPL5 mRNAs, but within the coding region of other SPL transcripts. In all cases the miR156-binding site is located downstream of the SBP box-encoding motif which is represented by the dark-blue box. Image taken from Yang (2011).

### 1.7.3 Discovery of the miR156-SPL module as a master regulator of vegetative phase change

Most of the mutants which affect vegetative phase change, such as *hasty, hyponastic leaves, serrate* and *squint*, also have pleiotropic effects on a wide range of morphological features (Telfer and Poethig, 1998; Clarke et al., 1999; Berardini et al., 2001; Li et al., 2012). On the other hand, *zippy/argonaute7 (zip/ago7), suppressor of gene silencing 3 (sgs3) and rna dependent polymerase 6 (rdr6)* represent a subset of mutations that have a much more specific effect on vegetative phase change (Hunter et al., 2003; Peragine et al., 2004) and thus were employed in initial studies searching for genes responsible for this developmental process. These studies yielded findings on two broad pathways regulating vegetative phase change, namely miR156-mediated...
repression of SPL genes (Wu and Poethig, 2006; Figure 6) and tasiR-ARF-mediated repression of AUXIN RESPONSE FACTOR 3 (ARF3) and ARF4 (Hunter et al., 2006; see also Section 1.7.6; tasiR-ARF: trans-acting short-interfering RNA-auxin response factor).

miR156 is amongst the most abundant miRNAs in Arabidopsis. Its abundance reaches highest levels in very young seedlings but declines rapidly within two weeks after germination and then gradually decreases over the next few weeks as the plant matures (Wu and Poethig, 2006; Jung et al., 2011, 2012; Wang et al., 2009; Wu et al., 2009; Xu et al., 2016a, b). The abundance of miR156 is repressed by sugar, the product of photosynthesis in leaves (Figure 6a; Yang et al., 2013; Yu et al., 2013; see also Section 1.7.5). High levels of miR156 promote juvenile characteristics of shoots, evidenced by the prolonged juvenile vegetative phase observed in transgenic lines overexpressing this miRNA. These plants produce many more juvenile leaves characterized by small and round blades with smooth margins and without abaxial trichomes (Schwab et al., 2005; Wu and Poethig, 2006). In contrast, sequestration of miR156 by introducing an artificial target mimic of this miRNA leads to diminished activity of the miRNA, hence increased levels of SPL transcripts and ultimately resulting in plants that virtually skip the juvenile vegetative phase completely (Franco-Zorrilla et al., 2007). Further evidence arguing for miR156 being a master controller of the juvenile vegetative phase comes from the analysis of mutants such as hasty, squint and hyponastic leaves, of which the precious adult phenotypes, among other morphological abnormalities, were shown to be attributed to reduced levels of miR156 or its activities (Park et al., 2005; Wu and Poethig, 2006; Smith et al., 2009; Li et al., 2012). The developmental decline of miR156 over shoot maturation and its temporal control of vegetative phase change were also demonstrated in other annual plants such as maize (Chuck et al., 2007), tomato (Zhang et al., 2011), rice (Xie et al., 2012), soybean (Yoshikawa et al., 2013), Chinese cabbage (Wang et al., 2014), tobacco (Feng et al., 2016) and many perennials including the herbaceous Cardamine flexuosa (Zhou et al., 2013) and some woody species such as Acacia confusa, Acacia colei, Eucalyptus globulus, Hedera helix, Quercus acutissima, and Populus x canadensis (Wang et al., 2011; Hudson et al., 2014).
Figure 6 Regulation of phase change in Arabidopsis. (a) Sugar promotes phase change by repressing miR156 accumulation. (b) Roles for miR156 and miR172 and their target genes in the control of phase change. After germination, plants enter a juvenile vegetative phase, producing leaves with juvenile characteristics. During this phase, the levels of miR156 are high, thereby repressing the abundance of SPL mRNA. As plants mature, sugar accumulation causes a steady reduction in miR156 levels (blue triangle), permitting SPL transcripts to gradually accumulate and promote the onset of the adult vegetative phase. Also, increases in SPL levels (SPL9, SPL10) lead to a gradual increase in miR172 levels (pink triangle), thereby allowing the plant to enter the reproductive phase via the downregulation of six AP2-like transcription factors. See text for more details. Abbreviations: AP2, APETALA2; SMZ, SCHLAFMÜTZE; SNZ, SCHNARCHZAPFEN; SPL, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE; TOE, TARGET OF EARLY ACTIVATION TAGGED. Image (a) sourced from the Wang lab website (http://wanglab.sippe.ac.cn) and (b) from Grotewold et al. (2015).

Consistent with being direct targets of miR156, SPL levels gradually increase over shoot maturation (Wu and Poethig, 2006; Wang et al., 2009; Jung et al., 2011, 2012).
These transcription factors are unique targets of miR156 and hence predicted to play key roles in promoting the adult vegetative phase. However, owing to extensive redundancy with this family, single mutants of SPL genes generally have weak or no obvious phenotypes and thus the contribution of single genes has been difficult to establish (Poethig, 2013). Functions of SPL genes have mainly been revealed in plants overexpressing miR156-resistant versions of their transcripts. SPL3 was the first miR156-target gene identified to have a role in controlling vegetative phase change, by which the miR156-SPL interaction was originally discovered as the main player in this phase transition (Wu and Poethig, 2006). SPL4 and SPL5 have overlapping functions with SPL3 in promoting adult patterns of epidermal differentiation, evidenced by the precocious initiation of leaf abaxial trichomes in transgenic plants carrying miR156-resistant forms of SPL3, SPL4 or SPL5 (rSPL3, rSPL4, rSPL5, respectively) (Wu and Poethig, 2006). These SPL3-clade genes were also proposed to regulate heteroblasty of leaf shape (petiole length and blade base angle; Wu and Poethig, 2006) and cell size and number (Usami et al., 2009). However, due to the early flowering phenotype of rSPL3, rSPL4, rSPL5 under long-day conditions and the absence of effects on leaf shape in rSPL3 under short-day conditions, Wu et al. (2009) attributed these effects to photoperiod-induced floral signals rather than changes in vegetative maturation.

Subsequent studies revealed that miR156-targeted SPL genes belonging to other clades play more diverse roles in controlling shoot maturation. The SPL9 clade is comprised of two likely paralogous genes SPL9 and SPL15 (Figure 5). SPL9 is the only member of the SPLs regulated by miR156 that has a loss-of-function phenotype on its own (Poethig, 2013). The spl9 single mutant showed a slight increase in leaf initiation rate and a weak juvenilized phenotype in which abaxial trichome production is delayed by 2.8 plastochrons (note: a plastochron is the time interval between the initiation of two consecutive leaves) and leaf blade is rounder, whereas the spl15 single mutant was very similar to wild-type (Schwarz et al., 2008; Wang et al., 2008; Wu et al., 2009). The spl9 spl15 double mutants exhibited more severe delay in abaxial trichome phenotype than both single mutants, however, leaf shape was indistinguishable from that of the spl9 single mutant, suggesting a less important role of SPL15 in regulating leaf morphogenesis even albeit these genes have overlapping functions (Wu et al.,
2009). This hypothesis is further supported by the observations that overexpression of miR156-resistant version of SPL9 (rSPL9) caused accelerated expression of almost all adult leaf traits such as elongated leaf blade, serrated leaf margin, short petiole and abaxial trichomes (Wu et al., 2009), whereas the gain-of-function mutant SPL15-1D showed the precocious adult phenotype of abaxial trichome production but no obvious effects on leaf morphology except for a larger blade area (Usami et al., 2009). Other phenotypes of the spl9 spl15 double mutant include increased rate of leaf initiation (or shortened plastochron length), increased number of leaves, increased number of branches and late flowering (Schwarz et al., 2008; Wang et al., 2008). Collectively, this double mutant strongly phenocopies transgenics that overexpress miR156, implying that, among eleven miR156-targeted SPL genes, redundant action of SPL9 and SPL15 importantly and primarily contribute to the miR156 overexpressing phenotype. This contribution is not entire however, which suggests overlapping functions from other SPL(s). Indeed SPL2 was shown to enhance the effects of SPL9 and SPL15 on shoot maturation in which the triple mutant plants showed a closer approximation to the miR156 overexpressing phenotype (Schwarz et al., 2008). It was also reported that SPL15 has a role in regulating the progression of heteroblasty of cell size and number (Usami et al., 2009).

SPL10 and SPL11, belonging to the SPL10 clade, have overlapping but distinct functions with SPL9 in regulating leaf morphology (Figure 6b; Wu et al., 2009). Leaves of rSPL9, rSPL10 and rSPL11 (plants transformed with miR156-resistant versions of SPL9, SPL10 and SPL11, respectively) have more elongated blade, more serrated margin and earlier abaxial trichome production than that of wild-type; however rSPL10 and rSPL11 leaves are more serrated, rounder and flatter than rSPL9 leaves (Wu et al., 2009). In contrast, observations by Shikata et al. (2009) suggest more limited roles for genes of the SPL10 clade in controlling phase-specific traits. These authors offered convincing evidence showing that SPL2, SPL10 and SPL11 have no effect on abaxial trichome initiation, which is a common marker for the onset of the adult vegetative phase. Furthermore, although altered expression of these genes caused changes in leaf shape, these effects were somewhat different from those reported by Wu et al. (2009). For example, overexpression of SPL2, SPL10 or SPL11 resulted in plants bearing adult-
and even cauleine-like rosette leaves in the early vegetative phase, with these cauleine-
like leaves having narrow and oblong blades and lacked petioles (Shikata et al., 2009).
Both Wang et al. (2008) and Shikata et al. (2009) showed similar effects of SPL10 on
plastochron length, suggesting overlapping functions of SPL9, SPL10 and SPL15 in
controlling this aspect of shoot maturation.

Little is known about roles for SPL6 in regulating vegetative phase change. It has
recently emerged that SPL6 is a novel positive regulator in activating the defense
transcriptome following its association with a nuclear-localized immune receptor
(Padmanabhan et al., 2013; see also Table 1). SPL13 has been proposed as a repressor
of the transition from the cotyledon to vegetative-leaf stage since the expression of a
miR156-resistant form of SPL13 triggered a delayed emergence of the first true leaves
(Martin et al., 2010a). Very recently, Xu et al. (2016b) have shown that SPL13 also plays
an important role in promoting the juvenile phase and delaying leaf initiation. These
authors also demonstrated that six genes, SPL2, SPL9, SPL10, SPL11, SPL13 and SPL15,
account for the effect of miR156 on vegetative phase change, among which SPL9,
SPL13 and SPL15 play dominant roles (Xu et al., 2016b).

1.7.4 Roles for the miR156-SPL module in regulating other aspects of plant
development

miR156-regulated SPLs have distinct roles in floral induction and the transition of floral
meristem identity (Xu et al., 2016b). SPL3, SPL4 and SPL5 have no effects on the former
process (Xu et al., 2016b) but regulate the latter by activating MADS box genes such as
FRUITFULL (FUL) and APETALA1 (AP1), and the meristem identity gene LEAFY (LFY)
(Yamaguchi et al., 2009; Xu et al., 2016b). Other SPL genes, particularly SPL9, SPL13
and SPL15, participate in both processes (Xu et al., 2016b). SPL9 was shown to directly
promote the transcription of MIR172b and SOC1 (Wang et al., 2009; Wu et al., 2009),
in addition to regulating FUL and AP1 (Wang et al., 2009). Recently, Hyun et al. (2016)
demonstrated that SPL15 also directly induced transcription of FUL and MIR172b, and
acts in parallel with SOC1 and gibberellin in short-day conditions to induce FUL.

In addition to post-embryonic phases of shoot maturation, miR156-mediated SPL
repression is known to also modulate the timing of embryonic development (Nodine
and Bartel, 2010). dcl1, a mutant defective in DICER-LIKE1, a key factor for miRNA biogenesis, displays growth arrest early in development. Microarray analysis of this mutant at the eight-cell stage revealed that SPL10 and SPL11 were de-repressed more than 150-fold. Subsequent experiments demonstrated that these two miR156-targeted genes were redundantly responsible for the defects observed in dcl1 embryos and that miR156, by preventing precocious expression of these differentiation promoting transcription factors, enables proper embryonic patterning (Nodine and Bartel, 2010).

Along with phase dimorphisms, the miR156-SPL module regulates a variety of other processes during plant development. These processes include the biosynthesis of anthocyanin (Gou et al., 2011), the development of stamens (Xing et al., 2010) and gynoecium (Xing et al., 2013), the distribution of trichomes on stems (Yu et al., 2010) and many more (see Table 1). The SPL9-group genes also have important roles in regulating both male and female fertility, and, interestingly, these miR156-targeted SPL genes act redundantly with SPL8, a non-miR156-targeted SPL, in this aspect of development (Xing et al., 2010, 2013).

SPL proteins are generally considered transcriptional activators; however, in several cases they can act as repressors. For example, SPL9 has been shown to exert dual molecular roles in regulating stem trichome number and anthocyanin levels, both of which are inversely correlated to SPL levels during shoot maturation (Yu et al., 2010; Gou et al., 2011). SPL9, by directly binding to their promoters, can activate the expression of TRICHOMELESS1 (TCL1) and TRIPTYCHON (TRY), two MYB transcription factor genes important for the repression of trichome cell fate (Yu et al., 2011). On the other hand, SPL9 indirectly binds to the promoter of DIHYDROFLAVONOL REDUCTASE (DFR) but prevents its expression. DFR is a key enzyme in anthocyanin biosynthesis, therefore, increased levels of SPL9 resulting from decreased miR156 activity lead to reduced anthocyanin accumulation (Gou et al., 2011). Another line of evidence suggesting a repressive role for SPL9 is in the case of B-type ARABIDOPSIS RESPONSE REGULATORS (ARRs) expression. SPL9 directly inhibits the transcriptional activity of B-type ARRs by binding to their transactivation domain, thereby exerting its role in reducing the regenerative capacity of the shoot (Zhang et al., 2015).
In conclusion, the miR156-SPL module has diverse roles in regulating various aspects of plant development. Most of these roles are summarized in Table 1.

### Table 1 Known functions of miR156-targeted SPL genes in Arabidopsis (modified from Wang, 2014)

<table>
<thead>
<tr>
<th>Developmental processes</th>
<th>SPL genes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic development</td>
<td>SPL10, SPL11</td>
<td>Nodine and Bartel (2010)</td>
</tr>
<tr>
<td>Cotyledon to vegetative-leaf transition</td>
<td>SPL13</td>
<td>Martin et al. (2010a, b)</td>
</tr>
<tr>
<td>Vegetative phase change</td>
<td>SPL3, SPL4, SPL5, SPL9, SPL15, SPL10, SPL11, SPL13</td>
<td>Wu and Poethig (2006); Shikata et al. (2009); Wu et al. (2009); Xu et al. (2016b)</td>
</tr>
<tr>
<td>Flowering time</td>
<td>SPL3, SPL4, SPL5, SPL9, SPL15</td>
<td>Wang et al. (2009)</td>
</tr>
<tr>
<td>Leaf initiation rate</td>
<td>SPL9, SPL15</td>
<td>Schwarz et al. (2008); Wang et al. (2008)</td>
</tr>
<tr>
<td>Branching</td>
<td>SPL9, SPL15</td>
<td>Schwarz et al. (2008); Wang et al. (2008)</td>
</tr>
<tr>
<td>Trichome distribution on stem</td>
<td>SPL9, SPL15</td>
<td>Yu et al. (2010)</td>
</tr>
<tr>
<td>Anthocyanin biosynthesis</td>
<td>SPL9, SPL15</td>
<td>Gou et al. (2011)</td>
</tr>
<tr>
<td>Pathogen resistance</td>
<td>SPL6</td>
<td>Padmanabhan et al. (2013)</td>
</tr>
<tr>
<td>Heat stress</td>
<td>SPL2</td>
<td>Stief et al. (2014)</td>
</tr>
<tr>
<td>Salt and drought stress</td>
<td>SPL9</td>
<td>Cui et al. (2014)</td>
</tr>
<tr>
<td>Stamen development</td>
<td>SPL9-group genes</td>
<td>Xing et al. (2010)</td>
</tr>
<tr>
<td>Gynoecium development</td>
<td>SPL9-group genes</td>
<td>Xing et al. (2013)</td>
</tr>
<tr>
<td>Shoot regenerative capacity</td>
<td>SPL9-group genes</td>
<td>Zhang et al. (2015)</td>
</tr>
</tbody>
</table>

#### 1.7.5 Regulation of miR156 levels

While the downstream events of miR156 expression have been well investigated and unveiled, research on immediate upstream regulator(s) of this miRNA remains in its infancy. Recently Yang et al. (2013) and Yu et al. (2013) proposed a role for glucose and sucrose in regulating vegetative phase change by repressing miR156 accumulation in leaves. In addition, trehalose-6-phosphate may also act upstream of miR156 in the
flowering pathway (Wahl et al., 2013). Interestingly, more than a century ago, Goebel (1900) also proposed that nutrients play a key role in these transitions.

### 1.7.6 Other genetic pathways regulating heteroblasty and vegetative phase change

miR172 and its AP2-like target genes have also been implicated in modulating vegetative phase change in maize (Chuck et al., 2007) and Arabidopsis (Wu et al., 2009). The accumulation of miR172 opposes to that of miR156, namely being initially low and then increasing over shoot development (Jung et al., 2007, 2011; Wu et al., 2009). In Arabidopsis, miR172 levels are regulated by SPL9, hence acting downstream of miR156. The primary function of miR172 in vegetative maturation is to promote adult epidermal identity, evidenced by the precocious appearance of abaxial trichomes in plants overexpressing this miRNA and by the delayed abaxial trichome production in the miR172a mutant (Wu et al., 2009). Among six miR172-target AP2-like genes, TARGET OF OVEREXPRESSION OF EAT1 (TOE1) and TOE2 were demonstrated to mediate this function of miR172 (Wu et al., 2009).

In addition to the two miRNAs miR156 and miR172, another class of small RNAs, tasi-ARF, also plays a role in regulating the progression of heteroblasty. tasi-ARF is a trans-acting small interfering RNA (ta-siRNA) that target both AUXIN RESPONSE FACTOR 3 (ARF3) and ARF4. Upregulation of ARF3 and ARF4 was shown to be responsible for the zip phenotype, typically recognized by the elongated and downwardly curled blades of the first two leaves and the accelerated initiation of leaf abaxial trichomes (Hunter et al., 2003, 2006). This zip phenotype is seen in zip/ago7, rdr6 and sgs3 mutants and is supposedly attributable to the absence of tasiR-ARF-mediated repression of ARF3 and ARF4 (Hunter et al., 2006).

It has recently emerged that the floral repressor FLOWERING LOCUS C (FLC) has effects on the timing and progression of vegetative phase change in Arabidopsis (Willmann and Poethig, 2011), which may be a consequence of the binding of FLC to the promoter of SPL15 (Deng et al., 2011). SPL3 is another FLC-targeted SPL identified by Deng et al. (2011); however, a role for SPL3 in mediating effects of FLC on vegetative maturation was excluded as there was no significant change of SPL3
expression in the *flc* mutant (Deng et al., 2011). These observations, together with the findings that miR156, miR172 and their respective targets function in both vegetative and reproductive maturation, imply tight connections between these two developmental transitions although they are regulated by distinct genetic pathways.

### 1.8 Project aims and significance

The functional and morphological specifications of TCs have made this fascinating cell type of great interest of plant biologists over the last five decades. The observation that TCs are also present in Arabidopsis - the leading model species for plant molecular research - has paved the way to elucidate the mechanisms underlying wall ingrowth formation in TCs. This progress has been slow, however, partly due to the lack of a robust means to access the abundance of TCs, which typically develop deep within inner tissues such as vascular bundles. To address this impediment, the first aim of this PhD project (Chapter 2) was to investigate approaches for fluorescence staining of PP TCs and develop a method of semi-quantitative assessment of wall ingrowth deposition in PP TCs at the whole leaf level.

Application of the mPS-PI staining technique (Truernit et al., 2008; Wuyts et al., 2010) for confocal imaging of plant cell walls enabled successful imaging of wall ingrowth deposition in PP TCs. The robustness of this staining approach enabled mapping the abundance and distribution of PP TCs along the shoot axis, from which the heteroblastic features of wall ingrowth deposition in PP TCs were recognized. As outlined above (see Section 1.7), heteroblasty is a component of shoot maturation in Arabidopsis that has been extensively studied at both anatomical and molecular levels, thus providing a promising avenue for investigating this aspect of PP TC development. Therefore, the second aim of this project (Chapter 3) was to demonstrate that (i) the extent of wall ingrowth deposition in PP TCs is a novel trait specific to heteroblasty in Arabidopsis, and that (ii) this novel heteroblastic trait is regulated by a developmental genetic program which controls vegetative phase transition, namely the miR156/SPL regulatory module.
CHAPTER 2

High-resolution confocal imaging of wall ingrowth deposition in plant transfer cells: Semi-quantitative analysis of phloem parenchyma transfer cell development in leaf minor veins of Arabidopsis

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Running title: Confocal imaging of TC wall ingrowths

Keywords: Transfer cells, Cell wall ingrowths, Confocal imaging, Pseudo-Schiff base, Propidium iodide, Phloem parenchyma, Arabidopsis, Companion cells


The text presented in this Chapter, including Supplementary Data, is the accepted manuscript submitted to BMC Plant Biology.
**Summary**

**Background**

Transfer cells (TCs) are *trans*-differentiated versions of existing cell types designed to facilitate enhanced membrane transport of nutrients at symplasmic/apoplastic interfaces. This transport capacity is conferred by intricate wall ingrowths deposited secondarily on the inner face of the primary cell wall, hence promoting the potential trans-membrane flux of solutes and consequently assigning TCs as having key roles in plant growth and productivity. However, TCs are typically positioned deep within tissues and have been studied mostly by electron microscopy.

Recent advances in fluorophore labelling of plant cell walls using a modified pseudo-Schiff-propidium iodide (mPS-PI) staining procedure in combination with high-resolution confocal microscopy have allowed visualization of cellular details of individual tissue layers in whole mounts, hence enabling study of tissue and cellular architecture without the need for tissue sectioning. Here we apply a simplified version of the mPS-PI procedure for confocal imaging of cellulose-enriched wall ingrowths in vascular TCs at the whole tissue level.

**Results**

The simplified mPS-PI staining procedure produced high-resolution three-dimensional images of individual cell types in vascular bundles and, importantly, wall ingrowths in phloem parenchyma (PP) TCs in minor veins of Arabidopsis leaves and companion cell TCs in pea. More efficient staining of tissues was obtained by replacing complex clearing procedures with a simple post-fixation bleaching step. We used this modified procedure to survey the presence of PP TCs in other tissues of Arabidopsis including cotyledons, cauline leaves and sepals. This high-resolution imaging enabled us to classify different stages of wall ingrowth development in Arabidopsis leaves, hence enabling semi-quantitative assessment of the extent of wall ingrowth deposition in PP TCs at the whole leaf level. Finally, we conducted a defoliation experiment as an example of using this approach to statistically analyze responses of PP TC development to leaf ablation.
Conclusions

Use of a modified mPS-PI staining technique resulted in high-resolution confocal imaging of polarized wall ingrowth deposition in TCs. This technique can be used in place of conventional electron microscopy and opens new possibilities to study mechanisms determining polarized deposition of wall ingrowths and use reverse genetics to identify regulatory genes controlling TC trans-differentiation.

Introduction

Transfer cells (TCs) are important for plant development as they form at nutrient transport bottlenecks where an apoplastic/symplasmic transport step is required for acquisition and/or delivery of nutrients [1]. TCs are anatomically specialized for this function as they develop extensive wall ingrowths which result in increased plasma membrane surface area which supports an increased density of nutrient transporters [1-3]. Seeds of many crop species develop TCs to facilitate seed filling [4], and TCs support both phloem loading and short and long distance transport via xylem/phloem exchange [5]. TCs develop by trans-differentiation of existing cell types in response to developmental or stress-induced signals [1], but despite the importance of TCs to plant development, little is known of the molecular processes responsible for their trans-differentiation. This situation is caused in part by TCs typically being located deep within tissues [6] and thus not readily accessible for experimental manipulation and study.

The trans-differentiation of TCs involves differential expression of hundreds of genes. The formation of nucellar projection and endosperm TCs in barley grains involves differential expression of at least 815 genes [7], while the development of epidermal TCs in Vicia faba cotyledons is predicted to involve up to 650 genes [8]. These and other observations have led to the proposition that wall ingrowth deposition in TCs involves hierarchical regulation of cascades of gene expression, presumably controlled by key transcription factors [9], a model based on the genetic regulation of secondary wall deposition in xylem tissue [10,11]. The identification of
such factors putatively regulating wall ingrowth deposition in TCs is best undertaken in a genetic model such as *Arabidopsis thaliana* (Arabidopsis).

In Arabidopsis, phloem parenchyma (PP) TCs are known to form in minor veins of leaves and sepals where they are proposed to function in apoplastic phloem loading [12-14]. Previous studies examining PP TCs in Arabidopsis have relied on transmission electron microscopy (TEM) to analyze these cells. Indeed, Amiard et al. [13] traced cell wall contours of PP TCs viewed by TEM to demonstrate a role for high light and jasmonic acid in signaling wall ingrowth development, and similar approaches were undertaken to demonstrate a relationship between photosynthetic capacity and PP TC development [15]. Analysis by electron microscopy, however, is time-consuming and clearly not compatible for high-throughput screening required to identify genetic factors controlling the trans-differentiation of TCs.

High-resolution imaging of cell walls by confocal microscopy has been achieved using a modified pseudo-Schiff base-propidium iodide (mPS-PI) staining procedure [16]. In this process, treatment of fixed plant tissue with periodic acid results in the formation of aldehyde groups in the carbohydrate moieties of cell walls. These aldehyde groups can then be reacted with various fluorescent pseudo-Schiff reagents, such as propidium iodide, resulting in strong covalent fluorophore labelling of cell walls [16]. The strong covalent labelling enables the tissue to be extensively cleared and mounted in high-refractive index mounting medium, giving strong and stable fluorescence labelling of cell walls and thus enabling extensive z-stack imaging of cellular organization in complex tissues [16,17]. Wall ingrowths of TCs are rich in cellulose and other polysaccharides such as pectins [18], a feature that may provide an opportunity to use the mPS-PI procedure to image wall ingrowth deposition in PP TCs.

Here we report the successful use of mPS-PI staining of Arabidopsis leaves to visualize wall ingrowth deposition in PP TCs in minor veins of leaves, cotyledons and sepals by confocal imaging. Wall ingrowths in these cells are discernable as highly localized thickenings of wall material deposited along the face of the PP TC adjacent to neighboring cells of the sieve element/companion cell (SE/CC) complex. Depending on tissue orientation, this deposition can often be seen as a central band running along each PP TC and superimposing an underlying SE or CC. We have used this procedure to
also image light-dependent wall ingrowth deposition in CC TCs of pea minor veins [19,20], and have developed a scoring method based on the extent of wall ingrowth deposition for semi-quantitative analysis of TC development. Furthermore, introduction of a simple post-fixation bleaching step as an alternative to extensive clearing procedures in the original technique has simplified the processing steps to enable more efficient staining of tissue. Collectively, this procedure now provides the opportunity to investigate the cell biology of wall ingrowth deposition of PP TCs in Arabidopsis in a semi-quantitative manner without resorting to electron microscopy, and will also enable high-throughput phenotypic screening of TC development to identify key transcriptional regulators of this process.

**Materials and Methods**

**Plant growth conditions**

*Arabidopsis thaliana* (Col-0) seeds were sown directly onto pasteurised soil mix and stratified for three days in darkness at 4°C. Plants were then transferred to a growth cabinet (100–120 μmol m$^{-2}$ sec$^{-1}$, 22°C day/18°C night, 16 h photoperiod) for 2–3 weeks or until stated, and cotyledons, rosette leaves, cauline leaves and sepals were then collected for analysis. Peas (*Pisum sativum*) were raised in potting mix in a glasshouse maintained at 20-24°C and approximately 800–900 μmol m$^{-2}$ sec$^{-1}$ during daytime. Nine days after sowing, some seedlings were covered with aluminium foil to provide dark treatment and then the second pair of true leaves from both control (full light) or dark-treated plants were harvested after 4 days further growth. For defoliation experiments, all rosette leaves except leaf 9, 10 and 11 were removed from 3-week-old Arabidopsis plants. After 5 days of additional growth, leaf 10 from both control and defoliated plants were collected and processed for mPS-PI staining.

**Pseudo-Schiff-propidium iodide staining of tissues**

Rosette and cauline leaves from Arabidopsis and the second pair of true leaves from pea seedlings were pressed firmly onto clear sticky tape and the abaxial epidermal layer and associated mesophyll tissue from each leaf was peeled away using Scotch 3M...
M™ magic tape. Sepal and cotyledon tissue was processed without epidermal peeling. Tissue was then fixed overnight at 4°C in ethanol:acetic anhydride (3:1), then washed in 70% (v/v) ethanol and processed at room temperature as described below or stored in 70% ethanol at 4°C for several months. Tissue was washed in chloroform for 10 min, then progressively rehydrated and cleared in 1% (w/v) SDS in 0.2 N NaOH for 10 min. The extracted tissue was washed extensively in water and then incubated overnight at 37°C in 0.5% (v/v) amylase and 0.5% (v/v) pullulanase (Sigma, Australia) to remove starch. Tissue was then washed in water and incubated in 1% (v/v) periodic acid for 15 min, then washed in water again and stained in pseudo-Schiff propidium iodide for 1 h (100 mM Na₂S₂O₅, 0.15 N HCl with propidium iodide added to a final concentration of 100 μg/mL at the time of staining). Stained leaves were washed briefly in water and then mounted in chloral hydrate (4 g chloral hydrate, 1 mL glycerol, and 2 mL water) with the abaxial surface of the leaf facing up. The mounted tissue was covered with a coverslip and left overnight in darkness at room temperature before viewing.

**Simplified extraction of tissue using sodium hypochlorite**

Tissue was processed and fixed as described above and washed in 70% (v/v) ethanol. Cellular content of tissue was cleared by extracting tissue in 25% (v/v) White King™ bleach (4% (v/v) effective hypochlorite concentration) with gentle shaking at room temperature for at least 2 h depending on the tissue type. Cleared tissue was washed extensively in water and subjected to mPS-PI staining and mounting as described above.

**Confocal microscopy and image acquisition**

Confocal imaging of stained tissues was performed using an Olympus FluoView FV1000 confocal microscope. Imaging used 488 nm Argon-ion laser excitation and a 60 × 1.35NA Olympus oil-immersion objective. Emission wavelengths were collected at 522–622 nm. Image pixel resolution was set at 1600 × 1600, and used pixel dwell time of 4 μs and one-way scanning. Kalman average filtering of 4 was used during image acquisitions to improve signal-to-noise ratio of the acquired images.
Results

A modified pseudo-Schiff staining technique using propidium iodide to visualize wall ingrowths in TCs

The development of high-contrast staining of cell walls in cleared plant tissue using a mPS-PI procedure has enabled improved confocal imaging throughout plant tissues generally [16] and leaf tissue in particular [17]. To develop a procedure for confocal imaging of wall ingrowths in PP TCs in Arabidopsis leaves we used the technique of Wuyts et al. [17], modified by first peeling away the abaxial epidermis of rosette leaves immediately prior to fixation. Removing the abaxial epidermal layer and most of the associated mesophyll tissue and viewing from the abaxial face of the leaf enabled clear viewing of vascular bundles (Figure 1D). Under these conditions, confocal imaging of mPS-PI-stained leaves clearly resolved bands of wall ingrowth material, seen as unevenly thickened and mottled staining, positioned along the face of PP TCs adjacent to cells of the SE/CC complex (Figure 1A). In these images PP TCs can be identified as relative thin, elongated cells sharing a common longitudinal wall with a larger bundle sheath cell and the opposite wall with neighboring cells of the SE/CC complex. The highly localized deposition of wall ingrowths in PP TCs is evidenced by their occurrence only along the wall shared with a cell of the SE/CC complex (Figure 1A, B). In Figure 1A, the PP TC labelled with a double asterisk shows localized ingrowth deposition on the two faces of the cell neighboring two different CCs, indicating that the localizing signal most likely emanates from cells of the SE/CC complex. A longitudinal y-z projection of a z-stack through the vascular bundle shown in Figure 1A resolved discrete finger-like projections of wall ingrowth material along the face of PP TCs neighboring two SEs (Figure 1B). This image is highly reminiscent of TEM views of finger-like wall ingrowth projections in these cells (see Figure six of [21]), thus supporting the conclusion that the structures being imaged are indeed wall ingrowths in PP TCs. An x-z projection of
the same z-stack showing the vascular bundle in transverse section, clearly resolved localized patches of wall ingrowth material deposited in PP TCs adjacent mostly to the smaller SEs but also to CCs (Figure 1C). A survey of PP TCs revealed that in most discernable instances, wall ingrowth deposition in a PP TC was initiated immediately opposite a SE, but consolidation of this deposition spreads to areas of the cell wall opposite CCs (data not shown). This observation supports the suggestion that the source of signals such as reactive oxygen species likely to drive wall ingrowth deposition in PP TCs is derived from SEs [5].

Figure 1 Confocal imaging of wall ingrowths in PP TCs of Arabidopsis leaf minor veins stained by the mPS-PI procedure. A. Single confocal section of a minor vein junction revealing polarized deposition of wall ingrowths (arrows) on the face of PP TCs (single asterisks) adjoining CCs. Polarized deposition of wall ingrowth material can be seen in other PP TCs, including the central PP TC (double asterisk) where ingrowth deposition is directed to opposite faces of the PP TC, each adjoining a different CC. The yellow dotted lines labelled y-z and x-z correspond to the projections shown in B and C, respectively. B. y-z projection of a z-stack of the image shown in A revealing finger-like projections (arrows) of wall ingrowth material extending from the face of two linearly-arranged PP TCs (asterisks) adjacent to neighboring SEs. C. x-z projection of a z-stack of the image shown in A revealing minor vein architecture in transverse section and the presence of highly-localized depositions of wall ingrowth material (arrowheads) adjacent to small SEs (asterisks) and larger CCs. D. Bright-field image of minor vein junction. The boxed area corresponds to the region shown in A and indicates the clarity of viewing vascular tissue when the abaxial epidermal layer is removed and the tissue is viewed from the abaxial surface of the leaf. BS, bundle sheath cell; CC, companion cell; SE, sieve element. Scale bar = 10 μm in A, B and C. Scale bar = 100 μm in D.
When rosette leaves are torn paradermally and viewed by scanning electron microscopy (SEM), wall ingrowth deposition can often be seen as a central band of reticulate ingrowth material running along the length of a given PP TC (Figure 2A; see [22]), or as discrete clumps of tangled, finger-like projections (Figure 2D). These features are also seen by confocal imaging of mPS-PI-stained leaf material, namely central bands of wall ingrowth material running along PP TCs (arrows, Figure 2B) and isolated clumps of wall ingrowths (arrows, Figure 2E, F). The central bands of ingrowth material reflect their highly localized deposition immediately adjacent to neighboring cells of the SE/CC complex (see [12,13]). This spatial relationship is clearly seen in Figure 2B where the focal plane in the middle of the image passes from a PP TC (double asterisk, Figure 2B) to an underlying SE, revealing how the band of wall ingrowth material in the PP TC superimposes the underlying SE (Figure 2B). This feature is particularly evident when viewed as a z-stack movie through these cells (Additional file 1: Movie S1). At higher magnification, confocal imaging clearly resolved the intertwined, finger-like projections of wall ingrowth material (arrow, Figure 2C), a feature that is readily evident when viewed by SEM (Figure 2A).

We surveyed different vein orders in mature leaves for the presence of PP TCs. Consistent with previous studies identifying phloem involved in assimilate loading in mature leaves [23], we observed typically substantial levels of wall ingrowth deposition in virtually all veins except the midrib and most of the secondary veins (data not shown). The exception to this observation was minor levels of wall ingrowth deposition seen in small terminating regions of secondary veins (Additional file 2: Movie S2). These observations are consistent with the conclusion that wall ingrowth deposition in PP TCs correlates with phloem loading capacity of minor veins [23], which in turn correlates inversely with vein size, as suggested by Haritatos et al. [12].
Figure 2 Comparison of wall ingrowths in PP TCs of Arabidopsis leaf minor veins by confocal imaging and SEM. A, D. SEM views of fresh leaf material torn paradermally then subjected to bleach extraction and viewed by SEM. B, C, E, F. Confocal imaging of minor veins from leaf material stained by the mPS-PI procedure. A. SEM image of a PP TC showing a central band of reticulate wall ingrowth material (arrows). B. Highly localized deposition of wall ingrowth material seen as a central band (arrows) running along the length of each PP TC (asterisks). The focal plane of the image passes from the PP TC on the right (double asterisk) into the underlying SE, indicating how the band of wall ingrowth material superimposes the underlying SE. C. Confocal image at higher magnification showing substructure (arrow) of the wall ingrowth material in a PP TC (asterisk). In this image the SE to the left of this PP TC is obscured. D. SEM view of a minor vein junction of two PP TCs showing examples of isolated patches of wall ingrowth deposition (arrows). E. Confocal image of minor vein junction showing discrete patches of wall ingrowth deposition (arrows). F. Higher magnification confocal image showing patches of wall ingrowth deposition (arrows) in two PP TCs (asterisks). BS, bundle sheath cell; CC, companion cell; SE, sieve element. Scale bars = 2 μm in A and D. Scale bars = 10 μm in B and E. Scale bars = 5 μm in C and F. The image in A is reproduced in part from Edwards et al. [22].
Imaging wall ingrowth deposition in CC TCs in leaf minor veins of pea
To test the general applicability of this method for imaging wall ingrowths in other species, we used the mPS-PI procedure of Wuyts et al. [17] to stain CC TCs in leaf minor veins of pea, where reticulate wall ingrowth deposition occurs on all faces of these cells [19,20,24]. Confocal imaging of minor veins showed mottled labelling across the full face of CC TCs (arrows, Figure 3A). In contrast, wall ingrowth deposition was not detected in PP cells neighboring cells of the bundle sheath (Figure 3A). A $y$-$z$ projection of a $z$-stack passing longitudinally through the vertically-orientated minor vein in Figure 3A revealed the presence of reticulate wall ingrowths along the longitudinal walls of a CC TC (inset A’, Figure 3A), and similarly, a $y$-$z$ projection passing transversely through the horizontal minor vein in Figure 3A revealed wall ingrowth deposition across all faces of the large, mostly circular CC TCs (inset A”, Figure 3A), consistent with TEM images of these cells [20,24]. To verify that these structures were indeed wall ingrowths, leaves were stained from light-grown plants subjected to 4 days of dark treatment, conditions known to cause reduced wall ingrowth deposition [19,20]. Accordingly, reticulate wall ingrowth deposition in CC TCs was also greatly reduced, as shown by the $y$-$z$ projection of a $z$-stack passing transversely through a vascular bundle (inset B’, Figure 3B). This result confirms that the CC TCs shown in Figure 3A contain reticulate wall ingrowths, and that these ingrowths can be detected by confocal imaging of mPS-PI-stained pea leaves.

A simplified extraction procedure for mPS-PI staining of PP TCs using sodium hypochlorite
The collective analyses described above established that the mPS-PI staining procedure adapted from Wuyts et al. [17] can be used to image wall ingrowth deposition in TCs involved in phloem loading in both Arabidopsis and pea. The Wuyts et al. procedure is lengthy, however, involving several extractions in organic solvents and clearing in SDS/NaOH, followed by overnight treatment with amylase and pullulanase prior to mPS-PI staining (see Methods). To circumvent these lengthy procedures, we tested bleaching of fixed and ethanol-washed leaf tissue in sodium hypochlorite to clear cellular content for subsequent mPS-PI-staining of cell walls. Sodium hypochlorite was
used by Sugimoto et al. [25] to extract cellular content from root tissue prior to viewing cellulose microfibrils by field emission SEM, and was used by Edwards et al. [22] to clear leaf tissue for fluorescence imaging of wall ingrowth deposition in PP TCs using Calcofluor White. In this current study, the use of bleach to clear tissue resulted in equivalent, if indeed somewhat improved, imaging of wall ingrowths in PP TCs (data not shown) compared to the procedure of Wuyts et al. The bleach method provided consistently good extraction of cellular content, with the minor exception of cotyledons (see below), and often yielded well defined cellular morphology as revealed by a z-series scan of vascular tissue (Additional file 3: Movie S3). Given this outcome, we subsequently adopted the bleaching of fixed and ethanol-washed tissue as our standard method for confocal imaging of mPS-PI-stained tissue.

**Figure 3 Confocal imaging of wall ingrowth deposition in CC TCs of leaf minor veins in pea.** A. Minor veins from second true leaf of 13 day-old full light-grown seedlings showing mottled wall ingrowth labelling across the full face of CC TCs (asterisks). No wall ingrowth deposition is seen in PP cells. A y-z projection of a z-stack through the vertical minor vein is shown in inset A’. Wall ingrowth deposition detected as fuzzy labelling can be seen along all longitudinal walls of the CC TC (asterisk). A y-z projection through the horizontal minor vein at the bottom right of A is shown in inset A”. Here, fuzzy labelling indicating wall ingrowth deposition is seen around all faces of the CC TCs seen in transverse view. The double asterisk in A and A” indicates a large CC TC. B. Minor veins from second true leaf of 9 day-old seedling subjected to 4 days of darkness. No wall ingrowth deposition is seen in CC TCs (asterisks) seen in B or when the minor vein is seen in transverse view as a y-z projection through this minor vein (inset B’). BS, bundle sheath cell; PP, phloem parenchyma; SE, sieve element; XE, xylem element. Scale bars = 10 μm in A, A’ and B. Scale bars = 5 μm in A” and B’.
Wall ingrowth deposition in PP TCs from different tissues of Arabidopsis

We used our modified mPS-PI-staining procedure to survey other tissues in Arabidopsis for the presence of PP TCs. In cotyledons, as typically seen in rosette leaves and other tissues (see below), the morphology of wall ingrowths can vary depending on age of the tissue. For example, early-stage wall ingrowth development in cotyledons of 7 day-old seedlings appears identical to early-stage wall ingrowth deposition in immature leaves of 14 day-old seedlings (data not shown). In cotyledons from 18 day-old seedlings, however, extensive deposition of wall ingrowths is seen along the face of PP TCs adjacent to cells of the SE/CC complex (Figure 4A-C). The morphology of wall ingrowth deposition in cotyledons from such plants was surprisingly varied, ranging from uniform deposition similar to that seen in rosette leaves (Figure 4A, Additional file 4: Figure S1A), to sharply pointed peaks of wall ingrowth material (Figure 4B), or very substantial deposition, albeit irregularly distributed along the length of a given PP TC and occupying a considerable volume of the cell (Figure 4B, C). This feature is similar to the manner in which dense fenestrated networks of ingrowth material protrude extensively into the outer periclinal cytoplasmic volume of abaxial epidermal TCs in *V. faba* cotyledons [26]. The images shown in Figure 4A-C are of PP TCs in vascular bundles located at the base, middle and tip regions of cotyledons, respectively, reflecting a basipetal gradient of wall ingrowth deposition which correlates with phloem loading capacity in cotyledons [27]. Variations in wall ingrowth development are also apparent in nearby veins as seen in Additional file 4: Figure S1A. The PP TC marked with an asterisk in Figure S1A and A’ developed very extensive and dense wall ingrowths, while in a nearby PP TC (double asterisk, Additional file 4: Figure S1A) wall ingrowth deposition was less developed, hence typical finger-like projections can be detected in a longitudinal view (double asterisk, Additional file 4: Figure S1A”) reconstructed from the z-stack image shown in S1A.
Figure 4 (See legend on next page)
Confocal imaging of TC wall ingrowths

Figure 4 Confocal imaging of wall ingrowth deposition in PP TCs in cotyledons, cauline leaves and sepal. A. Minor vein from the base of an 18 day-old cotyledon showing extensive wall ingrowth deposition (arrows) in PP TCs (asterisks). B. Minor vein from the mid-region of an 18 day-old cotyledon showing highly sculptured and extensive wall ingrowth deposition (arrows) in PP TCs (asterisks). C. Minor vein from the tip an 18 day-old cotyledon showing massive levels of wall ingrowth deposition (arrows) that occupy a considerable volume of each PP TC (asterisks). The fragments of fluorescent labelling seen in bundle sheath cells in both B and C correspond to remnant starch grains not completely extracted by the bleach treatment. D. Wall ingrowth deposition (arrows) in PP TCs (asterisks) in a fully expanded cauline leaf. E. Wall ingrowths in a minor vein of sepal, showing numerous localized patches of wall deposition (arrows) along each PP TC (asterisk). F. Wall ingrowths in a minor vein of sepal showing apparent consolidation or merging of localized patches of wall ingrowth material (arrows) in a PP TC (asterisk). BS, bundle sheath cell; CC, companion cell; XE, xylem element. Scale bars = 10 μm in A, B and C. Scale bars = 5 μm in D, E and F.

Wall ingrowth deposition in PP TCs was also detected in cauline leaves (Figure 4D, Additional file 4: Figure S1B) and sepals (Figure 4E, F, Additional file 4: Figure S1C). In cauline leaves deposition of ingrowth material is typically abundant, especially in veins in the tip region of the leaf (Figure 4D). In sepal tissue, wall ingrowths were often seen as discrete clusters of wall material positioned along the length of a PP TC (Figure 4E). These discrete clusters appeared in places to merge with neighboring clusters to form localized clumps of ingrowth material along a given PP TC (Figure 4F). These features were also seen in PP TCs in other tissues (e.g., Figure 5C), but were more common in sepals. Xylem elements were often detected adjacent to PP TCs in sepals (Figure 4E, F, Additional file 4: Figure S1C) due to the simple structure of vascular bundles in this tissue (see [14]).

Semi-quantitative assessment of wall ingrowth deposition in PP TCs

The clarity of confocal imaging by the bleach-modified mPS-PI procedure enabled semi-quantitative assessment of both the extent of trans-differentiation of PP TCs and the abundance of wall ingrowth deposition in a given cell. To facilitate this process, we developed a scoring procedure ranging across four categories of wall ingrowth deposition as defined by our observations. Class I represents PP cells with no detectable wall ingrowths (Figure 5A, Additional file 5: Figure S2A, B). These cells, defined by their elongated, rectangular shape and connection to a neighboring bundle sheath cell, were devoid of detectable wall ingrowths as evidenced by the thin, regular outline of their stained cell walls. In Class II, wall ingrowths were detected in early
stages of development as evidenced by limited regions of patchy, mottled staining along the wall of the PP TC opposite that of a bundle sheath cell (Figure 5B, Additional file 5: Figure S2F), or visualized as discrete dots of fluorescence in face view (Additional file 5: Figure S2C, D, E). For this class, not all PP cells in a given region of vein showed evidence of wall ingrowth deposition. In Class III, wall ingrowth deposition was more obvious as wider regions of mottled fluorescence and this level of deposition was commonly detected in most but not necessarily all PP cells in a field of view (Figure 5C, Additional file 5: Figure S2G, H, I, J). In Class IV, wall ingrowths in PP TCs were very abundant and seen as continuous thick bands of mottled fluorescence present in essentially all PP TCs in the field of view (Figure 5D, Additional file 5: Figure S2K, L).

Figure 5 Classification system for the extent of wall ingrowth deposition in PP TCs in minor veins of Arabidopsis leaves. A. Class I - no wall ingrowths visible in PP cells (asterisks), which are identified by their sharing a common wall with a large bundle sheath cell. B. Class II - evidence of discrete, punctate-like wall ingrowth deposition seen as discrete fluorescent dots (arrows) distributed along the face of PP TCs adjacent to either a CC or SE. Not all PP cells in a given field of view contain wall ingrowths. C. Class III - substantial levels of reticulate wall ingrowth deposition is seen as clusters or continuous stretches of fluorescent labelling (arrows) on the face of PP TCs (asterisks) neighboring cells of the SE/CC complex. This level of labelling is seen in most PP cells in a given field of view, but can be somewhat variable. D. Class IV - extensive wall ingrowth deposition seen as thick bands of fluorescence labelling (arrows) seen in essentially all PP TCs in a given field of view. BS, bundle sheath cell; CC, companion cell; SE, sieve element. Scale bars = 10 μm in A, B, C and D.

Using this classification system, we qualitatively surveyed the abundance of wall ingrowth deposition in PP TCs in terminating minor veins across leaf development, selecting leaf 11, 8 and 5 as representative of immature, intermediate and mature leaves, respectively (Figure 6A). A representation of this survey is shown in Figure 6B.
Immature sink leaves contain predominantly Class I PP cells with no wall ingrowths, while intermediate leaves are characterized by a basipetal gradient with Class III PP TCs in minor veins at the tip of the leaf and predominantly Class I PP cells at the leaf base. In contrast, mature source leaves are dominated by Class IV PP TCs with highly abundant wall ingrowths in minor veins across virtually the entire leaf (Figure 6B). The distribution of PP TCs seen in this analysis is consistent with the known sink-source transition that occurs in maturing leaves [28], as well as the development of apoplastic loading that occurs in a basipetal gradient within a dicot leaf [23,27]. The value of mPS-PI staining in this case, however, is that it provides a rapid means to assess the development of PP TCs across an entire leaf in response to different biotic or abiotic signals and within different genetic backgrounds, with high spatial resolution without relying on time-consuming procedures such as TEM.

Figure 6 Survey of wall ingrowth deposition in PP TCs in minor veins across leaf development in Arabidopsis. A. Leaf numbering of 3.5 week-old rosette leaf. B. Representation of wall ingrowth deposition in immature, intermediate and mature leaves as represented by color-coding of the four classes of wall ingrowth deposition as described in Results. The result shown here is representative of three replicate leaves for each stage of development. Immature leaves contain little if any wall ingrowth deposition in PP cells. Intermediate leaves show a basipetal gradient from typically Class III at the tip to Class I at the leaf base. Mature leaves contain Class IV wall ingrowth deposition in PP TCs of minor veins throughout the entire leaf, with the exception of a few examples of Class III at the very base of the leaf. Skeletonized images of leaves shown in Figure 6B were adapted from Alonso-Peral et al. [34].
Manipulating wall ingrowth deposition in PP TCs by defoliation

We next examined the response of PP TC development to altered sink-source status in Arabidopsis leaves as a test of the mPS-PI staining procedure to provide semi-quantitative assessment of wall ingrowth development. For this analysis we conducted a defoliation experiment on 3-week-old plants by removing all leaves except leaf 9, 10 and 11 (Figure 7A). After five days further growth, leaves from control and defoliated plants (Figure 7B) were harvested and stained by the bleach-modified mPS-PI procedure. In leaf 10 from control plants, typically no wall ingrowths were visible in PP cells (Class I; Figure 7C), but in a few cases Class II wall ingrowth deposition was occasionally seen (Figure 7D). In this case, the ingrowths were small discrete clusters of wall material. In contrast to the typical lack of ingrowth deposition in control leaves, the extent of wall ingrowth deposition in leaf 10 from defoliated plants was substantially increased (Figure 7E-G). In these cases, Class III deposition was commonly seen, either in early stages of deposition where discrete clusters of tangled wall ingrowths were detected (Figure 7E), or as more dense clusters or bulges of ingrowth wall deposition (Figure 7F). In the tip region of leaf 10 from a defoliated plant, numerous cases of Class IV deposition were detected in these leaves 5 days-post defoliation (Figure 7G).

To provide a semi-quantitative analysis of the response of wall ingrowth development to defoliation, we used a scoring system whereby Class I, II, III and IV were assigned 0, 2, 4 and 6 points, respectively. Using these values, we then scored PP TC development in at least five terminating veins within the base, middle and tip regions of each leaf on either side of the mid-vein (see Figure 6B). The results of this analysis showed a rapid and significant increase in wall ingrowth deposition in PP TCs in leaf 10 of defoliated plants relative to leaf 10 in control (non-defoliated) plants, with this response being more pronounced in PP TCs in minor veins in the middle sector of leaves (ca. 8.6-fold change) compared to the tip (ca. 2.2-fold change; Figure 7H).
Figure 7 Semi-quantitative analysis of wall ingrowth deposition in PP TCs of leaf minor veins in Arabidopsis following defoliation. A. All leaves except leaf 9, 10, and 11 were removed from 3 week-old plants. The picture shows a control plant (left) and a defoliated plant (right) at the beginning of the experiment. B. Control (left) and defoliated plant (right) after 5 days additional growth. C. Minor vein in leaf 10 from control plant. No wall ingrowth deposition is seen in PP TCs (asterisks). D. Minor vein in leaf 10 from control plant showing early stage wall ingrowth deposition (arrows) in a PP TC (asterisk). E. Wall ingrowth deposition seen as discrete clusters (arrows) in a PP TC (asterisk) in leaf 10 of defoliated plant. F. Denser clusters of wall ingrowth deposition (arrow) in leaf 10 of defoliated plant. G. Extensive wall ingrowth deposition (arrows) in PP TCs (asterisks) in vein near the tip of leaf 10 from a defoliated plant. BS, bundle sheath; CC, companion cell. Scale bars = 5 μm in D, E, F and G. Scale bar = 10 μm in C. H. Semi-quantitative analysis of defoliation on wall ingrowth deposition in PP TCs of leaf 10 measured in control and defoliated plants 5 days after defoliation. Wall ingrowth deposition in PP TCs is greatly enhanced as a consequence of defoliation, with this response being maximal in minor veins from the middle region of the leaf. Data shows mean ± SE of scores for wall ingrowth deposition in arbitrary units (AU); n = 4.

Discussion

We have developed a simplified mPS-PI staining procedure for confocal imaging of wall ingrowths in vascular TCs involved in phloem loading. This procedure, involving
clearing of fixed tissue using sodium hypochlorite followed by mPS-PI staining and mounting in high refractive index mounting medium provides a rapid means to image wall ingrowth deposition in TCs without the need to use more time-consuming electron microscopy techniques. The clarity of the mPS-PI staining enables high-resolution imaging of wall ingrowths in vascular tissue buried deep within the leaf, and the use of sodium hypochlorite as a clearing step simplifies the original methods of Truernit et al. [16] and Wuyts et al. [17] to enable high-throughput processing of samples to suite semi-quantitative assessment of wall ingrowth deposition in TCs. Furthermore, the ability to optically section throughout an entire vascular bundle and reconstruction from a series of z-axis images enables the three-dimensional reconstruction of wall ingrowth deposition to analyse the highly polarised nature of this process in TCs.

We used this procedure to analyse the distribution of PP TCs both within individual leaves and in leaves of different developmental stages. Haritatos et al. [12] noted that veins of different sizes have overall similar cellular structure and organization, and since most veins are in close proximity to mesophyll, they can be considered to participate in phloem loading and thus physiologically defined as “minor” veins. Our observations support this conclusion since virtually all veins examined, except the midrib and larger regions of the secondary vein network, contained PP TCs with typically substantial levels of wall ingrowth deposition. Furthermore, within individual developing leaves, a basipetal gradient of PP TCs was detected (Figure 6). Both observations are consistent with development of phloem loading capacity in leaves [27,28] and demonstrate a presumed correlation between wall ingrowth deposition in PP TCs and their role in phloem loading.

The general applicability of this method for confocal imaging of wall ingrowth formation in TCs was demonstrated by visualizing CC TCs in minor veins of pea leaves. In this case, light-dependent deposition of wall ingrowths to all faces of CC TCs was clearly detected (Figure 3), consistent with earlier studies using TEM [19,20]. Thus, it is likely that this approach can be used to investigate TC development in other species and tissue locations. In Arabidopsis, we observed PP TCs with extensive wall ingrowths in sepals, cotyledons and cauline leaves (Figure 4) as well as the first true leaves of
Confocal imaging of TC wall ingrowths

young seedlings (data not shown). Interestingly, in both cotyledons and cauline leaves, the presence of PP TCs with abundant wall ingrowths correlates with the high relative expression of both AtSWEET11 and AtSWEET12 (eFP Browser; bar.toronto.ca). These sucrose effluxers have recently been demonstrated to be involved in a two-step phloem loading strategy used in Arabidopsis leaves, namely unloading of sucrose into the apoplasm by PP TCs driven by AtSWEET transporters, followed by active uptake into cells of the SE/CC complex by AtSUC2 [14]. The extensive wall ingrowth deposition observed in PP TCs of cotyledons is also consistent with cotyledons acting as a strong source of photosynthesis-derived sucrose required to sustain root growth in response to light [29].

An intriguing observation seen most clearly in sepal tissue was the initial deposition of wall ingrowths as numerous discrete clusters along the length of a PP TC (Figure 4E). A similar pattern of deposition was seen in young leaves responding to defoliation (Figure 7E). These structures are presumably equivalent to the isolated patches of wall deposition observed by SEM (Figure 2D). These observations suggest that early stages of reticulate ingrowth deposition can be highly localized to discrete regions within an individual PP TC, and then continued deposition causes consolidation of these patches into more continuous regions of ingrowth deposition. The signals directing such localized patches of ingrowth deposition are unknown, but in non-vascular TC types the reactive oxygen species hydrogen peroxide has been implicated as a polarizing signal directing wall ingrowth deposition [5,30,31]. Recently, localized plumes of Ca\(^{2+}\) have been implicated in directing the highly localized deposition of individual papillae wall ingrowths in epidermal TCs of V. faba cotyledons [32]. A similar mechanism may operate in PP TCs, however the larger clusters of wall deposition seen by SEM and confocal imaging (Figures 2, 4, 7) imply a higher level of organization may be operating, possibly aggregation of Ca\(^{2+}\) channels, to direct the deposition of wall ingrowths into such clusters. The ability to clearly image wall ingrowths by confocal microscopy in Arabidopsis PP TCs will enable a genetic approach to investigate signalling mechanisms driving this process.

The defoliation experiment (Figure 7) illustrates the value of the bleach-modified mPS-PI method in combination with semi-quantitative scoring to provide high-
throughput and high-resolution assessment of PP TC development in leaves. The significant increase in wall ingrowth deposition in PP TCs in leaf 10 remaining after defoliation suggests a rapid switch from sink to source status within this leaf [33], and a requirement for wall ingrowth deposition for this to occur. The predicted concomitant changes in gene expression required for wall ingrowth deposition amid other processes associated with this transition provides an opportunity to identify these genes by transcriptional profiling.

Conclusion

We have developed a simple method for confocal imaging of wall ingrowth deposition in TCs using mPS-PI staining. This method was used to image wall ingrowth deposition in PP TCs in rosette leaves, cauline leaves, cotyledons and sepals of Arabidopsis as well as CC TCs in leaf minor veins of pea. The clarity of the staining provides cellular detail of wall ingrowth deposition in these diverse tissues, thus enabling future studies investigating the cellular mechanisms directing the highly polarized deposition of wall ingrowths in TCs without the need to use electron microscopy. The high-throughput potential of this procedure also offers the opportunity to apply reverse genetics to identify genes involved in wall ingrowth deposition in TCs.

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References


22. Edwards J, Martin AP, Andriunas F, Offler CE, Patrick JW, McCurdy DW: *GIGANTEA* is a component of a regulatory pathway determining wall ingrowth deposition in phloem parenchyma transfer cells of *Arabidopsis thaliana*. *Plant J* 2010, **63**:651-661.


Online supplementary data
*(Additional files 1-3 are included on a DVD disk attached to this thesis.)*

**Additional_file_1 as AVI** (see attached DVD disk)

**Additional file 1: Movie S1** z-stack scan of *Arabidopsis* leaf minor vein showing wall ingrowth deposition in PP TCs. The scan moves through the tissue in an abaxial to adaxial direction. Wall ingrowths (red arrows) showing polarized deposition to the face of two PP TCs neighboring a common companion cell (CC) are seen. In some regions of the minor vein, wall ingrowth deposition can be seen as a central band (yellow arrows) which co-aligns with a neighboring sieve element (SE).

**Additional_file_2 as AVI** (see attached DVD disk)

**Additional file 2: Movie S2** z-stack scan of *Arabidopsis* second order vein showing a rare example of wall ingrowth deposition (purple arrows) in a PP TC in the vein. The scan moves through the tissue in an abaxial to adaxial direction. Most second order veins do not contain PP TCs. Wall ingrowth deposition (yellow arrows) is seen in a PP TC in the third order vein at the top of the image. M, myrosin cell; SE, sieve element.

**Additional_file_3 as AVI** (see attached DVD disk)

**Additional file 3: Movie S3** z-stack scan of *Arabidopsis* leaf minor vein from fixed tissue extracted with bleach. The scan moves through the tissue in an abaxial to adaxial direction.
direction. Bleach extraction provides good preservation of cellular architecture and imaging of PP TCs with imaging of wall ingrowths in PP TCs being comparable if not superior to the more complex extraction procedures employed by Truernit et al. [16] and Wuyts et al. [17].

**Additional file 4 as TIFF**

**Additional file 4: Figure S1** Additional examples of wall ingrowth deposition in PP TCs in cotyledons, cauline leaves and sepal.

![Figure S1 Additional examples of wall ingrowth deposition in PP TCs in cotyledons, cauline leaves and sepal](image)

Figure S1 Additional examples of wall ingrowth deposition in PP TCs in cotyledons, cauline leaves and sepal. **A, A’ and A”**. Wall ingrowth deposition in cotyledons. **A.** Single confocal section of a minor vein junction revealing polarized deposition of wall ingrowths (arrows). The yellow dotted lines labelled $x$-$z$ and $x’$-$z$ correspond to the projection shown in A’ and A”, respectively. **A’.** $x$-$z$ projection of a $z$-stack of the image shown in A revealing minor vein architecture in transverse section and the presence of highly-localized and very substantial deposition of wall ingrowth material occupying nearly half the cell volume (asterisk). **A”.** $x’$-$z$ projection of a $z$-stack of the image shown in A revealing the longitudinal section of a PP TC (double asterisks) with less extensive wall ingrowth deposition but with finger-like projections (arrow).  **B, B’ and B”.** Wall ingrowth deposition in cauline leaves. **B.** Single confocal section of a minor vein junction showing polarized deposition of wall ingrowths (arrows). The yellow dotted lines labelled $y$-$z$ and $x$-$z$ correspond to the projections shown in B’ and B”, respectively. **B’.** $x$-$z$ projection of a $z$-stack of the image shown in B revealing minor vein architecture in transverse section and the presence of highly-localized wall ingrowth deposition (arrow). **B”.** $y$-$z$ projection of a $z$-stack of the image shown in B revealing finger-like projections (arrows) of wall ingrowths in a PP TC (double asterisks).  **C and C’.** Wall ingrowth deposition in sepals. **C.** Single confocal section of a minor vein revealing polarized deposition of wall ingrowths (arrows). Note that xylem elements (XE) were also detected in this small minor vein. The yellow dotted line labelled $y$-$z$ corresponds to the projections shown in C’. **C’.** $y$-$z$ projection of a $z$-stack of the image shown in C revealing finger-like wall ingrowth projections (arrows) in a PP TC (asterisk). Scale bars = 10 μm.
Additional file 5: Figure S2 Additional examples of the four classes of wall deposition in PP TCs in Arabidopsis leaf veins.

Figure S2 Additional examples of the four classes of wall deposition in PP TCs in Arabidopsis leaf veins. Asterisks in all figures represent PP or PP TCs. The double asterisks in I and J represent PP TCs with Class II deposition in a region of minor vein otherwise defined as Class III. Arrows point to wall ingrowth deposition in PP TCs. See Figure 5 for description of each class. Scale bars = 10 μm.

These supplementary data can be found online at http://bmcplantbiol.biomedcentral.com/articles/10.1186/s12870-015-0483-8.
CHAPTER 3

Heteroblastic Development of Transfer Cells in Arabidopsis is Controlled by the miR156/SPL Module

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Abstract

We report that wall ingrowth deposition in phloem parenchyma (PP) transfer cells (TCs) in leaf veins of *Arabidopsis thaliana* (Arabidopsis) represents a novel trait of heteroblasty. Development of PP TCs involves extensive deposition of wall ingrowths adjacent to cells of the sieve element/companion cell (SE/CC) complex. These PP TCs potentially facilitate phloem loading by enhancing efflux of symplasmic sucrose for subsequent active uptake into cells of the SE/CC complex. PP TCs with extensive wall ingrowths are ubiquitous in mature cotyledons and juvenile leaves, but dramatically less so in mature adult leaves, an observation consistent with PP TC development reflecting vegetative phase change (VPC) in Arabidopsis. Consistent with this conclusion, the abundance of PP TCs with extensive wall ingrowths varied across rosette development in three ecotypes displaying differing durations of juvenile phase, and extensive deposition of wall ingrowths was observed in rejuvenated leaves following prolonged defoliation. PP TC development across juvenile, transition and adult leaves correlated positively with levels of miR156, a major regulator of VPC in plants, and corresponding changes in wall ingrowth deposition were observed when miR156 was overexpressed or its activity suppressed by target mimicry. Analysis of plants carrying miR156-resistant forms of *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) genes showed that wall ingrowth deposition was decreased in *SPL9*-group but not *SPL3*-group genes, indicating that *SPL9*-group genes may function as negative regulators of wall ingrowth deposition in PP TCs. Collectively, our results point to wall ingrowth deposition in PP TCs being under control of the genetic program regulating VPC.

Introduction

Transfer cells (TCs) play critical roles in membrane transport of solutes at various sites within plants and between plants and their environment (Gunning and Pate, 1969; Offler et al., 2003; McCurdy, 2015). This transport capacity is conferred by wall protuberances which extend into the cell lumen, hence called wall ingrowths. These ingrowths, considered to be primary wall-like in composition (Vaughn et al., 2007), are
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deposited secondarily on the inner face of the primary cell wall and function to enhance the area of surrounding plasma membrane, therefore increasing surface-to-volume ratio of the TC and consequently promoting potential trans-membrane flux of solutes (Gunning et al., 1968; Gunning and Pate, 1974; Offler et al., 2003).

TCs have been reported widely across the plant kingdom, from seedless plants, e.g., algae, hornworts, mosses, etc., to gymnosperms and angiosperms, including both monocotyledons and dicotyledons (Gunning and Pate, 1969; Pate and Gunning, 1972; Gunning, 1977; Offler et al., 2003). Across the plant kingdom, the occurrence of TCs is proposed to be an indicator of anatomical locations where intensive transport occurs, however the existence of bottlenecks in nutrient exchange does not always ensure development of TC morphology. In Vicia faba seeds, for example, TCs develop in both maternal and filial interfaces whilst they are present at just the filial site of Zea mays seeds and completely absent in seeds of Phaseolus vulgaris (Offler et al., 2003). Interestingly, many species lacking TCs under normal growth conditions nonetheless have the capacity to produce these highly specialized cells when abnormal stimuli or stress are applied; an example of this scenario is where iron and phosphorous starvation bring about wall ingrowth formation in rhizodermal TCs of tomato (Schikora and Schmidt, 2002). A compilation of such sporadic but versatile and widespread occurrences of TCs suggests that all plant species possess the genetic information coding for their development, but this genetic competency may be repressed in some circumstances (Gunning and Pate, 1974; Gunning, 1977). This observation has raised a long-standing question of why and when a given tissue of a given species develops TCs with wall ingrowths if its genome encodes capacity to do so. This question implies that specific signals are required for TC development and thus response to these signals may vary depending on the developmental context of the tissue or its exposure to specific biotic or abiotic stresses.

In Arabidopsis thaliana (Arabidopsis), phloem parenchyma (PP) cells in the minor vein network of leaves (Haritatos et al., 2000) and sepals (Chen et al., 2012) can develop wall ingrowths to become PP TCs. Wall ingrowths in these PP TCs are typically bulky and predominantly abut sieve elements (SEs) and to a lesser extent companion cells (CCs) (Haritatos et al., 2000; Amiard et al., 2007). PP TCs have been assumed to
facilitate the efflux of photoassimilates into the apoplasm (Haritatos et al., 2000), and support for this notion was the recent discovery of SWEET11 and 12 effluxers in plasma membrane putatively associated with wall ingrowths in these PP TCs (Chen et al., 2012).

Previous studies of PP TCs in Arabidopsis veins have relied on using transmission electron microscopy (TEM) since these cells are buried deep within vascular bundles (e.g., Haritatos et al., 2000; Amiard et al., 2007; Maeda et al., 2006, 2008). Recently, we used a modified pseudo-Schiff-propidium iodide (mPS-PI) procedure for staining cell walls (Truernit et al., 2008; Wuyts et al., 2010) to image wall ingrowth deposition in PP TCs by confocal microscopy (Nguyen and McCurdy, 2015). This procedure enables more extensive and semi-quantitative assessment of PP TC development at the whole leaf level and across rosette development compared to using TEM, and was used to assess, amongst other criteria, the extent of wall ingrowth deposition in response to defoliation (Nguyen and McCurdy, 2015). In surveying PP TCs across individual leaf and rosette development, we identified that wall ingrowth deposition was highly abundant in early-emerged organs such as cotyledons (Nguyen and McCurdy, 2015) and juvenile leaves, and was dramatically less so in later-emerged adult leaves. These observations led us to speculate that PP TC development, as assessed by levels of wall ingrowth deposition, is specific to leaf identity.

Leaf identity is specified during shoot development through heteroblasty or vegetative phase change (VPC), a phenomenon where plants progress from a juvenile, to transition, and then an adult phase (Poethig, 1990; Kerstetter and Poethig, 1998). The progression of phase transition is mainly controlled by miR156-mediated repression of SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) genes, with this class of SPL involved in promoting a variety of adult traits (Wu and Poethig, 2006). In addition, a small subset of VPC traits are regulated by a second pathway, the trans-acting small interfering RNA (tasiRNA)/AUXIN RESPONSE FACTOR (ARF/tasiARF)-dependent pathway (Hunter et al., 2006). In this study, we investigated the hypothesis that PP TC development is regulated by the same genetic program, or elements thereof, that control VPC. We found that levels of miR156 across leaf maturation of both juvenile and adult leaves correlated strongly with PP TC development, and
conversely, levels of the miR156 target genes, namely *SPL3*, *SPL9*, *SPL10* and *SPL15*, showed a negative correlation with wall ingrowth deposition in PP TCs. Furthermore, disrupting VPC by either extensive defoliation or genetic manipulation of the miR156- *SPL-*specific pathway caused corresponding changes in levels of wall ingrowth deposition. Collectively, we conclude that PP TC development is controlled by the genetic program that regulates VPC in Arabidopsis. The implications of this finding in terms of the putative role(s) for PP TCs in Arabidopsis veins are discussed.

**Results**

**Classification and Observations of Wall Ingrowth Abundance in PP TCs of Arabidopsis Veins**

We recently reported semi-quantitative assessment of wall ingrowth deposition in PP TCs of Arabidopsis veins by confocal imaging of leaves stained by a modified pseudo-Schiff propidium iodide staining technique (Nguyen and McCurdy, 2015). This paper described four classes of PP TC development depending on abundance of wall ingrowth deposition, ranging from Class I with no wall ingrowths to Class IV with abundant deposition (Nguyen and McCurdy, 2015). By assigning an arbitrary value of 0, 2, 4 and 6 for Classes I, II, III and IV, respectively, a semi-quantitative assessment of wall ingrowth deposition in PP TCs across single leaves of developing and developed shoots could be generated (Nguyen and McCurdy, 2015). Following extensive observations of PP TCs in organs such as cotyledons and early juvenile leaves in this current study, we have added an additional Class V (8 points) to this scoring system, whereby Class V PP TCs show massively abundant wall ingrowth deposition which occupies a substantial volume of the PP TC. This extended classification scheme is outlined in Supplemental Fig. S1 and scoring of wall ingrowth deposition based upon this modified scheme was performed as described in Nguyen and McCurdy (2015). Based on this scheme, and herein, we use the term “PP TC development” to indicate levels of wall ingrowth deposition in these cells.

A survey of PP TC development at the whole leaf level throughout shoot development in Arabidopsis ecotype Columbia-0 (Col-0) revealed that early-emerged
juvenile leaves have abundant Class IV or V PP TCs distributed throughout the entire vein system, whereas in later-emerged adult leaves, PP TCs are much less abundant (Class II or III) and are distributed across the leaf in a distinctive basipetal gradient (Fig. 1A). Early transition leaves have relatively numerous Class IV PP TCs, whereas in late transition leaves PP TCs are predominantly Class III and display the basipetal gradient similar to the patterns seen in adult leaves (Fig. 1A). Collectively, in a mature plant, successive leaves differ in levels of PP TC development, with these levels gradually decreasing along the shoot axis (Fig. 1A). These observations are consistent with PP TC development representing a novel trait of heteroblasty in Arabidopsis, and thus encouraged us to further investigate heteroblastic features of PP TC development.

Figure 1. Heteroblastic development of PP TCs in ecotypes Col-0, Ws-2 and Ler-0. A, PP TC abundance and distribution in mature leaves 1 to 12 from 30-day-old Col-0 plants (n = 9). B, PP TC abundance and distribution in mature leaves 1 to 7 from 25-day-old Ws-2 plants (n = 4). C, PP TC abundance and distribution in mature leaves 1 to 7 from 28-day-old Ler-0 plants (n = 4). The colored dots in A-C represent PP TC development across each leaf as defined by Classes I-V (see Supplemental Fig. S1). Leaf template images were modified from Yang et al. (2013).
PP TC Development and Distribution in Mature Rosette Leaves Reflect Heteroblastic Differences Amongst Ecotypes

Telfer et al. (1997) reported that the ecotypes Col-0, Landsberg erecta (Ler) and Wassilewskija (Ws) display differing durations of juvenile phase. Therefore, we examined if changes in PP TC development in these ecotypes also reflects differences in heteroblasty accordingly. Under long day conditions, abaxial trichomes frequently appeared in leaf 6 of Col-0, leaf 4 of Ws-2 and leaf 5 of Ler-0 plants (Supplemental Fig. S2A). Leaves lacking abaxial trichomes are juvenile leaves, whereas leaves partially or completely covered with abaxial trichomes are defined as transition or adult leaves, respectively (Telfer et al., 1997; Willmann and Poethig, 2011). We noted that in Col-0, leaves 6 to 8 are transition leaves and leaves 9 to 12 are adult (Fig. 1A), whereas in Ws-2, leaves 4 and 5 are transition and leaves 6 and 7 are adult (Fig. 1B). The latest-emerged leaves 12 and 7 of Col-0 and Ws-2 plants respectively, are typical of adult identity with hundreds of trichomes covering the whole abaxial leaf surface (Supplemental Fig. S2A). In Ler-0 plants however, and in most instances, the latest-emerged leaf 7 has many fewer abaxial trichomes, which were frequently restricted to the midvein, making them more like transition leaves (Fig. 1C; Supplemental Fig. S2A).

Interestingly, PP TC development and distribution in leaf veins of all three ecotypes showed a negative correlation with that of abaxial trichomes. In mature juvenile leaves 1 and 2, which do not have abaxial trichomes, PP TCs with extensive wall ingrowth deposition (Class V for Col-0 and Ler-0, Classes IV and V for Ws-2) were extremely abundant and distributed throughout the whole leaf vein system (Fig. 1; Fig. 2A, E and I). Transition leaves in all three ecotypes frequently had Class IV PP TCs distributed across the apical half of the leaf and Class II to IV in basal regions of the leaf (Figs. 1, 2B, F and J). Latest emerged adult leaves 11 and 12 of Col-0 plants and leaf 7 of Ws-2 plants, which are completely covered by abaxial trichomes at maturity, had many fewer PP TCs with mostly Class II or III at the tip region, Class I or II in the middle third of the leaf and Class I at the base of the leaf (Figs. 1, 2C, D, G and H). Latest emerged adult leaf 7 of Ler-0 plants, which were partially covered by abaxial trichomes, had abundant PP TCs with Class IV at the tip, Class III in the middle and Class II or III at the base (Figs. 1, 2K and L). The gradual decrease and eventual absence of PP TCs in the
basal region of last-emerged adult leaves in Col-0 (Fig. 1A) and Ws-2 (Fig. 1B) is concomitant with a significant decrease in cell size, particularly cells of vascular bundles including PP cells (Supplemental Fig. S2B).

Although total leaf number of Ws-2 and Ler-0 plants were similar (6 or 7 leaves), there were striking differences in wall ingrowth deposition in PP TCs between leaf 6 of these two ecotypes, and these differences paralleled differences seen in abaxial trichome abundance and distribution. Leaf 6 of Ws-2 plants was typical of adult leaves with numerous abaxial trichomes distributed across the entire leaf (Fig. 1B; Supplemental Fig. S2A). Consistently, PP TCs in these leaves also showed adult characteristics with Class III PP TCs at the tip (Figs. 1B and 2G) and declining to Class I or II at the base (Figs. 1B and 2H), which resembled PP TCs in adult leaves 10 or 11 of Col-0 plants (Fig. 1A). PP TCs in leaf 6 of Ler-0 plants however, showed typical transition characteristics with Class IV observed throughout the vein network except for a few basal regions containing Class III PP TCs (Figs. 1C and 2J). Abaxial trichomes in these leaves were few in number and restricted to the midvein (Fig. 1C; Supplemental Fig. S2A). Also, leaf 6 of Ws-2 plants was highly serrated whereas leaf 6 of Ler-0 plants displayed smooth margins (Supplemental Fig. S2C). These results collectively support the conclusion that PP TC development differed across ecotypes in Arabidopsis in a manner similar to the known differences in vegetative phase length amongst these ecotypes.

(See figure on next page)

Figure 2. Confocal imaging of wall ingrowth deposition in PP TCs in mature leaves of different ecotypes. A to D, Col-0. E to H, Ws-2. I to L, Ler-0. A, E and I, Confocal images of veins from juvenile leaf 1 of Col-0 (A), Ws-2 (E) and Ler-0 (I) plants showing Class V PP TCs characterized by massive deposition of wall ingrowths (arrows) occupying a large volume of each PP TC (asterisks). B, F and J, Confocal images of veins from transition leaves, namely leaf 6 of Col-0 (B), leaf 4 of Ws-2 (F) and leaf 6 of Ler-0 (J) plants, showing Class IV PP TCs with extensive deposition of wall ingrowths (arrows). C, D, G, H, K and L, Confocal images of veins from adult leaves, namely leaf 11 of Col-0 (C and D), leaf 6 of Ws-2 (G and H) and leaf 7 of Ler-0 (K and L). Adult leaves of Col-0 and Ws-2 have Class III PP TCs (asterisks) with clusters of wall ingrowths (arrows) at the leaf tip (C and G) and have PP cells (asterisks) without wall ingrowths at the leaf base (D and H). Class IV PP TCs are seen at the leaf tip (K) and Class III PP TCs at the leaf base (L) in adult leaf 7 of Ler-0. BS, bundle sheath. Scale bar = 10 μm for all images.
Figure 2 (See legend on previous page)
Defoliation Confirms that Abundant PP TCs with Extensive Wall Ingrowth Deposition Signify Juvenile Identity of Leaves

It is well established that defoliation and severe pruning (Njoku, 1956; Libby and Hood, 1976; Yang et al., 2011), or culture of adult shoot apices in low-sugar medium (Orkwiszewski and Poethig, 2000), prolongs the production of juvenile traits in both herbaceous and woody plants. In this study we performed prolonged defoliation to examine if PP TC development occurs as part of whole-shoot rejuvenation. Ten-day-old plants were subjected to leaf ablation where leaves 1 and 2 were removed (Fig. 3A), and this process of leaf removal was continued at two- or three-day intervals until a total of eight leaves were ablated. Defoliated plants subsequently displayed a prolonged juvenile phase, produced approximately seven more juvenile leaves than control plants, but had a normal adult phase duration (Fig. 3B). Leaves 9 and 10 in control plants showed typical adult characteristics with numerous abaxial trichomes, short petioles and elongated, serrated and highly down-curved blades, whereas leaves produced at comparable nodes in defoliated plants were completely rejuvenated, evidenced by the absence of abaxial trichomes (Fig. 3C), long petioles and round, smooth and flat blades, resembling leaves 3 or 4 of control plants (Fig. 3D and E). In addition to these external morphological changes, internal anatomy of leaves 9 and 10 of defoliated plants also strikingly mirrored juvenile identity, evidenced by simple venation patterns and larger cell size (Supplemental Fig. S3). Of significance to this study, PP TC development in rejuvenated leaf 10, akin to PP TC development in juvenile leaves, was dramatically increased compared to that in leaf 10 of control plants (Fig. 3F-K). In addition, the distribution of PP TCs with abundant wall ingrowths did not display a basipetal gradient seen in control leaf 10, but rather was uniformly abundant from tip to base in these rejuvenated leaves (Fig. 3I and J). Collectively, these results show that changes in PP TC development paralleled changes in other phase-specific traits upon defoliation and that rejuvenated leaves displayed juvenile-like wall ingrowth deposition, thus indicating that abundant PP TCs with extensive wall ingrowths signify juvenile identity of leaves.
Figure 3. Effects of prolonged defoliation on PP TC development. A, Ten-day-old Col-0 seedlings before and after defoliation of juvenile leaves 1 and 2. Scale bar = 10 mm. B, Numbers of juvenile and adult leaves from control and defoliated plants. Leaf ablation delayed the production of adult leaves in defoliated plants; these plants produced approximately seven more juvenile leaves than control plants but the number of adult leaves was unaffected. Data is mean ± SE (n = 12). C, Leaf 10 of defoliated plants was completely rejuvenated, evidenced by the absence of abaxial trichomes. Data is mean ± SE (n = 12). D and E, Morphology of rejuvenated leaf 10 of defoliated plants resembled that of juvenile leaf 4 of control plants in terms of long petiole, round blade and narrower blade base angles, whereas leaf 10 of control plants was typical of adult characteristics with elongated blade, short petiole and wide blade base angles. Scale bars = 5 mm for all images in D. F to J, Confocal imaging of wall ingrowth deposition in PP TCs in mature leaves. Class V PP TCs with massive deposition of wall ingrowths were observed in both apical (I) and basal (J) regions of rejuvenated leaf 10 from defoliated plants, which is akin to PP TC development in leaf 4 of control plants (F). In contrast, leaf 10 of control plants had Class III PP TCs at the tip (G) and Class II PP TCs at the base (H). BS, bundle sheath. Arrows point to wall ingrowths; asterisks indicate PP TCs. Scale bars = 10 μm in all panels. K, Semi-quantitative analysis of PP TC development in rejuvenated leaf 10 of defoliated plants compared to leaves 4 and 10 of control plants. Defoliation experiments were performed three times (n = 9, n = 10 and n = 12) with the same outcomes. Results from one experiment are presented.
Chapter 3

PP TC Development Across Leaf Maturation Varies Depending on Heteroblastic Status of the Leaf and Is Independent of Day Length

In Arabidopsis, differentiated PP cells, characterized by a smooth primary cell wall, trans-differentiate to form PP TCs with wall ingrowths in vascular bundles of leaves and leaf-like organs (Haritatos et al., 2000; Nguyen and McCurdy, 2015, 2016). The striking differences in PP TC development between juvenile and adult leaves at their maturity (Figs. 1, 2A, C, D, E, G and H) imply differences in the trans-differentiation process of PP TCs in these leaves. Therefore, we examined the process of PP TC development across leaf maturation from expanding to fully expanded leaves. Our analysis of expanding leaves revealed that, regardless of leaf identity, PP TC fate was not determined during the ontogeny of vascular cells. Procambial cells, differentiating from the meristem, can follow either phloem or xylem cell fates to form phloem or xylem cell precursors, respectively. Phloem cell precursors then differentiate into SEs, CCs or PP cells (Fukuda, 2004; Schuetz et al., 2012). Therefore, the presence of SEs with distinct sieve plates can serve as an indicator of the differentiated state of other phloem cells. As shown in Fig. 4A, B and C, PP cells in vascular bundles of early expanding juvenile, transition and adult leaves are fully differentiated, similar to SEs with characteristic sieve plates, but because their primary walls appear smooth, similar to neighboring CCs, these PP cells have not formed papillate wall ingrowths. These expanding leaves are normally less than 20% of their final size, therefore can be considered as immature leaves.

Later in leaf development, PP cells express a second cell fate when they trans-differentiate to become PP TCs. The initiation of this process is the same in expanding juvenile, transition and adult leaves, as evidenced by the appearance of distinctive wall ingrowth material on the inner surface of the primary cell wall adjacent to cells of the SE/CC complex (Fig. 4A’, B’ and C’). However, the on-going process of building extensive networks of wall ingrowths was strikingly different among leaf identities, with this process occurring very rapidly across the maturation of juvenile leaves (Figs. 2A, E, I and 4D), but notably slower in transition leaves and strikingly slower in adult leaves (Figs. 2C, D, G, H and 4D). Significantly, we observed no major differences in xylem development, as revealed by mPS-PI staining, between mature juvenile,
transition and adult leaves (Supplemental Fig. S4), indicating that the differences seen in PP TC development at these different developmental states is a VPC-specific phenomenon.

We also examined heteroblastic development of PP TCs under short-day conditions, given that the onset of abaxial trichomes, the marker of VPC, is delayed in short-day plants (Telfer et al., 1997). By eight weeks, short-day Col-0 plants had produced at least 34 visible leaves ($n = 12$) and had 13 or 14 juvenile leaves ($n = 4$),
with these leaves bearing no abaxial trichomes, having long petioles, smooth leaf margins and rounder leaf blades (Supplemental Fig. S5A; compare to Fig. 1A and Supplemental Fig. S2). Wall ingrowth deposition in PP TCs also displayed juvenile characteristics in these leaves, namely an increased abundance in leaves 7 and 10 compared to that in long-day grown plants, and absence of a basipetal gradient of PP TC development in leaf 10 (Supplemental Fig. S5B). These results demonstrate that PP TC development reflects the heteroblastic status of leaves, regardless of day length.

**Strong Correlations Between Levels of miR156 and its SPL Targets and PP TC Development across Leaf, Shoot and Basipetal Maturation in Adult Leaves**

Because our data to this point was consistent with the interpretation that wall ingrowth deposition in PP TCs may be a component of the phase change program and hence potentially under the same genetic control as VPC, we examined changes in the abundance of the miR156 sRNA and expression of its target genes under the conditions where changes in PP TC development were observed. miR156 levels were measured by stem-loop RT-qPCR in representative juvenile (leaf 3 or 4), transition (leaf 7) and adult (leaf 11 or 12) leaves in mature (32-day-old) Arabidopsis shoots. Consistent with previous reports showing that miR156 levels at the whole shoot level decrease with shoot age (Wang et al., 2009; Wu et al., 2009), our data showed that in mature shoots, concomitant with a decrease in PP TC development (Fig. 5A), miR156 levels decreased 1.7- and 2.2-fold in transition and adult leaves, respectively, compared to that in juvenile leaves (Fig. 5B). Concomitant with a decline in miR156 abundance, the expression of several of its target genes, including SPL3, SPL9, SPL10 and SPL15, increased consistently across the juvenile to adult transition, with SPL15 showing the strongest response to decreased miR156 abundance (Fig. 5B). An increase in abundance across the juvenile to adult transition was also observed for miR172 (Fig. 5B), a positive regulator of VPC (Wu et al., 2009). Collectively, these results demonstrate correlations between miR156 sRNA abundance, miR156 target gene expression, and PP TC development in mature leaves (Fig. 5A and B), thus strongly implying a role for miR156 in promoting wall ingrowth deposition in PP TCs during the juvenile phase.
Figure 5. Strong correlations between PP TC development and abundance of miR156, miR172 and SPLs. A, C and E, PP TC development across shoot maturation (A), leaf maturation of juvenile leaf 1 and adult leaf 11 (C) and basipetal maturation in adult leaf 11 (E). B, D and F, RT-qPCR analysis of transcripts isolated from leaves at the same developmental stages as leaves subjected to mPS-PI staining and PP TC analysis as shown in A, C and E accordingly. Relative abundance levels were set to 1 for: (i) miR156 in juvenile leaves and miR172, SPL3, SPL9, SPL10 and SPL15 in adult leaves (B); (ii) miR156, SPL10 and SPL15 in 11-day-old leaf 1, miR172 and SPL3 in 35-day-old adult leaf 11, and SPL9 in 23-day-old adult leaf 11 (D); (iii) miR156, miR172, SPL3, SPL9, SPL10 and SPL15 in apical regions of adult leaf 11 (F). juv, juvenile; ad, adult. Data is mean ± se across at least five biological replicates for A, B, C, E and F, and three biological replicates for D.
To examine if the abundance of miR156, and that of its target genes, across individual leaf maturation also correlated with changes in wall ingrowth deposition in PP TCs, we measured their levels in representative juvenile leaf 1, and adult leaf 11, at different stages of maturation of each leaf. Leaf 1 and 11 were sampled from 11-, 17- and 23-day-old, or 23-, 29- and 35-day old plants, respectively, representing immature, intermediate and mature status of these juvenile or adult leaves. Consistent with an increase in PP TC development across leaf maturation (Fig. 5C), miR156 levels increased substantially from immature to intermediate and mature status, in both juvenile and adult leaves, with consistently higher miR156 abundance in juvenile leaves compared to adult leaves at the same maturation status of each leaf (i.e., immature juvenile versus immature adult leaves, etc.) (Fig. 5D). SPL9 and SPL15 expression showed an opposite pattern, that is, both transcripts were reduced in their abundance from immature to mature status for both juvenile and adult leaves, with their levels consistently lower in juvenile leaves compared to adult leaves at the same maturation status of each leaf (Fig. 5D). Interestingly, the most dramatic changes were again observed for SPL15, with transcript levels of this gene decreasing by 55-fold across immature to mature transition of juvenile leaf 11, and 5.7-fold across the same transition for adult leaf 11 (Fig. 5D). SPL10 levels reduced across maturation of juvenile leaves but remained unchanged across maturation of adult leaves, while SPL3 expression and miR172 abundance showed a similar trend, namely being stable during maturation of juvenile leaves but increasing from immature and intermediate to mature status in adult leaves (Fig. 5D). Again, these observations link temporal changes in wall ingrowth abundance in PP TCs to temporal changes in molecular phenotype of known regulators of VPC, including miR156, SPL9 and SPL15, during the maturation of individual leaves (Fig. 5C and D).

Given the distinct distributions of PP TCs between the tip and base of adult leaves (Figs. 1, 2 and 5E), we next assessed the levels of miR156 and miR172 and the SPLs in these regions. The apical third, and basal third (excluding the major vein), of leaf 11 were dissected and subjected to expression analysis. There was a 1.4-fold increase in SPL3 levels but no change in the abundance of miR156, miR172, SPL9 and SPL10. However, SPL15 expression was reduced by 3.1-fold in the apical
third, compared to the basal third (Fig. 5F), a finding which further strengthens the tight negative correlation observed for PP TC development and SPL15 expression (Fig. 5A, B, C and D).

**Altered Heteroblastic Development of PP TCs in Leaf Veins of sqn-6, 35S::MIM156 and 35S::MIR156 Plants Strongly Correlate with Altered Levels of miR156 and its Target Genes**

The strong correlation between PP TC development and miR156 abundance indicated a role for miR156 in regulating PP TC development. Therefore, we next examined PP TC development in Arabidopsis plant lines modified to have alterations in the miR156/SPL expression module. Numerous plant lines harboring mutations in the miRNA pathway, including *hasty* (Telfer and Poethig, 1998), *squint* (*sqn*) (Berardini et al., 2001), *serrate* (Clarke et al., 1999) and *hyponastic leaves1* (Lu and Fedoroff, 2000), have a shortened juvenile phase attributed to decreased miR156 abundance (Park et al., 2005; Lobbes et al., 2006; Li et al., 2012) or its activity (Smith et al., 2009). Additionally, some of these mutants also display altered reproductive competence and/or flowering time (Telfer and Poethig, 1998; Lu and Fedoroff, 2000). Several vegetative phase-specific traits are affected by floral induction (Willmann and Poethig, 2011), and it can be highly challenging in some instances to distinguish between flowering-dependent and flowering-independent effects on leaf morphology/anatomy. The Arabidopsis *sqn* mutant, a plant line defective in the activity of cyclophilin 40, displays altered vegetative phase transition, but flowering time and reproductive competence remains unchanged (Berardini et al., 2001). Therefore, in order to specifically examine effects of VPC on heteroblastic features of PP TC development, we analyzed PP TC development in leaf veins of the *sqn-6* allele. In addition to flowering at almost the same time as Col-0 plants, but with fewer leaves, *sqn-6* plants showed a precocious adult vegetative phenotype evidenced by an early appearance of abaxial trichomes and leaf margin serration (Supplemental Fig. S6A and B; see also Berardini et al., 2001; Usami et al., 2009). Leaf 5 of 30-day-old Col-0 plants displayed typical juvenile characteristics, namely the absence of abaxial trichomes and Class IV or V PP TCs seen in a majority of minor veins across the whole leaf (Figs. 1A and 6A). By comparison, PP TC development in leaf 5 of 30-day-old *sqn-6* plants was significantly reduced, with this
reduction being more obvious in the basal region of leaves (Fig. 6A), consistent with these leaves attaining a precocious adult vegetative phenotype (Supplemental Fig. S6C). PP TCs with extensive wall ingrowths were very abundant in the first two leaves of sqn-6 plants (Supplemental Fig. S6D and E), again in agreement with these leaves retaining juvenile identity (Supplemental Fig. S6A). This result is consistent with the heteroblastic status of PP TC development being independent of day length (Supplemental Fig. S5).

Constitutive expression of a miR156 target mimic transgene in Col-0 plants results in these plants, 35S::MIM156, displaying a severely altered VPC with precocious appearance of adult-like leaves replacing the juvenile phase (Franco-Zorrilla et al., 2007). In our study, leaf 1 and 2 of 35S::MIM156 plants developed typical adult-like features, including abaxial trichomes (Supplemental Table S1), serrated leaf margins and large and elongated blade (Supplemental Fig. S7A and C). However, inconsistent with previous reports (Franco-Zorrilla et al., 2007; Todesco et al., 2010), many of these plants flowered earlier than Col-0 and this flowering phenotype was frequently seen in plants with a reduced number of leaves (Supplemental Fig. S7A). Further, a considerable variation in leaf number was observed across the population of 35S::MIM156 plants assessed, ranging from 2-4 leaves (strong phenotype), 5-7 leaves (mild phenotype) or 8-9 leaves (weak phenotype), with the mild phenotype predominating (Supplemental Figs. S7B and C). We chose leaf 5 from 30-day-old 35S::MIM156 plants exhibiting the mild phenotype (5-7 leaves in total) to examine altered heteroblastic development of PP TCs in more detail. These leaves showed typical adult characteristics with numerous abaxial trichomes (Supplemental Table S1) and elongated blade (Supplemental Fig. S7D). PP TC development in minor veins of 35S::MIM156 plants was significantly reduced compared to leaf 5 of Col-0 plants at the same developmental stage (Fig. 6A), with this difference being more pronounced in the basal half of these leaves (Fig. 6A).

Overexpression of the miR156 precursor transcript greatly elevates miR156 levels and prolongs the juvenile phase of 35S::MIR156a plants, as evidenced by production of significantly more leaves displaying juvenile traits, including a lack of abaxial trichomes, long petioles and round and small sized leaf blades (Schwab et al.,
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2005; Wu and Poethig, 2006). Leaf 10 of 35-day-old 35S::MIR156a plants (Wu and Poethig, 2006), showed typical juvenile characteristics, whereas leaf 10 of 35-day-old Col-0 plants exhibited adult morphology (Supplemental Fig. S7E). In agreement with extensive wall ingrowth deposition signifying juvenile identity, we found a significant increase in PP TC development in veins of leaf 10 from 35S::MIR156a plants compared to that of Col-0, with this difference being most obvious in the basal half of the assessed leaves (Fig. 6A).

Figure 6. Effects of sqn-6, miR156 target-site mimic and overexpression of miR156 on PP TC development. A, PP TC development in leaf 5 of 30-day-old plants was reduced in sqn-6 and 35S::MIM156 (target-site mimic) lines compared to Col-0, whereas it was increased in leaf 10 of 35-day-old plants in 35S::MIR156a (over-expression) relative to Col-0. Effects on PP TC development were more significant in the basal-third compared to the apical-third of leaves. B, RT-qPCR analysis of transcripts isolated from leaves at the same developmental stages as leaves subjected to mPS-PI staining and PP TC analysis as shown in A. Relative abundance was set to 1 for leaf 5 of Col-0 for all transcripts. Data is mean ± SE across at least four biological replicates.
Levels of miR156 and its SPL targets have previously been measured in sqn-1 and sqn-6 mutants as well as 35S::MIM156 and 35S::MIR156a plants, however these measurements were made using whole seedlings and/or inflorescence tissues (Franco-Zorrilla et al., 2007; Smith et al., 2009), or pooled leaves from different nodes (Franco-Zorrilla et al., 2007; Usami et al., 2009). To more accurately correlate miR156 and SPL levels with PP TC abundance, we quantified miR156 accumulation, the expression of miR156 target genes SPL3, SPL9, SPL10 and SPL15, and miR172 abundance in: (i) leaf 5 from 30-day-old sqn-6, 35S::MIM156 and Col-0 plants, and; (ii) leaf 10 from 35-day-old 35S::MIR156a and Col-0 plants. Consistent with the findings of Smith et al. (2009) and Usami et al. (2009), our data showed no significant change in miR156 levels in the sqn-6 mutant compared to Col-0, but SPL3, SPL9 and SPL15 levels were elevated 2.4-, 2.6- and 4-fold, respectively, in leaf 5 of 30-day-old sqn-6 plants (Fig. 6B). This finding implies that the sqn mutation had major effects on miR156 activity, rather than influencing the level of this sRNA (Smith et al., 2009). The miR156 target-site mimic caused a 2.6-fold reduction in miR156 levels in leaf 5 of 35S::MIM156 compared to the same leaf of Col-0 plants, and we subsequently demonstrate that this reduction in miR156 abundance led to significant increases in levels of all examined SPLs, and miR172 abundance. Again, the most dramatic change was observed for SPL15 expression (7.4-fold increase) (Fig. 6B). These changes are in agreement with the appearance of precocious adult features observed in PP TC development. The increase in miR156 abundance and corresponding decrease in miR172 and SPL levels in leaf 10 of 35S::MIR156a plants overexpressing miR156 (Fig. 6B) is consistent with the juvenilized phenotype of PP TC development seen in this leaf (Fig. 6A).

**SPL9-Group, but not SPL3-Group Genes, Regulate Heteroblastic Development of PP TCs in Leaf Veins**

The consistently observed negative correlation between SPL levels and PP TC development suggested that these transcripts may negatively control heteroblastic development of PP TCs in leaf veins. In Arabidopsis, miR156-targeted SPLs can be divided into two broad classes, represented by SPL3 (including SPL3, SPL4, and SPL5) and SPL9 (including SPL2, SPL6, SPL9, SPL10, SPL11, SPL13, and SPL15) (Xing et al.,
We examined PP TC development in transgenic plants overexpressing miR156-resistant SPL3, SPL9 and SPL10, namely Pro35S:rSPL3 (rSPL3), ProSPL9:rSPL9 (rSPL9) and ProSPL10:rSPL10 (rSPL10), respectively (Wu and Poethig, 2006; Wang et al., 2008). PP TC development was significantly decreased in leaf 5 of rSPL9 and rSPL10 plants, which display precocious expression of all adult-specific leaf traits (Wu et al., 2009). However, this was not observed in rSPL3 plants compared to Col-0 (Fig. 7A). We also

**Figure 7.** Effects of overexpression of SPL3, SPL9, SPL10 and SPL15, and of spl9-4, spl15-1 and spl9-4/spl15-1 mutants, on PP TC development. A, PP TC development was reduced in leaf 5 of 30-day-old rSPL9, rSPL10 and SPL15-1D plants but was not significantly affected in rSPL3 compared to Col-0 (P < 0.01, Student’s t test). B, spl9-4 and spl15-1 single mutants did not show significant difference from Col-0 levels, whereas the spl9-4/spl15-1 double mutant showed a significant increase in PP TC development compared to Col-0 (P < 0.01, Student’s t test). Data is mean ± se across three or four biological replicates.
examined PP TC abundance in the gain-of-function mutant SPL15-1D, which has a C to T substitution in the miR156 target site of SPL15, which results in increased expression of SPL15 and hence a precocious adult phenotype (Usami et al., 2009). PP TC abundance in leaf 5 of SPL15-1D plants was significantly reduced compared to Col-0 (Fig. 7A), suggesting a negative role for SPL15 in regulating wall ingrowth deposition in PP TCs. This hypothesis is supported by the inverse correlation of SPL15 levels in circumstances where PP TC development was found to be altered accordingly (Figs. 5 and 6). Consistent with redundant effects of SPL9 and SPL15 in regulating vegetative phase transition and other aspects of plant development (Schwarz et al., 2008; Wang et al., 2008; Wu et al., 2009), we found no significant effect on PP TC development in spl9-4 and spl15-1 single mutants compared to the significant increase detected in leaf 10 of the spl9-4/spl15-1 double mutant compared to Col-0 (Fig. 7B). This increase in PP TC abundance in the double mutant was lower than that caused by overexpression of miR156 (35S::MIR156a; Fig. 6A), implying that other miR156 targeted SPLs may also contribute to repression of wall ingrowth deposition in PP TCs.

Heteroblastic Development of PP TCs is Unaffected in zip, rdr6 and sgs3

Unlike sqn mutations, in which the effects on VPC encompass both the onset of abaxial trichome production and total number of rosette leaves, another class of juvenile-to-adult transition mutations, including zip (ZIPPY; an allele of ARGONAUTE7), rdr6 (RNA DEPENDENT RNA POLYMERASE 6) and sgs3 (SUPPRESSOR OF GENE SILENCING 3), involved in the tasiARF pathway, accelerate the appearance of adult traits without altering total leaf number due to absence of effects on the length of the transition zone (Hunter et al., 2003; Peragine et al., 2004; Hunter et al., 2006). We examined if these mutations also showed altered heteroblastic development of PP TCs. Consistent with previous reports (Hunter et al., 2003; Peragine et al., 2004), zip-2, rdr6-11 and sgs3-11 mutants exhibited precocious adult vegetative traits such as an elongated and highly down-curved blade of leaves 1 and 2 in 15-day-old plants (Supplemental Fig. S8A), and accelerated abaxial trichome production (Supplemental Fig. S8B). Leaf 5 of these mutants displayed precocious adult features in several traits such as early appearance of abaxial trichomes, increased blade length-to-width ratio and slightly
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down-curled blades, but were not altered in other traits including leaf margin serration and blade base angle (data not shown). PP TC development in leaf 5 of these mutants was also not substantially different from that in leaf 5 of Col-0 plants (Fig. 8A), suggesting that zip-2, rdr6-11 and sgs3-11 have little, to no effect on heteroblastic development of PP TCs. This observation is consistent with a role for SPL9-group, but not SPL3-group genes, in repressing PP TCs development, as we found that levels of SPL3 increased 2.9- and 1.5-fold in leaf 5 of zip-2 and sgs3-11, respectively, but levels of miR156, SPL9 and SPL15 did not significantly change (Fig. 8B). Hunter et al. (2006) also reported that these mutations affected only a small subset of heteroblastic traits.

Figure 8. PP TC development was unaffected in zip-2, rdr6-11 and sgs3-11 mutants. A, In precocious adult leaf 5 of 30-day-old zip-2, rdr6-11 and sgs3-11 mutants, PP TC development showed typical juvenile characteristics with extensive wall ingrowths, similar to that in juvenile leaf 5 of Col-0. B, RT-qPCR analysis of transcripts isolated from leaves at the same developmental stages as leaves subjected to mPS-PI staining and PP TC analysis as shown in A. Relative abundance was set to 1 for leaf 5 of Col-0 for all transcripts. Data is mean ± se across four biological replicates.

Discussion

Heteroblastic Development of PP TC Wall Ingrowths Is Under Genetic Control of Phase Change

In this study, we have documented the novel finding that PP TC development in Arabidopsis leaf veins, as defined by the extent of wall ingrowth deposition, occurs across shoot development in a manner reflecting heteroblasty (Fig. 1), and that this process is under control of the same genetic program regulating VPC. This conclusion is based on our observations demonstrating that changes in PP TC development
paralleled changes in leaf abaxial trichomes and other well-defined phase-specific traits defining VPC in a predictable and coordinated manner across normal shoot ontogeny (Figs. 1, 2), as well as in conditions where shoot progression through ontogeny was delayed as a consequence of defoliation (Fig. 3). Furthermore, wall ingrowth deposition in PP TCs was altered in mutants and genetically modified plant lines having altered VPC, namely decreasing in abundance in mutants showing precocious adult phenotypes and increasing in abundance in mutants displaying juvenilized phenotypes (Figs. 6A and 7). Finally, measurements of the expression of miR156 and its SPL targets, the key players in genetic pathways regulating VPC, showed strong correlations, both positive and negative, respectively, between the abundance of these transcripts and that of wall ingrowth deposition in PP TCs. In all cases, miR156 levels were high in individual leaves where wall ingrowth deposition was abundant and low in conditions where wall ingrowth deposition was reduced or less developed. Significantly, the reverse correlation held true for the relationship between wall ingrowth abundance and levels of SPL9, SPL10 and SPL15, and to a lesser extent SPL3 and miR172 (Figs. 5A-D, 6 and 8). Collectively, these observations suggest that extensive wall ingrowth deposition signifies juvenile identity of leaves and that miR156, which is both necessary and sufficient for the expression of juvenile traits (Wu et al., 2009), promotes wall ingrowth deposition in PP TCs by repressing SPLs expression in the juvenile phase of shoot maturation. Importantly, the absence of apparent differences in xylem development across leaves of different vegetative phase identity (Supplementary Fig. S4) supports the conclusion that the responses seen in PP TC development represent a cell wall deposition event specifically reflecting leaf identity.

Temporal Changes in miR156 and SPL Expression Identify SPLs as repressors of PP TC development

In agreement with levels of miR156 being high in young seedlings and gradually decreasing with plant age (Wu et al., 2009; Xu et al. 2016a, b), we found that in mature shoots, juvenile leaves had significantly higher levels of miR156 compared to miR156 levels in transition and adult leaves (Fig. 5B). This result is also in agreement with
miR156 levels examined in leaves of *Acacia colei* (Wang et al., 2011), rice (Xie et al., 2006, 2012) and soybean (Yoshikawa et al., 2013). In individual leaves however, miR156 levels increased during maturation of either juvenile or adult leaves (Fig. 5D); this finding implies that miR156 levels in a given leaf, at a certain stage of development, is determined by both shoot age, i.e., the node of the leaf in the rosette, and leaf age.

Such temporal changes in miR156 levels led to a corresponding change in the abundance of the targeted *SPLs* (Fig. 5B and D). Changes in *SPL9* and *SPL15* expression, and to a lesser extent *SPL10*, were opposite to those seen for PP TC development, during both shoot (Fig. 5A and B) and leaf (Fig. 5C and D) maturation, implying a repressive role for these SPLs in temporally regulating PP TC development. SPL proteins are generally classified as transcriptional activators, however, in several cases they can act as transcriptional repressors. For example, SPL9 indirectly binds to the promoter of *DIHYDROFLAVONOL REDUCTASE*, a key enzyme in anthocyanin biosynthesis, repressing its expression and consequently leading to reduced anthocyanin accumulation (Gou et al., 2011). SPL9 and SPL10 have also been shown to directly bind to the transactivation domains of *B-type ARABIDOPSIS RESPONSE REGULATORS* (*ARRs*), which encode transcriptional activators of the cytokinin signaling pathway, thereby exerting a role in reducing the regenerative capacity of shoots (Zhang et al., 2015). Schwarz et al. (2008) also postulated that SPL9 and SPL15 may negatively regulate leaf maturation rate, consistent with our observation that levels of these two *SPL* transcripts decreased over leaf maturation in both juvenile and adult leaves (Fig. 5D). Collectively, these observations are consistent with SPLs, especially SPL9 and SPL15, functioning as negative regulators of PP TC development.

*SPL9*-group genes have also been shown to function in flowering time (Wang et al., 2009; Wu et al., 2009), with *SPL15* playing a more important role than other *SPL* genes under long-day conditions (Xu et al., 2016). In addition, other components of the flowering pathways including *FLOWERING LOCUS C* (*FLC*) and *FLOWERING LOCUS T* (*FLT*), also have effects on VPC and leaf morphology, and these effects can be either flowering-dependent or -independent (Willman and Poethig, 2011). Deng et al. (2011) have shown that FLC exerts its role in delaying the progression of VPC by binding to the
promoter of SPL15 and thus repress its expression. These observations collectively imply the existence of complex interactions between vegetative and reproductive phase transitions that influence the development of VPC-specific traits (Willman and Poethig, 2011), and also suggest the possibility that heteroblastic development of PP TCs could be affected by floral induction in addition to vegetative maturation. However, our results showing reduced PP TC development in the sqn-6 mutant (Fig. 6A; Supplementary Fig. S6), and that plants grown under short-day, non-photoinductive conditions, displayed the same heteroblastic development compared to that of abaxial trichomes (Supplemental Fig. S5), argues that PP TC development is tightly coupled to VPC. These results, however, do not formally rule out a role for floral induction on heteroblastic development of PP TCs, but this possibility, mediated by SPLs or other factor(s) such as FLC, await further investigation.

Trans-differentiation of PP TCs and Shoot Regenerative Capacity – Possible Connections via the miR156-SPL9 Module

The differentiation of PP TCs, like that for most TCs, occurs via trans-differentiation (Offler et al., 2003; Nguyen and McCurdy, 2016), which is defined as an irreversible conversion of a differentiated cell type into another cell type with distinct functional and morphological features (Okada, 1991). The clear reduction in PP TC development in adult leaves compared to juvenile leaves (Fig. 4), implies that the capacity to trans-differentiate into PP TCs is repressed in a majority of PP cells in adult leaves, particularly in cells at the base of the leaf. Interestingly, the trans-differentiation capacity of Zinnia mesophyll cells into tracheary elements was also shown to depend on the identity of leaves from which mesophyll cells were isolated (Fukuda and Komamine, 1980). When adult leaves of Zinnia plants were used, the trans-differentiation of tracheary elements occurred on day 6 of culture with just 10% of isolated cells forming tracheary elements. In contrast, when juvenile leaves were used, trans-differentiation was first observed on day 3 and the percentage reached 30% (Fukuda and Komamine, 1980). Collectively, these two examples of trans-differentiation, namely PP TCs in Arabidopsis and tracheary elements in Zinnia, link
shoot age and the competency of differentiated cells to *trans*-differentiate to a new cell fate.

Recently, Zhang et al. (2015) demonstrated that in Arabidopsis and tobacco, the capacity to regenerate shoots from explants of leaves progressively declined from juvenile to adult leaves, and that this process is regulated by *SPL9*-group genes. Consistent with a role for *SPL9*-group, but not *SPL3*-group genes in regulating shoot regeneration (Zhang et al., 2015), our data showed a stronger negative correlation between PP TC development and *SPL9, SPL10* and *SPL15* expression, compared to the levels of the *SPL3* transcript (Figs. 5, 7 and 8). *SPL9*-group genes exert their effects in repressing regenerative capacity of adult tissue by directly inhibiting the transcriptional activity of a group of *B*-type *ARR* genes (Zhang et al., 2015). Given the apparent correlation between *trans*-differentiation of PP TCs and shoot regenerative capacity of leaves and a role for *SPL9*-group genes in regulating these processes, investigating the cytokinin signaling pathways associated with *B*-type ARR is a promising avenue for future study of PP TC development in Arabidopsis.

**Establishment of Heteroblastic Variations in PP TC Development as a Reference for PP TC Study in Arabidopsis**

Previous studies of PP TCs in Arabidopsis (Maeda et al., 2006, 2008; Amiard et al., 2007) typically examined fully expanded or so-called “mature” leaves, without awareness of the heteroblastic aspects of PP TC development reported here. Consequently, in light of our findings, it is imperative that comparisons in PP TC development in various experiments, such as between control and treatment, or wild-type and mutants, need to be made in leaves harvested from the same node on the shoot given the differences seen in PP TC development even between successive leaves in the ecotypes assessed here (Fig. 1). Furthermore, careful analysis of the maturation status of individual leaves is an important consideration given that wall ingrowth abundance increases with leaf maturation, with the degree of such increase being different in individual leaf identities (Fig. 4). Given these observations, it is preferable to analyze PP TCs in mature leaves rather than developing leaves to minimize variations seen in the on-going formation of wall ingrowth networks. If
mature transition or adult leaves are selected for assessment of PP TC development, the location of the vascular bundles within the leaf also needs to be considered, since wall ingrowth abundance decreases from tip to base in these leaves, and does so more dramatically in adult leaves (Figs. 1, 2C, D, G, H, K, L and 5E). Furthermore, in a given PP TC, wall ingrowth abundance can also vary considerably in different areas along the cell wall that abuts the SE/CC complex (e.g., Figs. 2A, C, E and K). In this context, the robustness of the mPS-PI staining technique (Truernit et al., 2008) we have applied for confocal imaging of wall ingrowth deposition in PP TCs represents a substantially improved approach compared to ultrathin sectioning and TEM (Haritatos et al., 2000; Maeda et al., 2006, 2008; Amiard et al., 2007). Using this approach, we have mapped the distribution of PP TCs along the shoot axis (Fig. 1) and this map can now serve as a reference for future study of PP TC development in Arabidopsis.

Reevaluating Physiological Role(s) of Wall Ingrowth Deposition in PP TCs

It is widely accepted that immature leaves are sink tissues whereas mature leaves function as source organs delivering sugars to sinks via phloem loading (Turgeon 1989, 2006). Therefore, an increase in PP TC abundance in mature leaves relative to that in immature leaves was initially considered to support a role for wall ingrowths in PP TCs facilitating phloem loading (Nguyen and McCurdy 2015), as previously suggested by others (Haritatos et al., 2000; Maeda et al., 2006). However, the observations reported here show that PP TC development in mature adult leaves is dramatically reduced compared to that in mature juvenile leaves (Fig. 4), although these leaves of both identities were fully expanded and hence potentially acting as source organs. This observation implies that PP TC development may not simply correlate with the source/sink status of leaves. This said, however, it is unknown whether photosynthetic rate of a leaf, and hence phloem loading capacity of minor veins, is similar between mature juvenile and mature adult leaves. In soybean, Yoshikawa et al. (2013) showed that photosynthetic rates were comparable between juvenile leaves 1 and 2 and adult leaves 4 to 7, albeit for an unusual increase in leaf 3. In contrast, in rice (Asai et al., 2002) and ivy (Bauer and Bauer, 1980), photosynthetic activities are higher in adult leaves compared to juvenile leaves. As far as we are aware, there is no published work
in Arabidopsis comparing photosynthetic rates of juvenile and adult leaves. However, Stessman et al. (2002) reported a remarkable decline in photosynthetic rate across maturation of leaf 8 in plants grown under continuous light. This decline in photosynthesis with leaf maturation is opposite to the observed increase in wall ingrowth abundance in PP TCs in each individual leaf (Fig. 4). Therefore, taken collectively, these observations call into question a primary role for wall ingrowth deposition in PP TCs in influencing phloem loading capacity.

Several papers have suggested a role in pathogen defense for wall ingrowth deposition in PP TCs. PP is often the primary target of phloem-feeding pathogens (Ding et al., 1998; Zhou et al., 2002), hence the bulky and highly localized deposition of wall ingrowths in PP TCs adjacent to SEs may act as a physical barrier to impede systemic pathogen spread (Amiard et al., 2007). This possibility is supported by the observations that the extent of wall ingrowth deposition in PP TCs can be triggered by the defence hormone jasmonic acid (Amiard et al., 2007; Demmig-Adams et al., 2013), and that aphids, the major group of phloem-feeding insects, activate jasmonic acid-mediated defence pathways (Louis et al., 2012) and stimulate cell wall modifications (Divol et al., 2005). In contrast to this result, wall ingrowth deposition in CC TCs in pea leaf veins was not enhanced by exogenous methyl jasmonate, consistent with these wall ingrowths serving a primary role in enhancing phloem loading capacity (Wimmers and Turgeon, 1991; Amiard et al., 2007). Interestingly, of the large number of species showing TCs in collection phloem (either CC TCs, PP TCs or both), only about 3% of this number have PP TCs alone (Pate and Gunning, 1969). Of this small number, the two species in which PP TCs are well documented, namely Plantago lagopus (Pate and Gunning, 1969) and Arabidopsis (Haritatos, 2000), both display a rosette growth habit, with early emerged leaves (juvenile leaves in the case of Arabidopsis) being in close contact with soil and hence potentially prone to soil-borne bacterial and fungal infections. Consequently, the massive levels of wall ingrowth deposition seen in PP TCs in mature juvenile leaves (this study) and cotyledons of Arabidopsis (Nguyễn and McCurdy, 2015) may provide a physical means of defense against pathogen attack.
Materials and Methods

Plant Materials, Growth and Growth Analysis

*Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) was used as wild-type reference for all mutants and transgenic lines. Col-0, Ler-0, Ws-2, *spl9-4* (CS67866), *spl15-1* (CS67867), *spl9-4/spl15-1* (CS67865), *zip-2* (CS24282), *rdr6-11* (CS24285) and *sgs3-11* (CS24289) lines were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). *35S::MIM156* (Yu et al., 2013) and *35S::MIR156a* (Wu and Poethig, 2006) lines were kindly provided by J-W. Wang and R.S. Poethig, respectively. The *sqn-6* and *SPL15-1D* mutants (Usami et al., 2009) were kindly provided by H. Tsukaya. *rSPL3*, *rSPL9* and *rSLP10* (Zhang et al., 2015) were kind gifts from J-W. Wang and T.Q. Zhang. For all experiments, seeds were sown directly onto pasteurized soil mix and stratified for three days in darkness at 4°C. Plants were then transferred to a growth cabinet with light supplied at 100-120 μmol m⁻² sec⁻¹, 22°C day/18°C night, 16 h photoperiod. These long-day conditions were used in all experiments, except for that presented in Supplemental Fig. S5, where short-day conditions (8 h photoperiod) were used.

Number of juvenile and adult leaves and total leaf number were counted at bolting stage of plants. Abaxial trichomes were scored using a stereomicroscope at 3-4 weeks after planting and/or before leaves were subjected to fixation for mPS-PI staining. Leaf blade length and width, petiole length and blade base angle were measured in fully expanded leaves using ImageJ 1.47m ([https://imagej.nih.gov/ij/](https://imagej.nih.gov/ij/)).

Defoliation assays

Leaves 1 and 2 of 10-day-old Col-0 plants were removed using fine scissors and leaf removal was repeated at two- or three-day intervals until a total of 8 leaves were ablated. Leaf 9 or 10 from control and defoliated plants at maturation status of development were subjected to analyses of (i) PP TC development and (ii) phase-specific traits including abaxial trichomes, blade length-to-width ratio, petiole-to-blade length ratio and blade base angle. Defoliation experiments (*n* = 9, *n* = 10 and *n* = 12) were performed three times with the same outcomes and thus representative data from a single experiment is presented.
mPS-PI staining, confocal microscopy and PP/PP TC assays

mPS-PI staining of leaves was performed according to Nguyen and McCurdy (2015). In brief, leaves were fixed overnight at 4°C in ethanol:acetic anhydride (3:1), washed in 70% (v/v) ethanol and cleared in 25% (v/v) White King™ bleach (4% (v/v) effective hypochlorite concentration) for at least three hours depending on leaf age. Cleared tissue was washed extensively in water and stained in pseudo-Schiff propidium iodide (100 mM Na₂S₂O₅, 0.15 N HCl with propidium iodide added to a final concentration of 100 µg/mL) for at least one hour. Stained leaves were mounted in chloral hydrate (4 g chloral hydrate, 1 mL glycerol, 2 mL water). Confocal imaging of mPS-PI stained leaves was performed using an Olympus FluoView FV1000 laser scanning confocal microscope. Imaging used 488 nm Argon-ion laser excitation and emission wavelengths were collected at 522–622 nm. Semi-quantitative analysis of PP TC development was performed as described in Nguyen and McCurdy (2015; and see Supplementary Fig. S1). Diameters of PPs/PP TCs were measured using the scale bar tool of the FV1000 confocal microscope.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR (RT-qPCR) with SYBR Green detection is described in Supplemental Materials and Methods. In brief, total RNA extracted using TRIzol (Life Technologies) from leaves at developmental stages corresponding to those of mPS-PI stained leaves was subjected to RT-qPCR using a specific protocol for parallel quantification of small and large RNA species (Speth and Laubinger, 2014). miR156 and miR172 levels were quantified relative to U6, while SPL expression was determined relative to two novel reference genes, AT1G79810 (Peroxin 2) and AT2G20790 (clathrin adaptor complexes medium subunit family protein). Expression stability of these controls was validated using geNorm (Vandesomplele et al., 2002) and NormFinder (Andersen et al., 2004) algorithms. The quantitative cycle (Cq) was determined using the Cy0 method (Guescini et al., 2008); the amount of target transcript was related to that of internal controls using the Livak method (Livak and Schmittgen, 2001). A full description of these methods is provided in Supplemental Materials and Methods. Primers used for RT-qPCR are listed in Supplemental Table S2.
Supplementary data

The following supplemental materials are available.

Supplemental Figure S1. Confocal images of five classes of PP and PP TCs based on wall ingrowth abundance.

Supplemental Figure S2. Heteroblastic variations in three ecotypes Col-0, Ws-2 and Ler-0.

Supplemental Figure S3. Rejuvenation of internal anatomical traits in leaf 10 upon prolonged defoliation.

Supplemental Figure S4. Confocal imaging of tracheary elements in mature leaf veins.

Supplemental Figure S5. Heteroblastic features of Col-0 plants grown under short-day conditions.

Supplemental Figure S6. Effects of sqn-6 on VPC traits.

Supplemental Figure S7. Leaf morphology of 35S::MIM156 and 35S::MIR156 transgenic lines.

Supplemental Figure S8. Leaf morphology and the leaf position at which abaxial trichomes were first produced in Col-0, zip-2, rdr6-11 and sgs3-11 plants.

Supplemental Table S1. Variations on number of abaxial trichomes and total leaf number of 35S::MIM156 transgenic line.

Supplemental Table S2. List of primers used for RT-qPCR.

Supplemental Materials and Methods. Real-time quantitative RT-PCR.

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References


Chapter 3


Heteroblastic development of transfer cells


Heteroblastic development of transfer cells


Supplementary data

Includes:

- **Supplemental Figure S1.** Confocal images of five classes of PP TCs.
- **Supplemental Figure S2.** Heteroblastic variations in three ecotypes Col-0, Ws-2 and Ler-0.
- **Supplemental Figure S3.** Rejuvenation of internal anatomical traits in leaf 10 upon prolonged defoliation.
- **Supplemental Figure S4.** Confocal imaging of tracheary elements in mature leaf veins.
- **Supplemental Figure S5.** Heteroblastic features of Col-0 plants grown under short-day conditions.
- **Supplemental Figure S6.** Effects of sqn-6 on VPC traits.
- **Supplemental Figure S7.** Leaf morphology of 3SS::MIM156 and 3SS::MIR156a transgenic lines.
- **Supplemental Figure S8.** Leaf morphology and leaf position at which abaxial trichomes were first produced in Col-0, zip, rdr6 and sgs3 plants.
- **Supplemental Table S1.** Variations on number of abaxial trichomes and total leaf number of 3SS::MIM156 transgenic line.
- **Supplemental Table S2.** List of primers used for RT-qPCR.
- **Supplemental Materials and Methods.** Real-time quantitative RT-PCR.
**Supplemental Figure S1.** Confocal images of the five classes of PP or PP TCs based on extent of wall ingrowth deposition. Class I (●, 0 points) – no wall ingrowths; Class II (○, 2 points) – first evidence of discrete, punctate or linear regions of wall ingrowths appearing in some PP cells, defining them as PP TCs, in the field of view; Class III (■, 4 points) – more substantial clusters of wall ingrowths are evident, as shown here, or alternatively, more substantial linear regions of ingrowth deposition along the wall of the PP TC, typically occurring in all PP TCs in the field of view; Class IV (●, 6 points) – extensive levels of wall ingrowths appearing as a continuous thick band of deposition along the wall of all PP TCs; Class V (●, 8 points) – massive levels of wall ingrowth deposition that project into and occupy a considerable volume of each PP TC in the field of view. BS, bundle sheath. Asterisks indicate PP cells or PP TCs; arrows point to wall ingrowths. Scale bars = 10 μm for all images.
Supplemental Figure S2. Heteroblastic variations in three ecotypes Col-0, Ws-2 and Ler-0. A, Numbers of abaxial trichomes in individual leaves and total leaf number in Col-0 (n = 18), Ws-2 (n = 7) and Ler-0 (n = 9) plants. Data represents mean ± SD. B, Diameter of PP cells or PP TCs measured at tip, middle and base regions of juvenile leaf 1 (60 cells measured in total from 3 leaves), transition leaf 6 (60 cells measured in total from 3 leaves) and adult leaf 11 (146 cells measured in total from 9 leaves) from Col-0 plants. Data is mean ± se. C, Mature leaf 6 (arrows) in 25-day-old Col-0, 25-day-old Ws-2 and 28-day-old Ler-0 plants.
Supplemental Figure S3. Rejuvenation of internal anatomical traits in leaf 10 upon prolonged defoliation. A to C, mPS-PI-stained adult leaf 10 of control plants shows complex venation patterns; C, Higher magnification view of boxed region in B. D to F, mPS-PI stained rejuvenated leaf 10 of defoliated plants shows simple venation patterns; F, Higher magnification view of boxed region in D. Scale bars = 1 mm for all images. G, Diameter of PP cells or PP TCs measured at tip, middle and base regions of leaves shown in A (54 cells measured in total from 3 leaves) and B (96 cells measured in total from 5 leaves). Data is mean ± SE.
Supplemental Figure S4. Confocal imaging of tracheary elements in mature leaf veins. These images revealed no major differences in xylem development in mature juvenile leaves (A and B), mature transition leaves (C, G and H) and mature adult leaves (D to F). In minor veins of these leaves xylem was frequently comprised of two, and occasionally three, tracheary elements as revealed by single confocal sections (A to F), and x-z (G) and y-z (H) projections of a confocal z-stack. These tracheary elements were typical of protoxylem vessels with helical and annular thickenings. White asterisks in B, C, E, F and G indicate tracheary elements which were probably formed earlier during xylem development as indicated by the greater spacing of secondary wall thickenings compared to its neighboring tracheary element. White asterisks in H indicate two tracheary elements in a transverse view reconstructed from a series of confocal z-scans. Red asterisks in G and H indicate PP TCs with abundant wall ingrowth deposition (arrowheads). BS, bundle sheath; Scale bar = 10 µm for A-F, and 10 µm for G and H.
Supplemental Figure S5. Heteroblastic features of Col-0 plants grown under short-day conditions. A, Eight-week-old Col-0 plants; the insets showing the abaxial surface of leaves 7 and 10 (bearing no abaxial trichomes as observed by dissecting microscope). B, PP TC development in leaves 7 and 10 increased compared to that in long-day plants, and resembled PP TC development in leaf 4 or 5, or leaf 5 or 6, respectively, of long-day plants. Analysis of leaf 10 in short-day plants also shows no differences in PP TC development at the base vs tip of the leaf. LD: long day; SD: short day. Data is mean ± SE. n = 3 for leaf 7 and n = 4 for leaf 10.
Supplemental Figure S6. Effects of *sqn-6* on VPC traits. A, Number of abaxial trichomes in individual leaves and total leaf number of individual *sqn-6* plants. B, 25-day-old plants of Col-0 and *sqn-6* mutant. Leaf 3 of Col-0 was typical of juvenile morphology with smooth margins (arrow), whereas leaf 3 of *sqn-6* showed precocious adult traits with highly serrated margins (arrow). C, Leaf 5 in 30-day-old *sqn-6* plant was more elongated and serrated compared to Col-0. D and E, Confocal images showing PP TCs (asterisks) with massive wall ingrowth deposition (arrows) in veins of leaves 1 and 2 of *sqn-6*. BS, bundle sheath. Scale bar = 10 μm.
Supplemental Figure S7. Leaf morphology of 35S::MIM156 and 35S::MIR156a transgenic lines. A, 27-day-old Col-0 and 35S::MIM156 plants. B, Percent of 35S::MIM156 plants having 2-4 leaves (strong phenotype), 5-7 leaves (mild phenotype) and 8-9 leaves (weak phenotype); n = 71. C, 24-day-old plants of Col-0 and 35S::MIM156; arrows point to leaf 1 or 2. D, Leaf 5 from 30-day-old Col-0 and 35S::MIM156 plants. E, Leaf 10 from 35-day-old Col-0 and 35S::MIR156a plants. Images of leaf 10 from Col-0 were taken before and after the leaf being flattened to reveal leaf margins.
Supplemental Figure S8. Leaf morphology and leaf position at which abaxial trichomes were first produced in Col-0, zip-2, rdr6-11 and sgs3-11 plants. A, 15-day-old Col-0, zip-2, rdr6-11 and sgs3-11 plants; arrows point to leaf 1 or 2. B, Frequency distribution for the position of the earliest leaf bearing abaxial trichomes. In these plants the earliest leaf having abaxial trichomes was most frequently leaf 6 in Col-0, leaf 4 and 5 in zip-2, leaf 3 in rdr6-11 and leaf 5 in sgs3-11.
Supplemental Table S1. Variations on number of abaxial trichomes and total leaf number of 35S::MIM156 transgenic line.

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Supplemental Table S2. List of primers used for RT-qPCR. ^ indicates position of an exon-exon junction.

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Supplemental Materials and Methods: Real-time quantitative RT-PCR

Identification and validation of novel reference genes for quantification of mRNAs in Arabidopsis leaf tissue

We used the method of Hruz et al. (2011) to identify suitable reference genes for normalization of mRNA expression specifically across maturation of juvenile and adult leaf tissue. We selected potential reference genes based on public microarray data using RefGenes, available within Genevestigator (https://www.genevestigator.com).

From the 10615 Arabidopsis microarray datasets available in Genevestigator, we chose a set of 57 microarrays from 8 independent studies, in which the experimental conditions were as similar as possible to the experiments performed in this study. To minimize the effects of variation in amplification efficiencies between reference genes and target genes on the accuracy of qPCR results (Czechowski et al., 2005; Bustin et al., 2009; Svec et al., 2015), we defined the range of reference gene expression to be in the same expression range of our target genes, namely SPL3, SPL9, SPL10 and SPL15.

With these two criteria defined for RefGenes, Genevestigator returned the top 20 probe sets with the lowest variance, from which we chose AT1G76940 (RNA-binding (RRM/RBD/RNP motifs) family protein), AT1G79810 (PEROXIN 2), AT2G19950 (GOLGIN CANDIDATE 1) and AT2G20790 (clathrin adaptor complexes medium subunit family protein), all of which appear in the list of the 100 most stably expressed genes across various developmental series in Arabidopsis (see Czechowski et al., 2005). To empirically validate these potential reference genes, we performed RT-qPCR and used geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) algorithms to assess expression stability. Analyses by these two algorithms returned AT1G79810 and AT2G20790 as the most stably expressed genes across all our leaf samples, namely leaves from Col-0 at different developmental stages and from transgenic lines used in this study.

RT-qPCR primer design, specificity and efficiency

qPCR primers for mRNAs were designed using Primer-BLAST software (Ye et al., 2012) with the following criteria: $T_M$ of 60°C ± 1°C, PCR amplicon lengths of 60 to 150 bp, and at least one primer of a pair bridging an exon-exon junction if possible to prevent
amplification of potentially contaminating genomic DNA. As Oligo (dT) primers were used for cDNA synthesis, qPCR primers were also designed to amplify close to the annotated 3’ end of mRNAs (see Supplemental Table S2). In silico specificity screen of these primers was also performed by Primer-BLAST. Forward primers and universal reverse primers for miR156, miR172 and U6 were designed according to Varkonyi-Gasic et al. (2007), Kramer et al. (2011), Turner et al. (2013) and with modifications.

Specificity of amplicons was verified by (i) gel-electrophoretic analysis, (ii) melting-curve analysis post-amplification, and (iii) in silico prediction of amplicon melting temperature using OligoAnalyzer 3.1 (http://sg.idtdna.com/calc/analyzer). Primer efficiency was assessed for each primer pair in each experiment and listed as following: AT1G79810: 95% ± 1.8 (n = 6); AT2G20790: 91% ± 3.7 (n = 4); SPL3: 88% ± 1.7 (n = 5); SPL9: 95% ± 2.2 (n = 5); SPL10: 96% ± 1.6 (n = 4); SPL15: 97% ± 1.8 (n = 5); U6: 93% ± 1.0 (n = 6); miR156: 91% ± 1.8 (n = 6); miR172: 85% ± 3.1 (n = 4). Data is mean ± se.

Sample acquisition, total RNA extraction and quality assessment
At two hours into the light period of plant growth, leaf blades (not including petioles) were removed from plants and immediately snap-frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from these leaves using TRIzol reagent (Life Technologies) according to the manufacturer’s protocol and with in-house optimization of the procedures. RNA concentration was measured using a NanoDrop ND-1000 with two or three technical replicates for each RNA sample. On average, 100 mg of ground plant material from juvenile or adult leaves yielded of ~25 or ~70 µg total RNA, respectively. The purity of RNA was estimated by 260/280 and 260/230 ratios. On average, 260/280 ratios were in the range of 1.9-2, and 260/230 ratios were in the range of 2.2-2.5, indicating highly purified RNA. RNA integrity was confirmed by standard agarose gel electrophoresis, showing highly intact RNA (evidenced by intact 28S rRNA, 18S rRNA, 5S/sRNA bands and the presence of clear cytosolic and plastidic ribosomal RNA bands (Box et al., 2011) and the visible absence of genomic DNA contamination.
First strand cDNA synthesis using oligo (dT)\textsubscript{18} and miRNA-specific stem-loop primers

cDNA synthesis was performed with the RevertAid First Strand cDNA synthesis kit (Thermo Scientific). To minimize variations in cDNA synthesis efficiencies between miR156, miR172 and U6, and between miR156 and its SPL target genes, we employed the protocol of Speth and Laubinger (2014) to perform a multiplexed cDNA synthesis of miR156, miR172, U6 and mRNAs. Stem-loop primers were used for cDNA synthesis of miR156, miR172 and U6 and designed according to Varkonyi-Gasic et al. (2007), Kramer et al. (2011), Turner et al. (2013) and with modifications (Supplemental Table S2). Oligo (dT)\textsubscript{18} was used for priming cDNA synthesis from mRNA.

Prior to cDNA synthesis, total RNA was treated with DNaseI (Thermo Scientific) according to the manufacturer’s protocol to remove residual genomic DNA. Between 0.5-1.8 µg DNase-treated total RNA was used in a 20-µl RT reaction. Briefly, 1 µl of 100 µM oligo (dT)\textsubscript{18} and 0.5 µl of the stem-loop primer mixture (2 µM each primer) were added to DNase-treated total RNA and samples were incubated for 5 min at 65°C. Then 4 µl of 5x RT Buffer, 2 µl of 10 mM dNTPs, 20 units of Ribo-LOCK RNase Inhibitor and 200 units of RevertAid Reverse Transcriptase (RT) (Thermo Scientific) were added to primed-RNA samples. The RT reaction was perform with thermocycling conditions of 30 min at 16°C, followed by pulsed RT of 60 cycles of 30°C for 30s, 42°C for 30s and 50°C for 1s, followed by 85°C for 5 min to inactivate the RT. RT-minus reactions were performed for each corresponding RT-plus sample, and a negative control (NTC) and a positive control (provided by Thermo Scientific) were included in each experiment.

qPCR conditions and analysis

qPCR reactions were performed using a Rotor-Gene 6000 instrument (Qiagen) in technical duplicate for each biological replicate (n = 3-5). The cDNA equivalent of 7.5 ng of total RNA was used in a 10-µl PCR reaction, with the following components added: 5 µl of Maxima SYBR Green qPCR Master Mix 2x (Thermo Scientific), forward and reverse primers to the final concentration of 400 nM each, and nuclease-free water to a total volume of 10 µl. Samples were incubated at 95°C for 10 min, followed by a three step amplification by 40 cycles of 95°C for 10s, 57°C for 30s and 72°C for 20s. Melting curve analysis was performed post-amplification with a ramp from 60°C
to 95°C, increasing in increments of 1°C each 5 seconds. Serial 1:2 or 1:3 dilutions of cDNA were employed to create standard curves and subsequently calculate qPCR efficiency.

The quantitative cycle (Cq) was determined using the Cy0 method (Guescini et al., 2008, 2013; Sisti et al., 2010), which is ranked as the best publicly available curve analysis method in terms of algorithm precision, bias and resolution (Ruijter et al., 2013). Raw fluorescence data from each qPCR run was uploaded into the Cy0 website (http://www.cy0method.org/) and the Cy0 team analyzed and returned Cq values. These Cq values were subsequently used in all calculations, including (i) confirmation of the absence and/or no effects (if present) of contaminating genomic DNA (by RT-minus reactions) and primer-dimers (by NTC reactions), (ii) standard-curve analysis to calculate qPCR efficiency, (iii) evaluating the stability of reference genes, and (iv) the Livak formula (Livak and Schmittgen, 2001) to calculate relative abundance levels of transcripts of interest.

References


CHAPTER 4

General Discussion
4.1 Summary of principal findings

Research on TC biology over the last five decades has demonstrated a correlation between wall ingrowth deposition in TCs and enhanced transport capacity (see reviews by Pate and Gunning, 1972; Offler et al., 2003; McCurdy, 2015) in many major agricultural species such as peas (Wimmers and Turgeon, 1991), *V. faba* (Farley et al., 2000), cotton (Pugh et al., 2010) and maize (Zheng and Wang, 2010). Therefore, genetic manipulation of TC development, either by optimizing transport performance by increasing density of wall ingrowths and/or associated membrane transporters, and/or the induction of TC trans-differentiation in targeted cells/tissues, represents an innovative and promising approach to improve plant productivity and ultimately crop yield to address global food security. To achieve this long-term goal, elucidating the genetic control of TC development is of fundamental importance. The observation that TCs are also present in Arabidopsis (Haritatos et al., 2000) provides a fertile platform for TC research due to the readily accessible genetic resources of this model species (Meinke et al., 1998). On the other hand, this finding also brings new challenges for TC research in how to take full advantage of these increasing resources, given the historical difficulty to visualize and experimentally access TCs. The development of epidermal TCs in *V. faba* cotyledons overcomes some of these difficulties, but the lack of genetic resources in this legume species provides substantial roadblocks to generating detailed understanding of the mechanisms of wall ingrowth deposition and their genetic control.

The traditional methods of TEM and SEM used to study TCs are far from meeting the substantial demands of high-throughput analysis of mutants and transgenic lines that may be of use in studying PP TCs in Arabidopsis. In this context, the high-resolution imaging of wall ingrowth deposition using confocal microscopy as reported in Chapter 2 represents a major step forward for the study of TCs, not only those in Arabidopsis, but widely in other species as well.

Phloem TCs including PP TCs are located in the innermost tissue, namely vascular bundles, of leaves or leaf-like organs, and thus are not readily assessed. The long and narrow shape of vascular cells is another difficulty of imaging sub-cellular details of this cell type. Fortunately, recent advances in high-resolution confocal microscopy
combined with fluorophore labelling of plant cell walls using the mPS-PI staining procedure (Truernit et al., 2008) have provided a means to overcome these hurdles without the aid of traditional tissue sectioning and electron microscopy. We have successfully applied this technique to visualize wall ingrowth deposition in PP TCs in Arabidopsis and CC TCs in peas (Chapter 2). Confocal imaging of mPS-PI stained Arabidopsis leaves clearly resolved bands of, or in other cases, localized patches, of intertwined, finger-like projections of wall ingrowth material (Chapter 2). Particularly, the capacity to reconstruct y-z and x-z projections from series of confocal z-scans offers the opportunity to investigate the nature and hence the underlying molecular pathways regulating the highly polarized deposition of wall ingrowths in PP TCs. The high-throughput nature of the simplified version of the mPS-PI procedure is also highlighted by the use of bleach in place of lengthy extractions involving organic solvents, SDS/NaOH, amylase and pullulanase (Truernit et al., 2008; Wuyts et al., 2010). Furthermore, the scoring system developed in Chapter 2 based on the extent of wall ingrowth deposition in PP TCs enables semi-quantitative assessment of PP TC development at the whole leaf level, and thus provides a convenient, accurate and rapid way to analyze PP TC mutant phenotypes.

The robustness of the mPS-PI staining technique in combination with the scoring system for PP TC development led to the findings presented in Chapter 3. Using this approach, the distribution of PP TCs along the Arabidopsis shoot axis was mapped (Figure 4.1), from which the novel linkage of PP TC development and VPC was identified. This linkage is illustrated by the observations that the extent of wall ingrowth deposition varies substantially across the developmental transition of juvenile to adult leaves, and that this change can be seen in different ecotypes of Arabidopsis that display different juvenile phase lengths, and in leaves showing dramatically altered juvenile status in response to defoliation. These observations were extended by a series of molecular analyses showing a positive correlation between wall ingrowth abundance and the accumulation of miR156, the major regulator of VPC, measured either across the maturation of individual juvenile and adult leaves, or individual leaves of different vegetative phase status. Corresponding negative correlations with wall ingrowth deposition were seen with specific members
of the SPL gene family, which are targets of miR156. Various mutants or genetically modified lines showing altered VPC showed corresponding alterations in the levels of wall ingrowth deposition in PP TCs. Collectively, these results point to miR156-regulated SPL genes being repressors of PP TC development in Arabidopsis. This finding represents a significant advance in the study of the molecular pathways regulating the trans-differentiation of TCs.

Figure 4.1 The distribution of PP TCs along a mature Arabidopsis shoot axis. Cotyledons (cot) have Class V PP TCs with massive deposition of wall ingrowths; juvenile leaves have Class IV or V PP TCs. Adult leaves are characterized by much less developed PP TCs, with Class III or IV PP TCs at the apical region, Class II or III PP TCs at the middle and Class I or II at the base of the leaf. The abundance and distribution of PP TCs in cauline leaves (cau) are similar to that in adult leaves, namely a gradient of PP TCs ranging from Class III or IV at the tip to Class I at the base of the leaf. PP TCs are also present in sepals (not shown in this diagram); additionally, PP cells in vascular bundles of petals and stamens develop wall ingrowth-like structures (see Section 4.2.1). See Chapters 2 and 3 for the description of each class of PP TCs. The diagram of Arabidopsis shoot was taken from the website http://iasvn.org.
4.2 Implications of findings and future directions

4.2.1 Confocal imaging of diverse tissues using the simplified mPS-PI staining technique

In addition to the visualization of TC wall ingrowths in Arabidopsis and peas (Chapter 2), the simplified mPS-PI staining procedure can also be used to image cell walls in various Arabidopsis tissues including stems, roots, shoot apices, pollen, petals, sepals, stamens (Appendix Figures A1 - A5), etc., producing images with high resolution and hence clearly resolving sub-cellular structures such as the secondary wall thickening of the endothecium in anthers (Appendix Figure A2), sieve plates in transport phloem (Appendix Figure A4) or pit fields in various cell types (Appendix Figure A6). In particular, imaging of petals and stamens also revealed wall ingrowth-like structures in PP cells in these tissues (Appendix Figure A1). Consequently, this procedure should be widely applicable to image wall ingrowth deposition in TCs of other cell types in other species, thus facilitating experimental designs not restricted by the necessity to use electron microscopy.

4.2.2 Arabidopsis cotyledons as an experimental system to study PP TCs

Chapter 2 reported the presence of PP TCs in cotyledons of Arabidopsis, opening a new avenue for studying the development of PP TCs. PP cells in cotyledons initiate deposition of wall ingrowths by seven days in plants grown on soil (Appendix Figure A6 A-C) or by six days when grown on MS agar plates (Wu, Hou and McCurdy - unpublished), and deposition rapidly increases over the next four or five days (Appendix Figure A6 D-F). This profile of PP TC development in very young Arabidopsis seedlings facilitates the application of chemicals such as ROS-inducers (paraquat, H$_2$O$_2$), methyl jasmonate, auxin, ethylene, etc. to the culture medium to manipulate wall ingrowth deposition, given that these drugs and hormones were shown to play a role in the signaling pathways regulating TC development in Arabidopsis (Amiard et al., 2007) or V. faba (Zhou et al., 2010; Andriunas et al., 2011, 2012).

Secondly, forward genetic screening for altered PP TC development in cotyledons is a promising approach to identify genetic regulators of this process. Compared to true leaves, cotyledons are simpler organs with fewer layers of cells and simpler
venation patterns with typically larger-sized vascular cells, particularly PP cells, compared to leaves. These features make imaging of wall ingrowth deposition in PP TCs in cotyledons more readily assessable than that in true leaves. Consequently, in mPS-PI-stained cotyledons from two-week-old seedlings observed by differential interference contrast (DIC) microscopy, the fuzzy nature of wall ingrowth deposition in PP TCs is readily evident and easily recognized (unpublished observation). Thus, DIC microscopy could be employed for a high-throughput screen to identify aberrant wall ingrowth deposition in PP TCs in cotyledons and thus identify essential genes required for this process. Given the highly redundant roles of the two SPL transcription factors, SPL9 and SPL15, which may be acting as repressors of PP TC development (Chapter 3), and several NAC and MYB transcription factors identified as possible regulators of PP TC development (Chinnappa et al., 2013; see Appendix II), forward genetics promises to be a better approach for unbiased identification of genes essential for the trans-differentiation of PP TCs. Additionally, recent technical advances in next-generation sequencing (Austin et al., 2011; Schneeberger, 2014; Thole and Strader, 2015) and associated analysis tools (James et al., 2013) make forward genetic screens even more straightforward, timely and affordable, and hence highly feasible, even with a difficult cell type such as PP TCs.

Furthermore, the recent technical advance in rapidly isolating mesophyll, vasculature and epidermal tissue from Arabidopsis cotyledons (Endo et al., 2014) provides another experimental opportunity to use cotyledons to study PP TC development. Currently, the molecular data presented in Chapter 3 relies on the analysis of gene expression derived from whole leaves, of which only a small portion is represented by PP TCs compared to other cell types. Therefore, using isolated vasculature, or preferable phloem strands, will enable higher-resolution analysis of gene expression and corresponding wall ingrowth deposition in PP TCs. This approach is currently being established in the McCurdy lab.

4.2.3 Heteroblastic variations of PP TC development in Arabidopsis as a reference for TC research

The capacity to map the heteroblastic variations of PP TCs along the shoot axis of Arabidopsis (Figure 4.1) validated the robustness of the staining and imaging approach
described in Chapter 2. This map can now serve as a reference for future research on PP TCs, with emphasis lying on a cautious choice of which leaf identity, which status of leaf maturation or which location in a given leaf to perform PP TC analysis (Chapter 3). This map also brings a new view of TC biology in general, particularly vascular TCs in leaves. It will be interesting to know if the development of vascular TCs in other species, for instance, CC TCs in leaf veins of pea, *Genista sagittalis* or *Helianthemum spp.* (Pate and Gunning, 1969), also reflects heteroblasty; and if so, what is the fundamental significance of this phenomenon to plant development. For some species possessing more than one types of vascular TCs in leaf veins, such as *Anacyclus pyrethrum* with PP TCs, CC TCs, xylem parenchyma TCs and bundle sheath TCs, or *Pulicaria vulgaris* and *Osteospermum vaillantii* with both CC TCs and xylem parenchyma TCs (Pate and Gunning, 1969), knowing whether heteroblasty, if it occurs, is it seen in all TC types or only certain types, such as PP TCs, is also of great interest. Answering these questions will advance our understanding of ontogeny changes in shoot maturation and functional aspects of TCs in general.

### 4.2.4 Potential effects of floral induction on heteroblastic development of PP TCs?

As outlined in Chapter 1, vegetative phase transition and reproductive phase transition of shoots are developmentally uncoupled and regulated by separate genetic mechanisms (Diggle, 1999; Poethig, 2013), although these mechanisms share some common components such as miR156, miR172 and *SPL* genes (Huijser and Schmid, 2011; Poethig, 2013). Unlike woody plants, in herbaceous species such as Arabidopsis, these transitions occur very close together, so it is challenging to distinguish between the effects of vegetative phase change and those of floral induction on development of vegetative traits (Willmann and Poethig, 2011). Arabidopsis is a facultative long-day species, in which long-day conditions promote flowering more rapidly than short-day conditions (Gregory and Hussey, 1953). Therefore, to eliminate potential effects of floral induction on vegetative phase-specific traits, Wu et al. (2009) analyzed these traits in plants grown under short-day conditions. We analyzed PP TC development in wild-type plants grown under long- and short-day conditions, but due to time constraints, mutants/transgenic lines were examined under short-day conditions only.
(Chapter 3). However, our analysis of the sqn-6 mutant, a plant line displaying altered VPC but no effects of flowering time or reproductive competence, confirmed that reduced PP TC development in this mutant is specific to VPC (Chapter 3). Nonetheless, this observation does not necessarily exclude the effects of floral induction on this novel heteroblastic trait in other mutants/transgenic lines. Therefore, further experimental work is essential to address whether PP TC development is affected only by vegetative maturation, or by a combination of both vegetative and reproductive maturation. In this context, the latter possibility is supported by the observation that, despite having very similar total leaf number, Ws-2 plants flower earlier than Ler-0, and that PP TCs in leaves 6 and 7 of Ws-2 were much less developed than that of Ler-0 (Chapter 3).

Recently, Willmann and Poethig (2011) showed that the floral repressor FLOWERING LOCUS C (FLC), a major repressor of flowering in Arabidopsis (Michaels and Amasino, 1999), has effects on the progression of VPC in Arabidopsis, and that these effects can be either dependent or independent of its effect on flowering time. Consistently, this novel function for FLC in vegetative development was also reported by Deng et al. (2011), in which FLC was shown to strongly repress the expression of SPL15 by binding to its promoter and thus delaying the progression from juvenile to adult phase. FLC also targets SPL3; however, since there was no significant change of SPL3 expression in the flc mutant, a role for SPL3 in mediating effects of FLC on vegetative development was excluded (Deng et al., 2011). These observations are consistent with the findings that SPL3, SPL4 and SPL5 do not play a major role in vegetative phase transition (Xu et al., 2016b) and that PP TC development, the novel heteroblastic trait identify in this study, was not repressed by SPL3 (Chapter 3).

Interestingly, in all molecular analyses presented in Chapter 3, the expression patterns of SPL15 always showed the strongest negative correlations with PP TC development compared to that of the remaining SPLs, namely SPL3, SPL9 and SPL10. Particularly, in the experiment assessing basipetal maturation in adult leaf 11, SPL15 showed a 3.1-fold reduction in its expression in the apical third compared to the basal third, consistent with a 3.7-fold increase in PP TC development, although the abundance levels of miR172, SPL9, SPL10, and especially miR156, remained unchanged.
General discussion

(see Figure 5E and F in Chapter 3). In addition to cleaving SPL transcripts, miR156 also represses SPL gene expression by promoting their translational repression (Xu et al., 2016b), which can explain reduced SPL15 levels being independent of miR156 level in the basipetal maturation experiment. Alternatively, such differential expression of SPL15 in the base versus the tip of leaf 11 could be a consequence of the regulation of factor(s) other than miR156. In this context, FLC is a potential candidate due to its recently recognized function in VPC via mediating SPL15 expression (Deng et al., 2011; Willmann and Poethig, 2011) as described above.

Collectively, the data in Chapter 3 demonstrates a negative role for SPLs in regulating PP TC development in Arabidopsis; however, due to the complex and (in some cases) overlapping of vegetative and reproductive maturation, and the dual function of the miR156/SPL module in both developmental processes, potential effects of floral induction, either mediating by SPLs or other factor(s), on heteroblastic development of PP TC await further investigations. Answering this question is fundamentally important for future study of TCs with regards to identifying downstream targets of these SPLs.

4.2.5 A role for SPL9-group genes in the trans-differentiation process of PP TCs

Trans-differentiation is a biological phenomenon whereby one differentiated cell type irreversibly switches into another cell type with distinct morphological and functional features (Okada, 1991). In both plants and animals, trans-differentiation can be preceded by cell division, but this intermediate process is not obligatory in all cases (Beresford, 1990; Eguchi and Kodama, 1993). For example, in Eucommia ulmoides Oliv., during bark regeneration, immature xylem cells trans-differentiate to form sieve elements, companion cells and cambium cells with the intervention of cell division, whereas trans-differentiation of tracheary elements from isolated Zinnia mesophyll cells, from Zinnia pith parenchyma cells or from Arabidopsis bundle sheath cells, can occur without cell division (see references in Nguyen and McCurdy, 2016; Appendix III). It is unknown, however, whether cell division is a prerequisite for the trans-differentiation of TCs.
Since trans-differentiation of PP TCs is highly correlated with the regenerative capacity of shoots, and these processes are regulated by SPL9-group genes (Zhang et al., 2015; Chapter 3), investigating the downstream targets of SPL9-group genes associated with shoot regeneration is a promising avenue for future TC study. B-type ARABIDOPSIS RESPONSE REGULATORs (ARRs), which encode transcriptional activators of the cytokinin signaling pathway, were shown to mediate the effects of SPL9 and SPL10 on shoot regenerative capacity (Zhang et al., 2015). In Arabidopsis, ARR1, ARR2, AR10 and ARR12 are the four B-type ARRs which have important roles in cytokinin signal transduction (Mason et al., 2005; Ishida et al., 2008). The regenerative capacity of shoot tissue was markedly reduced in the arr2-4 arr12-1 double mutant and completely lost in the arr1-2 arr10-5 arr12-1 triple mutant compared to wild-type (Zhang et al., 2015). Preliminary results examining the arr1-3 arr10-5 arr12-1 triple mutant (Argyros et al., 2008) showed a reduction in PP TC development in cotyledons and leaves 1 and 2 relative to that in Col-0 (Figure A7). Leaves of the arr1-3 arr10-5 arr12-1 triple mutant had approximately half the number of cells compared to wild-type leaves, consistent with positive effects of cytokinin on cell division in the shoot being mediated by B-type ARRs (Argyros et al., 2008). Collectively, these observations suggest that reduced PP TC development in the triple mutant might be a consequence of impaired cell division, meaning that, cell division might be required for trans-differentiation of PP TCs. Further experimental work in this direction may elucidate specific role(s) for SPL9-group genes, B-type ARRs and cytokinin in regulating PP TC development.

4.2.6 Physiological role(s) of wall ingrowth deposition in PP TCs
As discussed in Chapter 3, the observation that PP TC development in mature adult leaves is dramatically reduced compared to that in mature juvenile leaves, seems to contradict a role for wall ingrowths in facilitating photoassimilate transport across the plasma membrane in these cells. Support for this possibility is data on the atsweet11/12 double mutant and suc2-1 mutant presented in Appendix I. ATSWEET11/12 and SUC2 are well-characterized proteins functioning in phloem loading as effluxers of sucrose from PP TCs into the apoplasm (Chen et al., 2012) and
importers of sucrose from the apoplasm into cells of the SE-CC complex (Truernit and Sauer, 1995), respectively. Phloem loading in *atsweet11/12* double mutants and particularly *suc2* was severely impaired, resulting in starch accumulation in leaves and ultimately stunted growth (Gottwald et al., 2000; Chen et al., 2012; Appendix Figure A8). Our analysis using mPS-PI staining revealed that wall ingrowth deposition in PP TCs in rosette leaves in these mutants was not detectably different compared to wild-type (Appendix Figure A9). Again this observation adds further evidence suggesting a role(s) for wall ingrowth deposition in PP TCs other than solely enhancing rates of photoassimilate transport.

Within the TC literature, the only published work supporting a link between phloem loading capacity in leaves and wall ingrowth deposition in PP TCs is the study by Maeda et al. (2006). These authors showed that in the *vte2* mutant, a mutant defect in vitamin E biosynthesis, the export of photoassimilates from leaves was significantly reduced upon transfer to low temperature conditions, and that this reduction was a consequence of callose deposition in wall growth regions of PP TCs. Additionally, cold treatment caused an increased and depolarized deposition of wall ingrowths in PP TCs of the *vte2* mutant (Maeda et al., 2006). However, the core observations reported by Maeda and colleagues were not repeated here. Firstly, aniline blue staining of leaves revealed that, even under permissive room temperature, callose deposition was readily evident in leaves of the *vte2* mutant, unlike that reported in Maeda et al. (2006), and these callose blobs were seen in all PP cells, regardless of these cells developing wall ingrowths or not (Appendix Figure A10A - D). Secondly, mPS-PI staining showed no increase in wall ingrowth deposition in PP TCs in *vte2* upon cold treatment (Appendix Figure A10E and F). It is important to note, however, that potential differences might exist in the choice of leaves analyzed in this study compared to that of Maeda et al. (2006). In light of our findings of heteroblastic variations in PP TC development, we compared PP TC development in leaves of the same node and maturation status across treatments, i.e., mature leaf 11 from control versus cold-treated *vte2* mutant, whereas Maeda et al. (2006) described using “mature leaf” without specifying leaf identity. Based on comparing equivalent leaf identities in our experiments, the conclusion that the results reported by Maeda et al. (2006)
support a role for wall ingrowth deposition in facilitating transport of photoassimilates therefore becomes doubtful.

Demonstrating a more rigorous correlation between PP TCs and their physiological roles in Arabidopsis may be addressed by several approaches. Leaf exudates to measure sucrose export could be collected from mature leaves of juvenile leaf 1, transition leaf 6 and mature leaf 11, and from juvenile leaf 1 and adult leaf 11 across maturation of each leaf identity. Similar measurements could be performed for leaf 5 from Col-0 versus the sqn-6, SPL15-1D mutants and the 35S::MIM156, rSPL9 and rSPL10 transgenic lines, given the observed reductions in PP TC development in these lines compared to Col-0 (see Chapter 3). The small leaf sizes may make these experiments technically challenging, but leaf exudate collection from Arabidopsis leaves has been reported (Maeda et al., 2006). Photosynthetic rate could also be measured for these leaves (Stessman et al., 2002). These experiments could be supplemented with similar measurements for pea leaves, given the potential role for CC TCs in peas in enhancing phloem transport (Wimmers and Turgeon, 1991).

It is also interesting to investigate the hypothesis that PP TCs may function in pathogen defense (Amiard et al., 2007; Demmig-Adams et al., 2013; see also Chapter 3). One approach to test this possibility could be to perform insect bioassays such as aphid choice and no-choice tests (Lei et al., 2014). For the choice test, adult aphids can be released at an equal distance between two plants of different PP TC phenotypes, such as between Col-0 and the sqn-6 or SPL15-1D mutant. Given the reduced PP TC development observed in these mutants (see Chapter 3), an anticipated outcome would be aphids preferably choosing to feed on these mutants. No-choice tests can be performed by inoculating age-synchronized second-instar nymphs on leaves of Col-0 and mutants, and the total aphid population (adult and nymph) on each plant can be recorded after seven days inoculation. Similarly, these tests could be performed between individual leaves of Col-0 plants to examine if adult leaves, which have much less PP TC abundance, are more susceptible to nymphs/aphids than juvenile leaves.
4.2.7 Novel reference genes for the analysis of gene expression in Arabidopsis leaves

A robust normalization strategy, namely to normalize using stably expressed reference genes, is one of the key factors determining the accuracy of qPCR results (Dheda et al., 2005; Nolan et al., 2006; Guenin et al., 2009). Putative “housekeeping genes” commonly used as normalizers can be variably expressed across tissue types and developmental stages, hence the need for systematic and empirical validation of reference genes specific to each experimental condition being examined (Gutierrez et al., 2008). A number of approaches have been used to identify reliable normalizers, among which the method of Hruz et al. (2011) outperforms others as it aims to choose reference genes specific to experimental conditions under study. Therefore, we used this method to identify suitable reference genes for normalization of mRNA expression specifically across leaf development. AT1G79810 (PEROXIN 2), and AT2G20790 (clathrin adaptor complexes medium subunit family protein) were found to be the most stably and comparably expressed genes across all our leaf samples, namely leaves from Col-0 at different developmental stages and from transgenic lines used in this study (Chapter 3). These two genes can therefore now be used in future gene expression studies across development of leaves in Arabidopsis.

4.3 Conclusions

The research reported in this thesis presents a significant step forward in the study of the molecular pathways underlying the trans-differentiation of PP TCs. We linked heteroblasty of shoots and associated genetic controls to PP TC development in leaves, which ultimately led to the conclusion that wall ingrowth deposition in PP TCs is under control of the miR156/SPL regulatory module. Such unexpected, but important findings were made possible by the robustness of a simplified version of the mPS-PI staining technique in combination with confocal microscopy to image wall ingrowth deposition in TCs. More importantly, future work derived from these findings promises to advance our understanding of the biology of this fascinating and important cell type.
REFERENCES

(For Chapters 1 and 4, and Appendix I)


References


References


References


References


References


APPENDIX I

Supplementary Data Accompanying Chapter 4
A1. Introduction

Following successful development of the mPS-PI staining procedure to enable confocal imaging of wall ingrowth deposition in TCs of Arabidopsis and pea (Chapter 2), several individual studies separate from those investigating the link between PP TC development and VPC in Arabidopsis (Chapter 3), were undertaken during the course of this PhD program. This Appendix reports on these investigations in the context of the general usefulness of the mPS-PI staining procedure for imaging cell wall deposition in different tissue and cell types in addition to using the mPS-PI procedure to investigate features of wall ingrowth deposition reported in PP TCs of Arabidopsis leaf veins in response to different abiotic stresses and altered developmental programs in both wild type and various mutants in which altered PP TC development has been reported based on TEM analyses of these cells.

A2. Material and Methods

A2.1 Plant growth conditions and cold treatment

*Arabidopsis thaliana* (L.) Heynh. ecotype Columbia (Col-0) was used as wild-type reference for the atsweet11/12 double mutant and vte2 mutant, whereas the suc2-1 mutant was on the Wassilewskija (Ws-2) background. Col-0, Ws-2, suc2-1 (CS3876) and arr1-3 arr10-5 arr12-1 (CS39992) seed were obtained from the Arabidopsis Biological Resource Center (Columbus, OH); the atsweet11/12 double mutant and vte2 mutant were kind gifts from Prof W. Frommer (Carnegie Institution for Science, USA) and Prof D. DellaPenna (Michigan State University, USA), respectively. Arabidopsis seeds were sown directly onto pasteurised soil mix and stratified for 3 days in darkness at 4°C. Plants were then transferred to a growth cabinet (100-120 µmol m⁻² sec⁻¹, 22°C day/18°C night, 16 h photoperiod). For cold treatment, 3-week-old vte2 plants were transferred to cold conditions (8.5 ± 2°C; 16 h light/8h dark; 75 µmol m⁻² sec⁻¹) and leaves were harvested after 24, 48 and 72 hours for Aniline Blue staining, and after 7 days for mPS-PI staining.
A2.2 Aniline blue staining and microscopy
Rosette leaves of Col-0 plants and vte2 mutant were cleared by placing the leaves in 25% (v/v) White King™ bleach (4% v/v effective hyperchlorite concentration) for at least 1 h with shaking until all chlorophyll had been extracted. Cleared leaves were washed thoroughly in MQ water and stained in 0.005% (w/v) Aniline Blue (Sigma Cat. #415049) in 70 mM sodium phosphate buffer, pH 9.0, for at least 1 h. Microscopy was performed with a Zeiss AxioScope fluorescence microscope using a DAPI filter and images were captured with a AxioCam MRC camera driven by AxioVision software (Zeiss).

A2.3 mPS-PI staining and confocal microscopy
mPS-PI staining of leaves, cotyledons, cross sections of inflorescence stem, roots, flowers and shoot apices of Arabidopsis and confocal microscopy of these tissues were performed as described in Chapter 3. Stem cross-sections (200-µm thickness) were obtained using a vibratome. To expose shoot apices, visible leaves (> 2 mm) were removed from young seedlings (one- to two-week-old).

A3. Results and Discussion
A3.1 High-resolution confocal imaging of diverse Arabidopsis tissues using the simplified mPS-PI staining procedure
Haritatos et al. (2000) and Chen et al. (2012) reported the presence of PP TCs in rosette leaves and sepals of Arabidopsis, respectively. In Chapter 2 we reported that veins of cotyledons and cauline leaves also developed PP TCs. Here, the simplified mPS-PI staining procedure has been applied to survey PP TCs in other organs/tissues of Arabidopsis shoots. The results from this analysis showed that PP cells in vascular bundles of fully-expanded petals and filaments also develop wall ingrowth-like structures (Figure A1). These structures resemble the discrete, punctate-like deposition of wall ingrowths seen in Class II PP TCs in rosette leaves (see classification of PP TCs in Chapters 2 and 3), which were highly localized to the wall face adjacent to SEs (Figure A1). However, further development of such wall material to form typical reticulate wall ingrowth networks was not observed in these PP cells. Therefore,
further investigation is necessary to confirm whether these structures are indeed TC wall ingrowths. It will be interesting to also determine whether they share any common function(s) with wall ingrowth deposition seen in leaves and other leaf-like organs. Given that they are small in size and few in number (Figure A1), the physiological role(s) of wall ingrowth-like structures in petals and filaments may not contribute significantly to the overall physiology of flowers. Nonetheless, the observation of such highly localized deposition of cell wall in these organs is not trivial as it might help to advance our understanding of the mechanism underlying the initial process of wall ingrowth deposition in TCs in general.

Confocal imaging of Arabidopsis anthers revealed the secondary wall thickening in endothecial cells (Figure A2). These lignified wall thickenings have an important role in the second phase of the two-step-dehiscence process of anthers, which ultimately leads to pollen release (Keijzer, 1987; Scott et al., 2004). Interestingly, these fibrous bands of thickened walls (Figure A2) are morphologically similar to the unlignified curvilinear bands of wall materials seen in TCs with flange wall ingrowth architectures (Gunning and Pate, 1974; Talbot et al., 2007a). In particular, the morphology of the endothecial cells as a whole shown in Figure A2, resembles, but with superior resolution, that of TCs in the leaf trace from a node of *Trollius europaeus*, as revealed by periodic acid-Schiff counterstained with toluidine blue (see Figure 13.2 in Gunning and Pate, 1974). Therefore, the confocal images of endothecial cells as presented in Figure A2 can serve as a reference for screening the presence of TCs with flange wall ingrowth morphology in mPS-PI stained tissues.

An example of this scenario could be to investigate whether the expression of *ZmMRP-1*, a maize endosperm TC-specific transcriptional activator (Gómez et al., 2002), in Arabidopsis can induce ectopic differentiation of flange-type TCs. Barrero et al. (2009) demonstrated that the promoter for *ZmMRP-1* drives strong GUS activity in intercotyledonary regions of young seedlings and at sites where new vascular bundles join the existing vascular network. Given that endosperm TCs in maize develop both flange and reticulate wall ingrowths with the former being predominant (Monjardino et al., 2013), and that TCs with flange wall ingrowth morphology, although more frequently encountered in monocot species, can also be seen in dicots, especially at
internodal regions (Gunning and Pate, 1974), it is conceivable that ectopic expression of \textit{ZmMRP-1} may induce flange wall ingrowth deposition in vascular cells at cotyledonary nodes of young Arabidopsis seedlings. Therefore, if one surveys the deposition of flange wall ingrowths in such instances using the simplified mPS-PI technique, the fibrous strands of secondary cell wall thickenings as shown in Figure A2 could be used for comparison.

The simplified mPS-PI staining procedure in combination with confocal microscopy was also applied to image other tissues in stems, roots and shoot apices (Figures A3 - A5). In all cases, the technique resolved sub-cellular structures in various cell types, such as starch grains in columella cells in the root tip (Figure A3) or sieve plates in the phloem of stems (Figure A4B and C). Additionally, the configuration of cell walls in mPS-PI-stained tissues was well preserved (Figures A3 - A5), especially in difficult tissues such as Arabidopsis shoot apical meristems (Figure A5). Consequently, smooth and intact cell walls of cell types that do not develop wall ingrowths was clearly resolved in these tissues (Figure A3 - A5), and thus the presence of wall ingrowth papillate, if they occur ectopically, would be easily recognized. This observation implies that this staining technique will enable high resolution assessment of the presence/absence of TCs in Arabidopsis as well as other species.

Confocal imaging of shoot apices stained by the mPS-PI procedure was used to identify developmental stages of leaf primordia in defoliation experiments. Data presented in Chapter 3 established that prolonged ablation of the first nine leaves starting in 10-day-old seedlings resulted in complete rejuvenation of leaf 10. We also performed similar leaf ablation experiments starting in 12- and 14-day-old seedlings and found that leaf 10 in these defoliated plants was only partially rejuvenated since this leaf still bore abaxial trichomes although the total number of these trichomes was reduced compared to that of leaf 10 in control plants. In cultured maize adult shoot apices, the degree of rejuvenation (partial or complete) occurring in an adult leaf depends on the length of the corresponding leaf primordia at the time of culture (Orkwiszewski and Poethig, 2000). Therefore, we examined if this phenomenon also applied for rejuvenation events occurring in defoliated Arabidopsis plants. Seedlings in which the defoliation regime was initiated at either 10-, 12- or 14-day-old, with leaves >2 mm being removed, were processed.
for mPS-PI staining and confocal imaging. We found that in a majority of shoot apices from 10-day-old seedlings, primordium leaf 10 had not initiated (Figure A5A), whereas primordium leaf 10 was readily visible and approximately 100 - 200 µm in length in 12-day-old shoot apices (Figure A5B) or > 300 µm in 14-day-old shoot apices (Figure A5C). These results are consistent with the observation by Orkwiszewski and Poethig (2000), and importantly imply that leaf identity can be changed even after the initiation of leaf primordia.

**A3.2 PP TC development in Arabidopsis cotyledons**

PP TC development was assessed in cotyledons of young Arabidopsis seedlings grown on soil. Cotyledons from seven-day-old seedlings had Class II PP TCs, evidenced by discrete, punctate or linear regions of wall ingrowth appearing in some PP TCs (Figure A6A - C). Over the next four or five days, this wall ingrowth deposition rapidly increased to form Class IV or V PP TCs in cotyledons of 11- or 12-day-old seedlings (Figure A6D - F). Similar to an increase in PP TC development across maturation of juvenile and adult leaves being independent of the differentiation of other vascular cells such as sieve elements or tracheary elements (Chapter 3), we observed no changes in xylem development in 12-day-old cotyledons compared to that in 7-day-old cotyledons (data not shown).

The massive density of wall ingrowths in 12-day-old cotyledons (Figure A6 D-F), in combination with simple cellular structure of these organs, makes these wall ingrowth materials readily evident and easily recognized when observed by differential interference contrast (DIC) microscopy (unpublished observation). This observation could be applied to perform high-throughput screening to identify aberrant wall ingrowth deposition in cotyledons, which then could be employed in a forward genetic screen approach to identify genes essential for the trans-differentiation of PP TCs.
Figure A1 Wall-ingrowth-like structures in PP cells in petals and filaments. A - C. Confocal images of vascular bundles in petals. D - F. Confocal images of vascular bundles in filaments. Arrowheads point to wall ingrowth-like structures in PP cells; asterisks indicate PP cells; SE, sieve elements; XE, xylem. Scale bar = 5 µm.
Figure A2 Secondary wall thickenings in endothecial cells in Arabidopsis anthers. **A,** Confocal image of the basal part of an anther, showing on the left transverse view of a single layer of epidermal cells (red arrows), a single layer of endothecial cells (red asterisks) and barely visible pollen grains (white asterisk) in the pollen sac. The right hand side of the image shows a longitudinal view of endothecial cells revealing fibrous strands of secondary wall thickenings, which can be seen as a continuous band (arrowhead) or part thereof (white arrow), depending on confocal planes. **B,** Higher magnification view of endothecial cells from another anther at a more advanced stage of development, showing fibrous strands of secondary wall thickenings (arrow and arrowhead) and a stoma (black asterisk) from the superimposed epidermis. Scale bars = 20 µm.

Figure A3 Confocal imaging of Arabidopsis root tip. Columella cells (arrow) are clearly resolved in this image, evident by the presence of starch grains.
Figure A4 Confocal imaging of Arabidopsis inflorescence stem. A, Transverse section of the stem. B and C, Higher magnification views of the vascular bundle in the boxed region in A, showing sieve plates (arrows) with visible holes. Asterisks indicate the same tracheary element imaged at different confocal planes in the three panels. The arrowhead in A points to a stoma.
Figure A5 Confocal imaging of Arabidopsis shoot apices in which defoliation had commenced at 10- (A), 12- (B) or 14-day-old (C) seedlings. Red asterisks indicate shoot apical meristems; single white asterisk indicates primordium leaf 8 in 10-day-old shoot apex; double white asterisks indicate primordia leaf 10 in 12- and 14-day-old shoot apices. S, stipules; T, adaxial trichome. Scale bars = 50 µm for all panels.
Figure A6 PP TC development in Arabidopsis cotyledons. A - C, Confocal images of veins from 7-day-old cotyledons, showing class II PP TCs. D - F, Confocal images of veins from 11- or 12-day-old cotyledons, showing class IV or V PP TCs. Arrowheads point to wall ingrowth deposition; asterisks indicate PP TCs. Arrows point to examples of pit fields. Scale bars = 10 µm for all images.
A3.3 PP TC development in the arr1-2 arr10-5 arr12-1 triple mutant
Chapter 3 reported a role for SPL9-group genes in regulating heteroblastic development of PP TCs and proposed possible connections between trans-differentiation of PP TCs and shoot regenerative capacity via the miR156-SPL9 module. Zhang et al. (2015) demonstrated that SPL9 and SPL10 exert their effects in repressing regenerative capacity of adult tissue by directly binding to the trans-activation domains of B-type ARABIDOPSIS RESPONSE REGULATORS (ARRs), which encode the transcriptional activators in the cytokinin signaling pathway. Regenerative capacity was completely lost in the arr1-2 arr10-5 arr12-1 triple mutant (Zhang et al., 2015), and consistent with this observation, PP TC development was significantly reduced in cotyledons and leaves 1 and 2 in this line compared to that in Col-0 (Figure A7). In cotyledons of mature arr1-2 arr10-5 arr12-1 plants, although a majority of PP cells do not develop wall ingrowths (Figure A7D), examples of PP TCs with extensive wall ingrowth deposition can be seen (Figure A7E). In addition, PP cells (and also bundle sheath cells) in veins of these cotyledons showed abnormal shape (Figure A7D) compared to the elongated shape typical of PP cells seen in Col-0. These observations imply a possible correlation between PP cell morphology/division and capacity to trans-differentiate into PP TCs.

A3.4 PP TC development in the atsweet11/12 double mutant and the suc2-1 mutant
ATSWEET11 and 12 are two sucrose effluxers localized specifically to PP cells and function in secreting sucrose into the apoplasm by a uniport mechanism (Chet et al., 2012). SUC2 is a sucrose importer located in the plasma membrane of companion cells (Truernit and Sauer, 1995; Stadler and Sauer, 1996). Defects in expression of these proteins cause impairment in phloem loading, ultimately leading to stunted growth of the atsweet11/12 double mutant (Chen et al., 2012) and the suc2 mutant (Gottwald et al., 2000). Therefore, to investigate whether expression of the sucrose transporters, AtSWEET11/12 and AtSUC2, correlated with wall ingrowth formation in PP TCs, PP TC development in these mutants was examined.
Figure A7 PP TC development in the arr1-2 arr10-5 arr12-1 triple mutant. A, 18-day-old plants of Col-0 and the arr1-2 arr10-5 arr12-1 triple mutant; at this stage, cotyledons (arrows) and leaves 1 and 2 (red arrowheads) of both genotypes had reached their final size. B - E, Confocal images of veins from cotyledons of (i) 15-day-old Col-0 (B and C) showing Class IV or V PP TCs (asterisks), and (ii) 25-day-old arr1-2 arr10-5 arr12-1 triple mutant (D and E) showing no wall ingrowth deposition in a majority of PP cells (asterisks in D) and Class IV PP TCs (asterisk in E) in a few locations. F - J, Confocal images of veins from leaves 1 or 2 of (i) 15-day-old (B) and 25-day-old Col-0 (G) showing Class IV or V PP TCs (asterisks), and (ii) 25-day-old arr1-2 arr10-5 arr12-1 triple mutant (H - J) showing a dramatic reduction in wall ingrowth deposition in PP TCs (Class II or III; asterisks in D) and no detectable wall ingrowth deposition in a majority of PP cells (asterisks in J). Arrowheads point to wall ingrowth deposition; asterisks indicate PP cells or PP TCs. Scale bars = 10 µm for B - J.
Prior to leaf fixation, to reduce starch levels and hence improve imaging quality, 25-day-old plants of Col-0, atsweet11/12 double mutant, Ws-2 and suc2-1/+ heterozygous mutant were covered with aluminum foil for 8 hours starting from the end of the 8-h night period of plant growth. Leaf 4 from these plants were harvested and subjected to mPS-PI staining and confocal imaging. Consistent with previous reports (Chen et al., 2012), the rosette size of the atsweet11/12 double mutant was approximately 30% smaller than that of Col-0 plants, whereas the Ws-2 plants were around 5-fold larger in size compared to the suc2 heterozygous mutant (Gottwald et al., 2000) (Figure A8). However, as revealed by mPS-PI staining and confocal imaging of these lines, there was no detectable differences in the extent of wall ingrowth deposition in these mutants compared to that in their corresponding wild-type (Figure A9). This initial result suggests that expression of the membrane transporters required for phloem loading in Arabidopsis (AtSWEET11/12 and AtSUC2) are not coupled with wall ingrowth deposition and vice versa. To validate this claim, further work examining expression levels of AtSWEET11/12 in the suc2, and SUC2 in the atsweet11/12 double mutant is warranted, in addition to measuring gene expression levels in mutant/transgenic lines with altered levels of PP TC development, such as sqn-6, SPL15-1D, 35S::MIM156 and 35S:MIR156 (see Chapter 3). These initial observations, however, add another layer of evidence arguing against a role for wall ingrowth deposition in PP TCs solely in facilitating the export of photoassimilate from collection phloem in leaves (see Chapter 3).

Figure A8 Growth phenotype of 25-day-old plants of Col-0, atsweet11/12 double mutant, Ws-2 and suc2-1 mutant. Note that the leaves in all lines were hyponastic due to the additional 8 h of dark treatment imposed to reduce starch levels in the two mutants.
Figure A9 Confocal imaging of veins in leaf 4 from 25-day-old plants of Col-0 (A), atsweet11/12 double mutant (B and C), Ws-2 (D) and suc2-1 mutant (E and F), showing Class IV PP TCs with extensive wall ingrowth deposition. Residual starch grains are seen in mesophyll cells of the atsweet11/12 double mutant and the suc2-1 mutant but not in their corresponding wild-type. Arrowheads point to wall ingrowth deposition; asterisks indicate PP TCs. Scale bars = 10 µm for all images.
A3.5 Cold treatment of vte2 mutant

Maeda et al. (2006) proposed a role for wall ingrowth deposition in Arabidopsis PP TCs in enhancing phloem loading based on changes specific to PP TCs upon cold treatment of the vte2 mutant, a mutant defective in vitamin E biosynthesis. Consistent with the observations reported by these authors, cold treatment caused impairment of photoassimilate export, evident by abundant starch grains in mesophyll and bundle sheath cells in leaves of cold-treated vte2 plants (Figure A10F). Maeda et al. (2006) attributed this phenomenon of reduced sugar export from leaves (and hence starch accumulation) to low-temperature-inducible callose deposition occurring selectively in PP TCs. However, aniline blue staining of veins from leaf 6 of 24-day cold treated plants showed that such callose deposition was not only seen in PP TCs but also in PP cells, evidenced by intense fluorescence signals seen in midvein and secondary veins (Figure A10C and D), where it is known from mPS-PI staining that PP cells do not develop wall ingrowths (Chapter 3). More surprisingly, under permissive temperature, callose deposition was readily evident (Figure A10A and B). Such deposition, however, significantly increased by 24 and 48 hours cold treatment (data not shown), and did so more dramatically after 72 hours (Figure A10C and D), similar to the observation made by Maeda et al. (2006). However, unlike Maeda et al. (2006), we did not observe an increase in wall ingrowth deposition in PP TCs in either adult (Figure A10E and F) or juvenile leaves (data not shown) of vte2 plants subjected to cold treatment, compared to that in control plants. Collectively, our data demonstrate that morphological changes seen in vascular cells of cold-treated vte2 plants did not occur exclusively in PP TCs; therefore, the conclusions associated with “PP TC-specific events” made by Maeda et al. (2006) can be questioned. As a result, the link between wall ingrowth deposition and the capacity of PP TCs to enhance photoassimilate export from leaves proposed by these authors based on such conclusions becomes doubtful.
Figure A10 Morphological changes upon cold treatment of the vte2 mutant. A - D, Aniline Blue staining of leaf veins from leaf 6 of 24-day-old vte2 plants grown under permissive room temperature (A and B) and after 72 hours of cold treatment (C and D). Arrowheads point to callose blobs in PP cells. E and F, mPS-PI staining of leaf 11 from 28-day-old vte2 plants grown under permissive room temperature (E) and after 72 hours of cold treatment (F). Note the abundance of starch grains in vte2 plants subjected to cold treatment (F) compared to the permissive control temperature (E). This result indicates that vte2 is responding to cold treatment as reported by Maeda et al. (2006), but unlike this paper, no wall ingrowths are detected in PP cells of the cold-treated vte2 line (F) compared to control (E). Asterisks indicate PP cells. Scale bars = 50 µm in A and C, = 20 µm in B and D, and = 10 µm in E and F.
APPENDIX II

Mini-Review

Phloem parenchyma transfer cells in Arabidopsis – an experimental system to identify transcriptional regulators of wall ingrowth formation

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The text presented in this Appendix is the accepted manuscript submitted to Frontier in Plant Science.
Abstract

In species performing apoplastic loading, phloem cells adjacent to sieve elements often develop into transfer cells (TCs) with wall ingrowths. The highly invaginated wall ingrowths serve to amplify plasma membrane surface area to achieve increased rates of apoplastic transport, and may also serve as physical barriers to deter pathogen invasion. Wall ingrowth formation in TCs therefore plays an important role in phloem biology, however the transcriptional switches regulating the deposition of this unique example of highly localized wall building remain unknown. Phloem parenchyma (PP) TCs in Arabidopsis veins provide an experimental system to identify such switches. The extent of ingrowth deposition responds to abiotic stress, enabling bioinformatics to identify candidate regulatory genes, and simple fluorescence staining of PP TCs in leaves enables phenotypic analysis of relevant mutants. Combining these approaches resulted in the identification of *GIGANTEA* as a regulatory component in the pathway controlling wall ingrowth development in PP TCs. Further utilization of this approach has identified two NAC-domain and two MYB-related genes as putative transcriptional switches regulating wall ingrowth deposition in these cells.

Introduction

The plant cell wall profoundly defines cell shape and functioning. This observation is particularly acute for transfer cells (TCs) which develop extensive wall ingrowths to aid nutrient transport. These cells trans-differentiate from various differentiated cell types at sites where nutrient distribution pathways encounter apoplastic/symplasmic discontinuities (Pate and Gunning, 1969; Offler et al., 2003). The increase in plasma membrane surface area resulting from wall ingrowth deposition enables increased densities of nutrient transporters to facilitate localized flux of nutrients across these apoplastic/symplasmic junctions.

TCs are prominent at anatomical sites required for phloem loading and post-phloem unloading processes. In species that perform apoplastic phloem loading, vascular cells adjacent to sieve elements (SEs) often develop extensive wall ingrowths. Well known examples include companion cells (CCs) in pea (Gunning and Pate, 1969;
Henry and Steer, 1980; Wimmers and Turgeon, 1991), phloem parenchyma (PP) in Arabidopsis (Haritatos et al., 2000; Amiard et al., 2007), and both CCs and PP in Senecio vulgaris (Pate and Gunning, 1969; Amiard et al., 2007). In pea, the onset of assimilate export from young leaves coincides with the differentiation of leaf minor vein TCs (Gunning & Pate, 1974), and in Arabidopsis, sucrose export from leaves is affected if wall ingrowth abnormalities occur in the PP TCs (Maeda et al., 2006). TCs are also commonly observed in cells involved in post-phloem unloading pathways (Patrick, 1997), particularly in seed of cereal crops such as wheat and barley (Thompson et al. 2001). Wall ingrowth formation therefore plays an important role in efficient phloem loading and post-phloem unloading strategies in many species, however the genetic pathways which regulate wall ingrowth deposition in TCs remain largely unknown.

TC development occurs across normal developmental windows but also in response to biotic and abiotic stress (Offler et al., 2003). Recent studies using epidermal TCs of Vicia faba cotyledons have established that auxin (Dibley et al., 2009), ethylene (Zhou et al., 2010; Andriunas et al., 2011) and reactive oxygen species (ROS) (Andriunas et al., 2012) function as inductive signals for TC development. Furthermore, expression profiling in epidermal TCs of V. faba cotyledons (Dibley et al., 2009) and in endosperm TCs in barley (Thiel et al., 2008, 2012) indicates that wall ingrowth deposition involves differential expression of hundreds of genes. The missing link in this developing molecular understanding of TC biology, however, is the identity of key transcriptional regulators which respond to inductive signals and switch on the downstream cascades of gene expression required to build wall ingrowths. A genetic approach is well suited to identify such transcription factors. In this mini-review we discuss the features of PP TCs in Arabidopsis that enabled a combined bioinformatics and reverse genetics approach to be undertaken to discover that GIGANTEA (GI) is a component of a pathway regulating wall ingrowth deposition in PP TCs. Further, we describe preliminary results using this approach to identify previously uncharacterized members of the NAC-domain and MYB-related gene families as putative transcriptional regulators of wall ingrowth deposition in PP TCs.
Phloem parenchyma transfer cells in Arabidopsis

TCs in Arabidopsis are known to occur in PP of the minor vein network in both leaves (Haritatos et al., 2000) and sepals (Chen et al., 2012). These PP TCs are defined as Type B TCs (Gunning and Pate, 1969), characterized by having bulky wall ingrowths predominantly abutting SEs and to a lesser extent CCs (Haritatos et al., 2000; Amiard et al., 2007). These three cell types together constitute phloem tissue of the minor vein in Arabidopsis, with proportionate numbers of cells of each type relatively consistent throughout the vein system regardless of vein order (Haritatos et al., 2000). SEs are smaller than CCs, as is typical of collection phloem described by van Bel (1996), and PP cells are larger than CCs (Haritatos et al., 2000). Vein order in Arabidopsis leaves typically extends to three or four (Haritatos et al., 2000) or sometimes to five orders (Kang et al., 2007). This number is lower than the typically six or seven vein orders seen in most dicot species, and may in part account for the suggestion that both major and minor veins, being in close proximity to mesophyll tissue, are likely to be involved in phloem loading and thus functionally defined as “minor veins” (Haritatos et al., 2000).

A role for PP TCs in phloem loading is based on structural and molecular observations. Prominent symplasmic connections occur between PP and neighboring bundle sheath cells (Haritatos et al., 2000), providing a symplasmic delivery pathway for sucrose from photosynthetic mesophyll cells. Prominent wall ingrowths deposited adjacent to abutting cells of the SE/CC complex infers that the symplasmically delivered sucrose is effluxed across the plasma membrane of PP TCs into the apoplasm (Amiard et al., 2007). Subsequent movement of sucrose into the SE/CC complex occurs via carrier-mediated uptake by SUC2, a sucrose/H⁺ co-transporter localized to the plasma membrane of CCs in Arabidopsis (Truernit and Sauer, 1995; Gottwald et al., 2000). The machinery responsible for sucrose efflux from PP TCs into the apoplasm was recently identified as members of the AtSWEET family of sugar transporters (Chen et al., 2012). AtSWEET11 and 12 function as sucrose uniporters that facilitate sucrose efflux, and both localize to the plasma membrane of PP TCs (Chen et al., 2012). An atswee11 atswee12 double mutant shows various physiological traits consistent with
impaired sucrose export from leaves (Chen et al., 2012). These authors concluded that PP TCs participate in a two-step phloem loading strategy in Arabidopsis – unloading of sucrose from PP TCs into the apoplasm, followed by active uptake of this apoplastic sugar into the SE/CC complex by SUC2. Interestingly, Chen et al. (2012) propose that the highly localized deposition of wall ingrowths in PP TCs adjacent to cells of the SE/CC complex enables restricted diffusion of sucrose into the apoplasm, thus potentially reducing access to this apoplastic sugar by pathogens. Others have suggested that the extensive deposition of bulky and highly localized wall ingrowths in PP TCs adjacent to SEs provides a physical barrier to protect against pathogen infection of sieve tissues which commonly target PP cells of the vascular network (Amiard et al., 2007).

Haritatos et al. (2000) observed that PP TCs also form asymmetric plasmodesmatal connections with adjacent CCs in Arabidopsis veins, implying that phloem loading in this system may also occur passively via plasmodesmatal pathways under certain physiological conditions. This observation implies that phloem loading strategies in different scenarios may be developmentally plastic, switching alternately from active, apoplastic loading, to passive, symplasmic loading, even along a single vascular bundle (Slewinski and Braun, 2010). The molecular signals that may control such plasticity are unknown, however the identification by Chen et al. (2012) that the promoter for AtSWEET11 drives expression in leaf tissue specifically in PP cells provides a valuable addition to the molecular tool box to investigate such processes.

Arabidopsis phloem parenchyma transfer cells as an experimental system to investigate genetic control of wall ingrowth deposition

Importantly for genetic analysis of TCs in a model species, wall ingrowth deposition in Arabidopsis PP TCs is responsive to various stresses. The extent of wall ingrowth invaginations in PP TCs of leaf minor veins was significantly increased in response to stress caused by high light or exposure to methyl jasmonate (Amiard et al., 2007). Furthermore, the high-light response was reduced in the jasmonate-deficient double
mutant *fad7-1 fad8-1* (Amiard et al., 2007), implying the unexpected conclusion that chloroplast-derived jasmonates signal wall ingrowth deposition in PP TCs in response to oxidative stress. In support of this conclusion, a *npq1-2 lut2-1* double mutant showed increased levels of wall ingrowth deposition compared to wild-type when subjected to high-light stress (Demmig-Adams et al., 2013). The double mutant lacks zeaxanthin and its isomer lutein, photoprotective agents which suppress lipid peroxidation and most likely oxylipin (methyl jasmonate and its precursors jasmonic acid and 12-oxo-phytodienoic acid) formation. The absence of this suppression in the *npq1-2 lut2-1* double mutant presumably leads to higher levels of jasmonic acid when plants are switched from low to high light, thus the observed increase in deposition of wall ingrowths in PP TCs (Demmig-Adams et al., 2013).

Wall ingrowth deposition in PP TCs is also responsive to cold stress. As part of their study investigating the role of tocopherols in photoprotection, Maeda et al. (2006) reported that growth of wild-type plants at low temperature caused increased deposition of polarized wall ingrowths in PP TCs. In contrast, at low temperature the vitamin E-deficient mutant, *vte2*, displayed greatly increased levels of abnormal wall ingrowth deposition, including loss of polarized deposition and substantial accumulation of callose in and around the wall ingrowths (Maeda et al., 2006). Not surprisingly, the *vte2* plants showed reduced sugar export and consequently increased levels of soluble sugar in leaves of cold-treated plants (Maeda et al., 2006). This result indicates not only that low temperature in itself causes increased wall ingrowth deposition, but at low temperature the signal(s) causing localized wall ingrowth deposition are lost or over-ridden in the *vte2* mutant. Irrespective of this issue, however, the study by Maeda et al. (2006) adds low temperature to high light and exposure to methyl jasmonate (Amiard et al., 2007) as stress signals causing wall ingrowth deposition in Arabidopsis PP TCs. From the perspective of identifying transcriptional regulators of wall ingrowth deposition, the importance of these observations is that they enable bioinformatics approaches to be used to identify candidate genes.
Fluorescence staining of phloem parenchyma transfer cells in Arabidopsis leaves

TCs typically occur deep within tissue systems and consequently have mostly been studied by electron microscopy, a process which is not compatible for high throughput genetic screening using Arabidopsis. Wall ingrowths lack lignin but are abundant in cellulose and hemicelluloses (DeWitt et al., 1999; Dahiya and Brewin, 2000; Vaughn et al., 2007), therefore Edwards et al. (2010) used Calcofluor White staining of cleared leaf tissue as a means to rapidly assess the abundance of PP TC development across whole leaves. Staining showed strong patches of fluorescence in terminating minor veins but also more continuous, linear regions of fluorescence often seen as one or two rows of staining within each vein (Figure 1A, B). Higher magnification views revealed that the Calcofluor White staining showed a distinctive mottled appearance, a characteristic consistent with staining the patchy and tangled wall ingrowths seen in leaf PP TCs by scanning electron microscopy (Figure 1C, D; Edwards et al., 2010). The non-continuous staining pattern for PP TCs along a given vein is consistent with observations by transmission electron microscopy that not all PP cells contain wall ingrowths (Amiard et al., 2007), a situation possibly reflecting potential plasticity in phloem loading mechanisms as discussed by Slewinski and Braun (2010). Furthermore, the ability to survey whole leaves for the presence of PP TCs clearly established that these cells are prominent in both minor and major veins of the vascular network, an observation consistent with the conclusion that both vein types in Arabidopsis are likely to be involved in phloem loading (Haritatos et al., 2000).

A recent improvement for fluorescence staining to detect PP TCs in Arabidopsis leaves has been the use of Aniline Blue rather than Calcofluor White. Callose is an abundant component of the electron translucent outer layer of wall ingrowths in both epidermal TCs of *V. faba* cotyledons (Vaughn et al., 2007) and Arabidopsis PP TCs (Maeda et al., 2006, 2008). Other than being deposited in sieve plates, callose is mostly absent from other tissues in unwounded leaves, thus giving superior signal-to-noise staining of PP TCs compared to Calcofluor White (Figure 1E, F). Double labeling experiments have shown that Aniline Blue gives the same mottled patterns of staining
for PP TCs as does Calcofluor White (J. Hou – unpublished observation and see Edwards et al., 2010), thus confirming that Aniline Blue can be used as a convenient and high throughput fluorescence stain for wall ingrowth deposition in PP TCs.

Figure 1 Imaging of PP TCs in Arabidopsis veins using fluorescence staining and scanning electron microscopy. Calcofluor White staining of cleared leaf tissue (A-C) showing presence of PP TCs in a terminating minor vein (arrow in A) and as more continuous linear strands of staining running along major veins (arrows in B). Higher magnification reveals a central band of mottled fluorescence (arrows in C, asterisks mark cell edges) in a PP TC which corresponds to the deposition pattern of reticulate wall ingrowths seen by scanning electron microscopy in these cells (arrows in D). Staining of PP TCs by Aniline Blue (E, F) shows the same patterns of staining as revealed by Calcofluor White, albeit with superior signal-to-noise properties (see F). Punctate staining indicating the non-continuous development of PP cells into PP TCs along a given length of vein is particularly evident in E. The images in A-D are reproduced from Edwards et al. (2010) and E and F are unpublished data. Staining with Aniline Blue was performed identically to that of Calcofluor White, except that 0.01 (w/v) Aniline Blue in 70 mM phosphate buffer, pH 8.5, was used to replace 0.05% (w/v) Calcofluor White. Scale bars: A, B, E = 100 µm; F = 200 µm; C = 5 µm; D = 2 µm.
Identification of GIGANTEA as a component in the regulatory pathway controlling wall ingrowth deposition in PP TCs

Combining the experimental features of PP TCs as described above, Edwards et al. (2010) performed a hierarchical bioinformatics analysis of publically available microarray datasets and identified GI, a well-known regulator of flowering time (Koornneef et al., 1991; Fowler et al., 1999), as one of about 46 genes commonly up-regulated in leaves subjected to either high-light or cold stress. Phenotypic analysis using Calcofluor White staining of leaves revealed that in both gi-2 and gi-3 plants, the abundance of PP TCs in veins was reduced up to 15-fold compared to wild-type. Over-expression of GI in the gi-2 mutant background restored PP TC abundance back to wild-type levels, whereas rescue of wall ingrowth deposition in gi-2 did not occur after exposure to high light, methyl jasmonate or cold. Based on these outcomes, Edwards et al. (2010) proposed that GI may be regulating wall ingrowth deposition downstream of inputs from stress signals, possibly through detoxification of ROS (see Cao et al., 2006). In epidermal TCs of V. faba cotyledons, extracellular H$_2$O$_2$ is known to act as a polarizing signal to direct aspects of wall ingrowth deposition (Andriunas et al., 2012; Xia et al., 2012). In Arabidopsis, however, H$_2$O$_2$ is abundant in leaf vasculature, even in the absence of stress (Mullineaux et al., 2006), hence its ability to act as a local signal directing polarized wall ingrowth formation in PP TCs needs further investigation.

Identification of NAC-domain and MYB-related transcription factors as putative regulators of wall ingrowth deposition

Based on the successful approach used by Edwards et al. (2010), we recently performed an extended bioinformatics analysis to identify transcription factors commonly up-regulated in leaf tissue in response to high light, methyl jasmonate and cold. Phenotypic analysis using Aniline Blue staining of leaves from homozygous T-DNA insertional mutants from this list identified several previously uncharacterized NAC-domain (At3g04420 and At1g33060) and MYB-related genes (At1g25550 and
At1g49560) which showed significantly reduced abundance of PP TCs in veins of mature leaves compared to wild-type (Table 1). The levels of reduced abundance in each line, while significant, were not comparable to that seen for the gi-2 mutant (Table 1), indicating the possibility that these transcription factors may be acting redundantly with unidentified orthologs in controlling wall ingrowth deposition. In silico expression data (eFP and Genevestigator) shows that all four genes are expressed at very low levels in leaves, and qPCR confirmed this observation directly for both expanding and fully expanded leaves (J. Hou, Y. Wu – unpublished observations). Low expression might be expected for genes operating as putative regulators of wall ingrowth deposition specifically in PP TCs, since the number of these cells relative to most other cell types in the leaf is exceedingly low (Haritatos et al., 2000, Edwards et al., 2010), and many plant transcription factors are expressed at low levels (Czechowski et al., 2004). Given these factors, we are using both constitutive (CaMV-35S promoter) and PP-specific (AtSWEET11 promoter) over-expression to test the role of these transcription factors as regulators of wall ingrowth deposition in Arabidopsis.

Interestingly, ectopic over-expression of VND6 or VND7, both NAC-domain transcription factors, causes trans-differentiation of non-vascular cells into metaxylem- and protoxylem-like vessels elements, respectively (Kubo et al., 2005), a process involving localized secondary wall deposition. Over-expression of various MYB transcription factors such as AtMYB46 (Zhong et al., 2007) and AtMYB83 (McCarthy et al., 2009) also causes ectopic secondary wall formation, leading to the conclusion that hierarchical transcriptional pathways, with NAC-domain and MYB transcription factors acting as either first- or second-level “master switches”, co-ordinate the gene expression programs required for localized secondary wall deposition (Zhong et al., 2010). Building wall ingrowths in TCs is also an example of highly localized wall deposition (McCurdy et al., 2008), thus our finding that two NAC-domain and two MYB-related genes are putative regulators of this process in PP TCs may indicate evolutionarily-conserved roles for members of these two large gene families in regulating transcriptional cascades involved in localized wall deposition. Further support for this proposition is that ZmMRP-1, a transcription factor which regulates
basal endosperm TC development in maize, is a member of the MYB-related family of transcription factors in plants (Gómez et al., 2002, 2009).

**Table 1.** Phenotypic analysis showing reduced abundance of PP TC staining for two NAC-domain and two MYB-related genes identified by bioinformatics as candidate transcriptional regulators of wall ingrowth deposition in PP TCs of *Arabidopsis* leaf veins.

<table>
<thead>
<tr>
<th>Gene number</th>
<th>Mutant allele</th>
<th>% vein length showing staining for PP TCs§</th>
</tr>
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<tbody>
<tr>
<td>WT (Col-0)</td>
<td></td>
<td>45.3 ± 3.6</td>
</tr>
<tr>
<td>NAC-domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At3g04420</td>
<td>FLAG_009F02</td>
<td>18.2 ± 3.8**</td>
</tr>
<tr>
<td>At1g33060</td>
<td>SALK_085596</td>
<td>27.5 ± 2.9*</td>
</tr>
<tr>
<td></td>
<td>SALK_024241</td>
<td>31.5 ± 4.7*</td>
</tr>
<tr>
<td>MYB-related</td>
<td></td>
<td></td>
</tr>
<tr>
<td>At1g25550</td>
<td>SALK_144656</td>
<td>16.3 ± 2.4**</td>
</tr>
<tr>
<td>At1g49560</td>
<td>SALK_085182</td>
<td>20.5 ± 2.9*</td>
</tr>
<tr>
<td></td>
<td>SALK_095775</td>
<td>15.6 ± 2.7**</td>
</tr>
<tr>
<td>GIGANTEA</td>
<td>gi-2</td>
<td>3.3 ± 1.0***†</td>
</tr>
</tbody>
</table>

§ This value was measured from mature, Aniline Blue-stained leaves according to our previously published method (Edwards et al., 2010). Data is presented as mean ± SE from two leaves from each of three plants per line. *P<0.01, **P<0.001.

† Data for gi-2 taken from Edwards et al. (2010).
Conclusions and future directions

The formation of wall ingrowths in TCs impacts on phloem loading and post-phloem unloading processes in many species, with corresponding impacts on plant development and reproduction. Development of an experimental system to investigate PP TCs in Arabidopsis has proven useful to identify candidate genes operating as putative transcriptional regulators of wall ingrowth deposition in TCs. The discovery of GI as a component in the pathway regulating wall ingrowth deposition, and identification of NAC-domain and MYB-related genes as putative “master switches” involved in controlling this process, provides new lines of investigation to understand the genetic control of TC development and the cell biology of localized wall ingrowth deposition. Ultimately, identifying master switches which respond to various inductive signals to coordinate wall ingrowth deposition in TCs may provide new opportunities for improving crop yield by manipulating this process.

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References


APPENDIX III

Book chapter

Transdifferentiation: a plant perspective

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Keywords: Arabidopsis thaliana; Dedifferentiation; Mesophyll cells; Redifferentiation; Regeneration; Tracheary elements; Transdifferentiation; Transfer cells; Waddington’s epigenetic landscape; Xylogenesis; Zinnia elegans


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Introduction

Transdifferentiation is defined as the irreversible switch of one differentiated cell type into another (Okada 1991). This process occurs in both plants and animals, but transdifferentiation as a biological phenomenon has attracted much greater attention in animal systems compared to plants. In animals, this process, together with dedifferentiation and reprogramming, reflects the flexibility in cell differentiation and morphogenesis and has brought a new view of differentiated cells and their determined states. Traditional understanding of embryonic development favoured the concept of lineage-based differentiation, where a differentiated cell was viewed as the last of a progressive sequence of binary choices and once acquired, a stable differentiated state could not change its phenotype (Okada 1991, Grafi 2004, Tosh and Horb 2013). Therefore, transdifferentiation represented a reversal of developmental progression and hence received much criticism in the early literature. To some critics, examples of such cell type switching were attributed to tissue culture artefacts or cell fusion, or merely an exceptional phenomenon and thus of little significance for further investigation. However, since the first discovery of transdifferentiation, known as Wolffian lens generation (formation of a lens from the iris of the eye) was reported by G. Wolff in 1895 (see Okada 1991 and references therein), increasing numbers of reliable systems of transdifferentiation have been demonstrated, thus arguing for biological reality of this process (Eguchi and Okada 1973, Itoh and Eguchi 1986, Okada 1991). Since then, numerous studies have been devoted to transdifferentiation and other related subjects of flexibility in animal cell differentiation, not only because of their increasingly recognized significance in developmental biology, but more importantly their therapeutic potential in regenerative medicine (Tosh and Slack 2002, Burke and Tosh 2005, Jopling et al. 2011).

Although the occurrence of transdifferentiation either in nature, during regeneration or in tissue culture has been unequivocally established, it is still a matter of some controversy as to how individual cases can be confirmed or dismissed as a true transdifferentiation event. A set of prerequisites proposed by Eguchi and Kodama (1993) has been widely accepted among reproductive biologists to distinguish
transdifferentiation from other types of modulation in cell differentiation. First, the differentiated states before and after the transdifferentiation must be clearly distinguished from one another based on morphological criteria accompanied by molecular and/or biochemical characterization. Second, the cell lineage relationship between the two cell types must be substantiated (Okada 1986, Eguchi and Kodama 1993). Transdifferentiation frequently involves the intervention of dedifferentiation and cell division, but these intermediate processes are not obligatory in all cases (Okada 1986, Beresford 1990, Eguchi and Kodama 1993).

To the best of our knowledge, there has been no comparable set(s) of criteria to evaluate a transdifferentiation process in plants. This is not surprising because plant cells are considered to have much greater developmental plasticity compared to animal cells, therefore observations of switches between two differentiated cells types, namely transdifferentiation, does not necessarily invoke criticism among plant biologists and thus the use of the term “transdifferentiation” tends to be easily accepted without much attention to the nature of the actual process. Further, apart from the contribution toward general understanding of plant cell differentiation, transdifferentiation in plants seems not to have significant practical implications such as the therapeutic potential in regenerative medicine resulting from transdifferentiation in animals. Therefore, in some cases, transdifferentiation in plants is exchangeably described as redifferentiation or simply differentiation (Grafi et al. 2011b, Bhojwani and Dantu 2013), without significant impacts on developmental outcomes of the organism.

In this Chapter, we review representative examples of transdifferentiation in plants and evaluate whether they represent true examples of this phenomena in light of the criteria used in animal research, and further, whether these criteria are indeed relevant to plant biology. We also discuss current understanding of molecular pathways underlying these processes, and describe other examples occurring naturally or in regeneration which fulfil the criteria of transdifferentiation but have been more or less neglected as examples of this process in plants.
Historical review: xylem cells as a spotlight for research on plant cell transdifferentiation

The conversion of various differentiated non-vascular cells into vascular cells observed in nature or in experimental systems was well documented long before the phenomenon was referred to as transdifferentiation. In 1908 Simon first demonstrated the regeneration of xylem strands resulting from a switch of existing parenchyma cells into tracheary elements (TEs) around wounding sites of Coleus internodes (see Jacobs 1952 and references therein). Since then, wound-induced vascular regeneration, particularly xylem regeneration, has been systematically studied in Coleus (Jacobs 1952, Aloni and Jacobs 1977a and 1977b) and other species including tobacco (Sussex et al. 1972), pea (Robbertse and McCully 1979, Rana and Gahan 1983) and pine (Kalev and Aloni 1999). The in vitro differentiation of TEs from, for example, cell suspension cultures of Centaurea (Torrey 1975), pith parenchyma cells of cultured lettuce (Dalessandro and Roberts 1971; Wilson et al. 1982), Jerusalem artichoke (Phillips and Dodds 1977) or carrot explants (Mizuno et al. 1971), and in particular mesophyll cells of Zinnia elegans (Fukuda and Komamine 1980a; Church and Galston 1988), are even more dramatic examples of this process and have been extensively studied in the early literature. The remarkable Zinnia system, first reported in 1975 by Kohlenbach and Schmidt (see Fukuda and Komamine 1980a and references therein), has been the main focus for research on this phenomenon in plant physiology, with numerous publications appearing from 1980 to 1989 from laboratories studying different aspects of the in vitro conversion of isolated Zinnia mesophyll cells into TEs. Over this period, this phenomenon was variously referred to as “formation”, “transformation”, “redifferentiation”, “in vitro differentiation” or simply “differentiation” of TEs.

Despite the long history of investigation of such switches from various differentiated cell types into TEs either in regeneration or in vitro, it was not until 1990 when the term “transdifferentiation” was first used in the plant literature by Sugiyama and Komamine to describe the phenomenon of isolated Zinnia mesophyll cells converting to TEs, but without, however, any definitive definition of the term or explanation of its usage (Sugiyama and Komamine 1990). The term “transdifferentiation” has since been accepted
and used, relatively widely but not systematically, in subsequent studies of this phenomenon.

The importance of terminology
Transdifferentiation is generally categorized as either direct or indirect. Originally this subdivision was based on the absence or presence of cell division. Cells not dividing before altering their differentiated state were considered to undergo direct transdifferentiation, while indirect transdifferentiation required the intervention of cell division (Okada 1986, Beresford 1990). This subdivision is still widely applied in both animal (Tosh and Slack 2002, Wang et al. 2015) and plant research (Sugiyama and Komamine 1990, McManus et al. 1998, Reusche et al. 2012). However, a more recent definition in the animal literature for direct transdifferentiation describes conversion of a differentiated cell without undergoing dedifferentiation back to a pluripotent state or progenitor cell type (Jopling et al. 2011, Ma et al. 2013, Xu et al. 2014, Cai et al. 2007). These two definitions would coincide if dedifferentiation is coupled with cell division; however, in several cases cells can undergo dedifferentiation independent of cell division (Grafi 2004, Srivastava 2002, Sugiyama 2015), hence causing potential confusion when using these terms. For example, a transdifferentiation process occurring without cell division but with dedifferentiation (to a progenitor cell type) is defined as direct transdifferentiation according to Tosh and Slack (2002), but as indirect transdifferentiation according to the definition of Jopling et al. (2011). This confusion in terminology can be elevated when transdifferentiation is uncoupled from the dedifferentiation process entirely (Hanna et al. 2010, Sugimoto et al. 2011, Mora and Raya 2013), or to a lesser extent cell division (McManus et al. 1998, Reyes and Verfaillie 2004), where the term “transdifferentiation” is applied to describe only direct conversions of cell fate that does not involve dedifferentiation or cell division, respectively.

In our view, the definition of direct transdifferentiation by Jopling et al. (2011) and others, i.e., the conversion of differentiated cells without dedifferentiation, provides a more precise description of this process, incorporating current
understanding of stem cells and reprogramming. Therefore, as stated previously, we apply this definition to review examples of transdifferentiation in plants. We also have adapted the elegant approach of biophysicists (Wang et al. 2011, Xu et al. 2014) with regard to cell development and differentiation, where Waddington’s epigenetic landscape (Waddington 1957) was explored to describe normal cell differentiation as well as the possibility of reverse differentiation of differentiated cells. Figure 1 is a combined modification of the original epigenetic landscape (Waddington 1957) and the schemes of cell type switching proposed by Xu and colleagues (see Figure 1A in Xu et al. 2014). In this figure, coloured marbles represent cells at different states of differentiation occupying different valleys of the landscape; the deeper the valley the more highly differentiated state of the cell. The red marble corresponding to a stem cell or totipotent cell sits at the top of the landscape, follows the chreode-paths, gradually loses its totipotency and ultimately gives rise to a differentiated (singlepotent) cell (blue marbles) at the bottom of the landscape. Normal differentiation of cells is depicted by the black arrows, whereas blue and orange arrows illustrate dedifferentiation and direct transdifferentiation, respectively. The fate of a plant cell can be determined to be at, for example, the differentiated state $S_B$ following normal differentiation. In tissue culture or regeneration in response to injury or pathogen attack, differentiated cell state $S_B$ can be formed via direct and/or indirect transdifferentiation. Indirect transdifferentiation of $S_B$ from $S_C$ involves dedifferentiation of $S_C$ to a progenitor or pluripotent state (orange marble) followed by normal differentiation to the new cell identity ($S_B$). The stepwise dedifferentiation requires the cell to move to a higher level on Waddington’s landscape to reach less differentiated (multipotent/pluripotent) states represented by the green/orange marbles, and this can occur with or without cell division. In other cases $S_C$ can transdifferentiate directly (orange arrow) to $S_B$ skipping the dedifferentiation step, probably through an explicit intermediate stable state $S_0$ (light blue marble, Figure 1) (Xu et al. 2014).
Figure 1 Transdifferentiation process in the Waddington’s epigenetic landscape shows cell populations with different developmental potentials. Coloured marbles correspond to differentiation states: red (totipotent), orange (pluripotent), green (multipotent) and dark blue (singlepotent). The four dark blue marbles depict four differentiated cell states $S_A$ – $S_D$. Normal differentiation is illustrated by black arrows. Indirect transdifferentiation involves a dedifferentiation step (blue arrows) requiring differentiated cells reverting back to a pluripotent state before undergoing a process of redifferentiation (black arrows). Direct transdifferentiation does not require cells to undergo dedifferentiation but potentially transit through an explicit intermediate stable state $S_0$ (light blue marble) as proposed by Xu et al. (2014).

Examples of transdifferentiation in plant biology

In vitro transdifferentiation of tracheary elements

The Zinnia elegans system

The best studied example of transdifferentiation is that of isolated mesophyll cells from Zinnia elegans leaves which transdifferentiate in culture to form TEs (Fukuda and Komamine 1980a 1980b). This transdifferentiation process occurs in the absence of cell division, hence has been referred to as “direct transdifferentiation”, first by the animal biologist Beresford (1990), and subsequently by Sugiyama and Komamine (1990) and others in the plant literature (McManus et al. 1998, Reusche et al. 2012). However, transdifferentiation in the Zinnia system requires a dedifferentiation step in which cells revert to a pluripotent procambial-like state (Fukuda 1996 1997);
therefore, according to the use of the term as described above, we propose that “indirect transdifferentiation” is a more appropriate term to describe this phenomenon.

The in vitro system for TE transdifferentiation in Zinnia was first established as an effective experimental system with high transdifferentiation frequency by Fukuda and Komamine (1980a) and has been used widely by various laboratories with minor modifications. Single mesophyll cells of Zinnia can be mechanically isolated by macerating pieces of surface-sterilized leaves. After extensive washing, the isolated cells are placed in a culture medium in the presence of auxin and cytokinin, and by three to four days culture, while some cells transdifferentiate to become xylem parenchyma, 30% of the population transdifferentiate to form TEs as defined by characteristic secondary wall thickenings (Figure 2A) (Fukuda and Komamine 1980a). Early work on this system reported differential requirements for the hormones, with Church and Galston (1988) showing that auxin was required for the first 56 h of culture but cytokinin for only the first 24 h, whereas Fukuda and Komamine (1985) demonstrated that auxin and cytokinin were not required at all in the first 12 h of culture. Milioni et al. (2001) conducted a detailed time-course study of this response and made the remarkable discovery that the isolated mesophyll cells responded maximally to added auxin and cytokinin across a window from 46 to 50 h of culture, and within this window the hormones need only be present for 10 min to achieve a maximum response of 50% of the cells transdifferentiating to form TEs.

Further experimentation established that the transdifferentiation process occurred across three discrete stages. Figure 2B depicts these three stages on the Waddington’s landscape in terms of dedifferentiation (Stage I), restriction of developmental potential (Stage II), and specific TE development (Stage III) (Fukuda 1996 1997 2004). Stage I can be regarded as a preparation stage to establish the competency of mesophyll cells becoming responsive to inductive signals to undergo redifferentiation (stages II and III). Mesophyll cells possess a highly differentiated phenotype with a large central vacuole and numerous lens-shaped chloroplasts occupying most parts of the cytoplasm and functioning in photosynthesis. These
Figure 2 Transdifferentiation of tracheary elements from isolated mesophyll cells in the *Zinnia elegans* culture system. (A) Morphological changes of transdifferentiating mesophyll cells when cultured in semisolid medium. The same two mesophyll cells were monitored over time, with secondary wall deposition clearly seen by 4 days culture. Remarkably, one cell transdifferentiated into a protoxylem-like TE (PX), whereas the other into a metaxylem-like TE (MX). The culturing in semisolid medium established that the transdifferentiation process occurred without cell division. Scale bars = 10 µm. Figure reproduced from Beňová-Kákošsova et al. (2006) with permission. (B) TE transdifferentiation from isolated mesophyll cells is illustrated in the Waddington’s epigenetic landscape. Stage I corresponds to the dedifferentiation process, whereby isolated mesophyll cells adapt to a new environment in the culture medium, lose mesophyll identity and acquire pluripotent potential to progress further differentiation. During this reverse process, isolated mesophyll cells are proposed to transition through intermediate cell states, including wound-activated cell (WAC) and/or dedifferentiated cell (DD) states (Fukuda 2004). The transition from Stage I to Stage II is marked by the addition of auxin and cytokinin to the culture medium, which can be as little as a 10-min window at 48 h culture (Milioni et al. 2001, 2002), inducing DD cells to commit to a procambial-like cell (PC) state. Stages II and III can be referred to as a redifferentiation process, where PC can differentiate to form a xylem precursor-like cell (pXC) state, which then differentiates to form a tracheary element (TE), or less commonly xylem parenchyma cell (XP). In other culture systems, different cell types such as epidermal cells (EP) can transdifferentiate to become TEs.
morphological and functional specifications remain stable during the lifespan of the cells *in planta*, hence in the Waddington’s landscape (Figure 2B) mesophyll cells metaphorically occupy a valley comparable to one of the four valleys representing four differentiated cell states (SA to SD) illustrated in Figure 1. In order to regain pluripotency mesophyll cells rely upon a sufficient push to escape their differentiated valley and progressively climb up the Waddington’s landscape. Once directed to follow the correct reverse paths (dashed arrows, Figure 2B) to reach a less-differentiated state, these mesophyll cells, now regarded as dedifferentiated cells (orange marble DD, Figure 2B), are competent to respond to the inductive signals auxin and cytokinin in the culture medium to further progress differentiation. The transition from Stage I to Stage II is determined by a combination of these signals, whereby dedifferentiated cells become re-specified to a new fate, in this case xylem cell fate, via a pluripotent procambial-like state (orange marble PC, Figure 2B). Stage II corresponds to the process of differentiation from procambial-like cells into xylem cell precursors (green marble pXC, Figure 2B), and Stage III involves dramatic changes in cell morphology such as deposition of patterned secondary cell walls and the autolysis of cell content, ultimately resulting in xylem cell precursors becoming dead TEs characterized by hollow water-conducting tubes (blue marble TE, Figure 2B). Collectively, stages II and III resemble the process of *in planta* xylem cell fate determination, in which procambial cells, differentiating from the meristem via procambial initials, metaphorically roll down the valley in the Waddington’s landscape, gradually lose their pluripotency and finally reach the zone of single potency of xylem parenchyma (XP) cells or tracheary elements (TE) (Figure 2B). In this example, meristematic stem cells are represented by the red marble sitting at the top of the Waddington’s landscape, and procambial initials are not shown.

If one is satisfied with the Waddington’s landscape as a depiction of TE transdifferentiation, then Stage I can be interpreted as being associated with a gravity-defeating push which directs mesophyll cells to move higher up the landscape, in a process known as dedifferentiation. Very little is known about the molecular events constituting this “push”, despite the fact that cell dedifferentiation in general has long
attracted interest from both animal and plant biologists. It is believed that this process is a prerequisite for the whole transdifferentiation process to occur (Fukuda 1997, McCann 1997) but seems not to be specific to the step of TE cell fate determination (Fukuda 1994). Indeed, auxin and cytokinin, which are absolute requirements for dedifferentiated cells restricting their potency of differentiation to a TE cell fate in the Zinnia system, are not required in the first 12 h (Fukuda and Komamine 1985) or more precisely the first 48 h (Milioni et al. 2001) of culture. Milioni and colleagues (2001) have proposed that the first 48 h correspond to a developmental course in which mesophyll cells adapt to a new environment and acquire competency to respond to inductive signals. They found that a narrow window of 10 minutes of exposure of auxin and cytokinin at 48 h is both necessary and sufficient to induce TE differentiation by 96 h. Furthermore, by adding these two growth hormones at 48 h instead of 0 h of culture, they improved the synchrony of the Zinnia system significantly (Milioni et al. 2001). These findings suggest different levels of competency in different mesophyll cells undergoing the dedifferentiation process. This situation can be interpreted based on the Waddington’s landscape: it is critical for dedifferentiating mesophyll cells to reach a certain height on the landscape, here illustrated by the position of a dedifferentiated cell (DD - orange marble, Figure 2B), to be competent to respond to auxin and cytokinin. Any cell states below this threshold height are not sufficiently competent to respond to these signals. Different isolated mesophyll cells in the culture may climb the landscape at different rates, or may start this process later than neighbouring cells, which collectively may account for the relatively asynchronous TE formation if inductive signals are present at the start of the culture. By 48 h, most mesophyll cells in the culture become fully competent. In an induction-free medium, all cells that can attain competency have done so by 48 h, and thus subsequent exposure to inductive signals induces this population to collectively commit to a new fate TE fate. Delaying the addition of auxin and cytokinin for more than 48 h reduced the final number of TEs, although a small number of cells remain competent for TE formation at even 90 h of culture (Milioni et al. 2001). This observation suggests that cells may be able to hold the dedifferentiated state for only a defined period of time,
after which the “gravity” force pulls these cells down the Waddington’s landscape, probably to the valleys of apoptosis if no hormone(s) is added.

Interpretation of transdifferentiation based on this uphill-climbing model may raise a question about the nature of the gravity-defeating push, which bought about the dedifferentiated state of mesophyll cells in Stage I. Wounding, auxin and cytokinin are the three factors involved in initiating the transdifferentiation of TEs from mesophyll cells in the Zinnia system (Fukuda 1997, McCann 1997). The work done by Milioni et al. (2001), as discussed above, plus other evidence (Fukuda and Komamine 1985), seems to exclude the participation of auxin and cytokinin in early events associated with cell adapting to new environments and acquiring competency, hence pointing to wounding as potentially the only factor controlling dedifferentiation in this system. However, this hypothesis has not been investigated further at the molecular level, partially due to the fact that the redifferentiation process, corresponding to stages II and III, has attached more interest compared to dedifferentiation occurring during Stage I. Indeed Milioni and colleagues took advantage of their highly synchronous Zinnia system to perform large-scale expression analysis based on a cDNA-amplified fragment length polymorphism approach, however, just focusing on the time points at 48 h onward and overlooking the dedifferentiation process presumably occurring before the addition of auxin and cytokinin (Milioni et al. 2002). The only molecular work published on Stage I was done by Demura et al. (2002), where a comprehensive analysis using a large cDNA array with more than 8000 Zinnia cDNA clones was performed to examine transcriptional profiles throughout the transdifferentiation process. This study revealed two main groups of events during Stage I; wound-induced events and those of down-regulation of photosynthetic activity. Wound-induced events were believed to occur during the 1 h preparation of isolated mesophyll cells and to be induced by wound stress. These events are potentially associated with genes encoding six protein kinases and three transcription factors (Demura et al. 2002). Down-regulation of photosynthetic activity is evidenced by the down-regulation of 29 out of 33 cDNAs potentially encoding plastid ribosomal proteins and chlorophyll a/b-binding proteins (Demura et al. 2002), which presumably
accounts for mesophyll cells losing the ability to perform photosynthesis (Fukuda 1997). In this culture system, however, unlike the system used by Milioni and colleagues (2001, 2002), auxin and cytokinin were administered to isolated mesophyll cells at the beginning of culture, hence the influence of these hormones was not necessarily separated from events occurring during Stage I. Therefore, it still remains unclear if the down-regulation of photosynthetic genes, among other early events associated with cells adapting to a new environment and acquiring competency, is attributable solely to wounding or the combination of wounding, auxin and cytokinin. Further analysis of gene expression focusing on Stage I (from 0 to 48 h of culture without the addition of auxin and cytokinin; Milioni et al. 2001 2002) may shed light on the dedifferentiation process, of which understanding at the molecular level is still in its infancy.

Although it has been unequivocally established that cell division or replication of DNA in the S-phase of the cell cycle is not a prerequisite for the dedifferentiation step of TE transdifferentiation in the *Zinnia* system (Fukuda and Komamine 1980b 1981a), some minor amount of DNA synthesis is essential for the sequence of transdifferentiation (Fukuda and Komamine 1981b; Sugiyama and Komamine 1990). This minor DNA synthesis was found to be DNA-repair events including repair-type synthesis of DNA, the synthesis of poly(ADP-ribose) and the rejoining of DNA strand breaks (Sugiyama et al. 1995, Shoji et al. 1997). This conclusion was further supported by the fact that culturing *Zinnia* mesophyll cells with PhT, an inhibitor of repair-type DNA synthesis, inhibited TE transdifferentiation via preventing the expression of TED (tracheary element differentiation) genes in Stage II (Demura and Fukuda 1993).

While various events occurring during the dedifferentiation process (Stage I) seem not to be specific to TE transdifferentiation, the transition from Stage I to Stage II can be regarded as the first specific step of TE cell fate determination. This transition presumably corresponds to the 10-minute window of auxin and cytokinin exposure in the system developed by Milioni et al. (2001, 2002) as discussed above (Figure 2B). Withdrawal of auxin and cytokinin from the culture medium at 48 h after 10 minutes exposure does not affect the later processes of transdifferentiation (Milioni et al. 2001,
This is strong evidence of a true transdifferentiation as it is consistent with observations of transdifferentiation in animals; for example, in Caenorhabditis elegans, brief expression of a single transcription factor can induce stable transdifferentiation of intestinal cells from fully differentiated pharynx cells (Riddle et al. 2013). This is an important characteristic of transdifferentiation, namely the phenotype of newly differentiated cells must remain stable (Okada 1991). This observation is also another testament to the excellence of the highly synchronous Zinnia system developed by the McCann group. It is also reasonable to envisage that, if another hormone or sets of hormones other than auxin and cytokinin are added at this specific window, mesophyll cells may be diverted to a different developmental pathway such as xylem parenchyma cells or even phloem sieve elements.

Stages II and III mimic in planta xylogenesis (Fukuda 2004) and thus have attracted greater interest from plant biologists. Thus, molecular events occurring in these two stages have been more thoroughly investigated compared to Stage I. Stage II corresponds to the in vivo differentiation from procambial initials to xylem cell precursors and is featured by the accumulation of TED2, TED3 and TED4 transcripts - novel markers for early stages of vascular differentiation (Demura and Fukuda 1993 1994, Fukuda 1994). In situ hybridization revealed that these three transcripts accumulate in cells involved in vascular differentiation in intact plants. TED2 transcripts, which encode a probable quinone oxidoreductase, were restricted to immature phloem and immature xylem cells and procambial cells of roots, whereas TED3, which codes a putative cell wall protein (Demura and Fukuda 1994), and TED4 which encodes a lipid transfer protein, were expressed mainly in TE precursor cells and immature xylem cells, respectively. Both in situ and in vitro analyses confirmed that TED transcripts were expressed sequentially, namely TED2, TED4 and then TED3 (Demura and Fukuda 1994). These markers were also identified in the cDNA-AFLP (Milioni et al. 2002) and microarray (Demura et al. 2002) analyses of gene expression programs during TE transdifferentiation in Zinnia. These two comprehensive studies also revealed several genes involved in auxin signalling were expressed during Stage 2 (Demura et al. 2002), or within 30 minutes of the addition of auxin and cytokinin
A NAC transcription factor was also upregulated within 30 minutes (Milioni et al. 2002).

Much later after the expression of TED transcripts, dramatic morphological changes including secondary wall formation and autolysis occur in transdifferentiating cells, indicating the commencement of Stage III. As noted by Demura et al. (2002), early Stage III gene expression is characterised by a large number of genes associated with secondary wall formation such as CesA and EGase genes as well as primary wall degradation including genes encoding O-glucosyl hydrolases. Later in Stage III, genes involved in lignin biosynthesis such as laccases- and peroxidases-genes were differentially expressed. This lignification process is believed to proceed after cell death, when autolysis is completed (Demura et al. 2002; Turner et al. 2007). Programmed cell death-specific genes, such as genes encoding cysteine proteases, serine proteases, aspartic peptidase and lipolytic acyl hydrolase, are differentially expressed during Stage III (Demura et al. 2002).

**The Arabidopsis thaliana system**

To take advantage of the wealth of publicly accessible molecular resources of the model plant Arabidopsis thaliana, various Arabidopsis xylogenic culture systems have been established. When Arabidopsis suspension cells were cultured with auxin, cytokinin, brassinosteroid and high concentration of boric acid, around 50% of cells transdifferentiated to form TEs after 7 days culture (Kubo et al. 2005). Utilizing subsequent GeneChip microarrays these workers identified a family of seven NAC transcription factors, VND1-7, which were upregulated during TE transdifferentiation. Among these genes, VND6 and VND7 were identified as master switches for the formation of metaxylem and protoxylem vessels, respectively (Kubo et al. 2005). Overexpression of either VND6 or VND7 causes ectopic TE development (Kubo et al. 2005; Yamaguchi et al. 2010). To examine microtubule dynamics during TE formation, Oda et al. (2005) developed a culture system using transformable Arabidopsis cell suspension cells. After 96 h culture approximately 30% of suspension cells of the AC-GT13 line (expressing a green fluorescent protein-tubulin fusion protein) were induced to form TEs by removal of auxin from and addition of brassinosteroid to the culture.
media (Oda et al. 2005). TEs derived from these cells were morphologically indistinguishable from those derived from non-transformed Col-0 cells, and also had distinct patterns of secondary wall deposition similar to those of mesophyll cell-derived TEs in the *Zinnia* system (Oda et al. 2005). Instead of hormones, master-switch transcription factors can be used as effective triggers to induce TE transdifferentiation (Oda et al. 2010), presumably involving direct transdifferentiation because no up-regulation of procambial-related genes was detected (Kondo et al. 2015).

Very recently a novel system for TE transdifferentiation using *Arabidopsis* leaf disks has been described (Kondo et al. 2015). In this system, bikinin was used to promote xylem cell differentiation via its inhibitive effects on glycogen synthase kinase 3 proteins, the central regulators for differentiation of procambial cells into xylem cells (Kondo et al. 2014). Leaf disks of *Arabidopsis* rosette leaves were cultured with auxin and cytokinin in the presence of bikinin. Three days after culture a large number of mesophyll cells transdifferentiated to form TEs in a bikinin dose-dependent manner (Kondo et al. 2015). This cell-type switching occurred via a dedifferentiation step to form procambial cells before converting to xylem cells, hence, according to our criteria described above, is an indirect transdifferentiation process. Bikinin was found to accelerate the formation of procambial cells, however, the underlying mechanism is unknown (Kondo et al. 2015). The advantages of this leaf-disk culture, as stated by the authors, are its synchronous differentiation of both procambial cells and TEs suitable for exploring the whole process of xylem transdifferentiation, its utilization of various *Arabidopsis* mutants and *Arabidopsis* transgenic lines harbouring vascular cell markers enabling spatiotemporal monitoring of cell fate switching, and its applicability to different species besides *Arabidopsis*. Such advantages promise a greater understanding of development of vascular tissues, particularly procambial cell formation and xylem cell differentiation.

**Transdifferentiation of transfer cells**

Transfer cells (TCs) represent a particularly striking and rare case of transdifferentiation associated with programmed development. TCs are anatomically specialised to achieve enhanced rates of nutrient transport across
apoplastic/symplasmic bottlenecks throughout the nutrient acquisition, distribution and utilization pathways in plants. TCs develop extensive wall ingrowths (McCurdy et al. 2008, McCurdy 2015) which provide a scaffold to increase plasma membrane surface area and thus enable enhanced densities of nutrient transporters to facilitate their capacity for enhanced rates of solute exchange (Offler et al. 2003). TCs also develop localised densities of mitochondria and organelles of the endomembrane system to accommodate increased energy demands to drive nutrient transport and vesicle secretion for wall ingrowth building, respectively (Gunning and Pate 1969, Pate and Gunning 1972). Numerous differentiated cell types, such as companion cells, xylem and phloem parenchyma cells, root cortical cells and epidermal cells can develop TC characteristics (Pate and Gunning 1972). This process is considered to be an example of transdifferentiation, whereby a given cell type, for example a phloem parenchyma cell, transdifferentiates to become a phloem parenchyma TC (Offler et al. 2003). It is important to clarify, however, that not all TCs originate via a transdifferentiation process. TCs in the basal endosperm of cereal seeds, such as barley, are a component of the triploid endosperm, and develop as part of a cell fate specification pathway during endosperm cellularization (Thiel 2014), a process which is distinct from transdifferentiation. Also, giant cells and syncytia resulting from nematode infection of roots have been described as TC-like due to the presence of wall ingrowth-like structures (Jones and Northcote 1972a 1972b). In this case, however, these cells form from undifferentiated procambial cells and pericycle, and involve the fusion of many cells into a single, larger giant cell or syncytia, a process which seems to be quite different from transdifferentiation of a single cell from one type to another of different function.

Two prominent examples of the transdifferentiation of TCs in plant biology are considered here. Abaxial epidermal cells of *Vicia faba* cotyledons transdifferentiate to become epidermal TCs and thus facilitate nutrient transport across the maternal/filial interface of developing seeds (Harrington et al. 1997). Importantly from an experimental perspective, adaxial epidermal cells, which normally do not form TCs *in planta*, can be induced to transdifferentiate into adaxial epidermal TCs by placing
isolated cotyledons adaxial-surface down on culture medium (Farley et al. 2000, Offler et al. 2003). This process induces a semi-synchronous development of a reticulate wall ingrowth network (Figure 3) and ultimately TC functionality (Farley et al. 2000). This experimental induction of adaxial epidermal TCs has been used extensively to study the morphology of reticulate wall ingrowth deposition (Talbot et al. 2001 2002 2007), signalling pathways that induce wall ingrowth deposition (Zhou et al. 2010, Andriunas et al. 2010 2011) and transcriptional changes associated with this process (Dibley et al. 2009, Zhang et al. 2015). Upon transfer of cotyledons to culture, cell division is transiently induced in about 10% of the adaxial epidermal cells (Dibley et al. 2009, Zhang et al. 2015), but nearly 90% of all adaxial epidermal cells go on to form a TC morphology by 24 h of culture (Wardini et al. 2007). This observation indicates that cell division is not a mandatory step in this example of transdifferentiation, and questions whether dedifferentiation to a mitotic state is a prerequisite event (Dibley et al. 2009). Epidermal-specific changes in expression of numerous transcription factors occurs across TC transdifferentiation (Dibley et al. 2009; Zhang et al. 2015), including genes associated with chromatin remodelling (Dibley et al. 2009), but definitive molecular evidence for dedifferentiation is absent. Consequently, the transdifferentiation of epidermal TCs in *V. faba* cotyledons may represent a case of direct transdifferentiation not involving cell division.

Phloem parenchyma cells also transdifferentiate into phloem parenchyma TCs in veins of *Arabidopsis* leaves (Haritatos et al. 2000, Amiard et al. 2007) and cotyledons (Nguyen and McCurdy 2015). Wall ingrowths in phloem parenchyma cells are deposited across the region of cell wall abutting adjacent cells of the sieve element/companion cell (SE/CC) complex (Figure 3). These phloem parenchyma TCs are proposed to be involved in a two-step phloem loading strategy whereby sucrose delivered by an apoplastic pathway from mesophyll cells is unloaded into the apoplasm by AtSWEET11/12 uniporters located on the plasma membrane of the phloem parenchyma TCs. This apoplastic sucrose is subsequently uploaded into the SE/CC complex via companion cell-localised AtSUC2 (Chen et al. 2012). The highly localized deposition of wall ingrowths to the face of phloem parenchyma cells directly
Figure 3 Transdifferentiation of transfer cells. (A, B) Scanning electron microscopy views of the cytoplasmic face of adaxial epidermal cells of cultured *Vicia faba* cotyledons. The cytoplasmic face of the outer periclinal wall of epidermal cells of cotyledons was revealed by a dry-cleaving procedure (Talbot et al. 2001). (A) Adaxial epidermal cells at 0 h culture. In normal development of the cotyledons, adaxial epidermal cells do not transdifferentiate into transfer cells, as evidenced by their smooth primary cell walls. Remnant anticlinal walls remaining after the dry-cleaving procedure reveal the outline of each epidermal cell. (B) Adaxial epidermal cells after 15 h culture undergoing transdifferentiation to form transfer cells, as evidenced by numerous individual papillate wall ingrowths (arrowheads) deposited on the cytoplasmic face of the outer periclinal primary wall. Note that the extent of wall ingrowth deposition varies in each epidermal cell, with one cell (asterisk) yet to initiate this process. S - starch grains. Scale bar = 20 µm. Images provided by Dr Mark Talbot. (C, D) Phloem parenchyma cells in *Arabidopsis* leaf minor veins. (C) In immature juvenile leaves phloem parenchyma (PP) cells in vascular bundles have not developed wall ingrowths. (D) In mature juvenile leaves phloem parenchyma cells transdifferentiate into TCs, as evidenced by the polarised deposition of an extensive layer of reticulate wall ingrowths (arrowheads) along the face of PP TCs adjacent to cells of the SE/CC complex. PP - phloem parenchyma; BSC - bundle sheath cell; CC - companion cell; TC - transfer cell; SE - sieve element. Scale bar = 5 µm.

neighbouring cells of the SE/CC complex is proposed to spatially restrict the unloading of apoplastic sucrose to sites immediately adjacent to the SE/CC complex, thus minimizing access to this sucrose by pathogens (Chen et al. 2012). In addition to this role in facilitating phloem loading, Amiard et al. (2007) have proposed that the wall ingrowths may provide a physical barrier to pathogen entry into SEs. This conclusion is based in part from the observation that wall ingrowth deposition in phloem parenchyma TCs is enhanced in response to stresses involving jasmonic acid signalling (Amiard et al. 2007). However, while it appears that wall ingrowth deposition in
phloem parenchyma TCs can be enhanced in response to stress hormones, the signals that cause induction of the transdifferentiation process itself are not known. No evidence is available suggesting that a preceding step of cell division of phloem parenchyma occurs prior to the formation of wall ingrowths, therefore, similar to the epidermal cells of *V. faba* cotyledons, this transdifferentiation process may result from direct transdifferentiation without cell division.

**Transdifferentiation in regeneration**

Transdifferentiation occurring as part of regeneration of plant tissues represents a common example of transdifferentiation in plants. The disturbance of the microenvironment of cells is a critical trigger for transdifferentiation to occur (Okada 1991). Sources of this disturbance may come from mechanical injury or attack by pathogens, potentially providing positional signals to convert existing differentiated cells to form new cell types to compensate for loss of functional cells. Investigation at the molecular level of transdifferentiation associated with regeneration, however, is difficult due to the highly asynchronous nature of regeneration. Unlike isolated cell culture systems (*Zinnia, Arabidopsis*) or culture of *V. faba* cotyledons or *Arabidopsis* leaf disks, in which reasonably synchronous transdifferentiation of a single cell type occurs, transdifferentiation associated with regeneration may involve various cell types, often occurring in a sequential manner and thus compromising the interpretation of molecular events associated with the transdifferentiation process specific to each cell type. Therefore, current understanding of molecular pathways regulating transdifferentiation occurring during regeneration is limited.

Since vascular strand continuity is essential for plant development, plants employ several strategies including transdifferentiation to re-establish this continuity when it is interrupted by various disturbances. Consequently, transdifferentiation of vascular cells from various cell types is the most frequently studied example of transdifferentiation in regeneration. Transdifferentiation of vascular cells, particularly of primary vascular tissues, has a long history of investigation in a variety of species (although not always being referred to as a transdifferentiation process in the early literature; see Historical Review section). When the primary vascular tissue in a stem
or petiole is severed, for example by a wound, new xylem and phloem cells are generated by transdifferentiation of existing parenchyma cells, bridging new vascular tissue with old to overcome the discontinuity. Presumably the molecular mechanisms of wound-induced transdifferentiation of vascular cells, *in vitro* transdifferentiation of TEs and *in planta* differentiation of TEs during normal vascular development, are similar if not identical. Indeed the processes of transdifferentiation of TEs from pith parenchyma cells induced *in situ* by vascular bundle interruption in *Zinnia* internodes (Nishitani et al. 2002) and from isolated mesophyll cells in suspension culture (Demura and Fukuda 1993), both involve the expression of TED3, a molecular marker of TE precursor cells in *Zinnia* (Demura and Fukuda 1994). When vascular strands of the first internodes of *Zinnia* seedlings are injured with a blade, transcripts of TED3 accumulate in pith parenchyma cells surrounding the wound site 48 h after wounding (Nishitani et al. 2002). Similarly, TED3 transcripts accumulated by 36 h culture of isolated mesophyll cells (Demura and Fukuda 1993). Nishitani et al. (2002) also reported that transcripts of ZeHB3 (*Zinnia elegans* homeobox gene 3), a molecular marker of early stages of phloem differentiation in intact plants (Nishitani et al. 2001), increased by 36 h post-wounding in immature phloem layers in and between severed vascular bundles (Nishitani et al. 2002). It is interesting to note that, in severed *Zinnia* internodes, wound-induced transdifferentiation of pith parenchyma cells into TE precursors, akin to the *in vitro* TE transdifferentiation from isolated mesophyll cells, does not involve the intervention of cell division, whereas wound-induced transdifferentiation of pith parenchyma cells into immature phloem cells requires cell division (Nishitani et al. 2002). Whether a dedifferentiation step is a prerequisite of these transdifferentiation processes is currently unknown.

Transdifferentiation of vascular cells has also been observed in secondary vascular tissue during bark regeneration. When bark of *Eucommia ulmoides* Oliv. and many other species is removed at a suitable developmental stage of the tree, new bark is rebuilt within 1-2 months (Pang et al. 2008, Zhang et al. 2011, Chen et al. 2014). During this regeneration of secondary vascular tissue in *E. ulmoides*, immature xylem cells in several cell surface layers formed callus at six days after girdling (DAG),
whereas immature xylem cells in layers beneath these surface layers transdifferentiated to form phloem cells including SEs and CCs at 12 DAG, and cells in the deeper layers of immature xylem cells dedifferentiated into cambium cells at 2-3 weeks after girdling (Pang et al. 2008). Similar processes were also observed during bark regeneration upon girdling in *Populus tomentose* (Zhang et al. 2011). Although the transdifferentiation of phloem cells from xylem cells in both *E. ulmoides* and *P. tomentose* is preceded by cell division, it is unclear if re-entry into the cell cycle is a prerequisite of this process. It has been proposed that, in *P. tomentose*, epigenetic regulation and cell cycle re-entry may play important roles in the switching of xylem cell fate into phloem or cambium cell fate, evidenced by the significant changes in gene expression of DNA methyltransferases, histone methyltransferases, chromatin remodelling-related proteins, polycombs (PcG) proteins, type A and type B cyclins (CYCs, CYCBs), cyclin-dependent kinases (CDKs) and CDK-like (CKL) genes (Zhang et al. 2011). The authors also reported down-regulation of xylem-specific genes as well as an activation of phloem and cambium development during bark regeneration (Zhang et al. 2011). It is not easy, however, to separate differential gene expression associated with the transdifferentiation process of phloem cells from that of the dedifferentiation of cambial cells, whereby progenitor cells of both examples are differentiating xylem cells left on the trunk after bark girdling. Mimicking these processes under more controlled laboratory conditions may provide a means to investigate the transdifferentiation process with higher resolution. An *in vitro* system of secondary vascular tissue regeneration in poplar has recently been established (unpublished data in Chen et al. 2014), promising a leap in our understanding of secondary vascular development, particularly in regeneration, in the near future.

In addition to physical assaults, pathogen attacks also induce transdifferentiation of vascular cells to substitute infected and non-functional vascular cells. A striking example of this scenario is in leaves of *Arabidopsis* seedlings following inoculation with *Verticillium longisporum* whereby chloroplast-containing bundle sheath cells transdifferentiate to form TEs characterized by secondary cell wall modifications (Figure 4) (Reusche et al. 2012). Loss of chloroplasts in bundle sheath cells surrounding
Figure 4 Transdifferentiation of tracheary elements from bundle sheath cells in Arabidopsis leaf veins upon Verticillium infection. (A, C) Vascular bundles in leaves of non-infected plants. (B, D) Vascular bundles in leaves of Verticillium-infected plants. (A, B) Bright-field images of leaf vein. Leaves were stained with trypan blue. (A) Leaf vein of non-infected plant showing clear bundle sheath cells (bsc) at the periphery of vascular bundles. (B) Leaf vein of infected plant at 21 days after infection showing bundle sheath cells transdifferentiated into protoxylem-like (pxl) and metaxylem-like (mxl) cells. (C, D) Epifluorescence images of leaf vein of non-infected plant (C) and leaf vein of infected plant at 21 days after infection (D). Arrowheads in (D) show de novo-formed TEs. bsc - bundle sheath cell; mxl - metaxylem-like; pxl - protoxylem-like. Scale bars = 50 µm for A and B, and 25 µm for C and D. Images reproduced from Reusche et al. (2012) with permission.

the vascular bundles in leaves results in a phenomenon known as vein clearing (Fradin and Thomma 2006), among other above-ground symptoms of infected plants including stunted growth, loss of fresh weight and chloroses (Reusche et al. 2012). Verticillium-infections frequently lead to wilting (Fradin and Thomma 2006); however, that is not the case for the invasion of V. longisporum in its Brassicaceae hosts including Brassica napus (Floerl et al. 2008) and Arabidopsis (Reusche et al. 2012). In these species, the infection of V. longisporum brought about enhanced drought tolerance of infected plants, which was attributed to de novo xylem formation resulting from the transdifferentiation of bundles sheath cells and xylem parenchyma cells and the
reinitiation of cambial activity (Reusche et al. 2012). Histological analysis of bundle sheath cell-derived TEs revealed that some of these elements featured annular thickenings typical of protoxylem vessels while others exhibited reticulate thickenings typical of metaxylem vessels. Consistent with the finding that VND6 and VND7, two NAC-domain transcription factors, are necessary and sufficient to induce transdifferentiation of various types of cells into metaxylem- and protoxylem-like vessel elements, respectively (Kubo et al. 2005), Reusche et al. (2012) showed that expression levels of VND6 and VND7 and their positive feedback loop regulators ASL19 and ASL20, were significantly induced in infected plants at 14 day after inoculation. Moreover, in recently transdifferentiated bundle sheath cells, enhanced transcriptional activity of VND7 in response to V. longisporum infection was also observed (Reusche et al. 2012). It is interesting to note that in this system the transdifferentiation occurred exclusively in bundle sheath cells located on the adaxial side (abutting xylem) but not on the abaxial side (abutting phloem) of vascular bundles of leaves, and that this transdifferentiation was not caused by in situ fungal colonization. These observations collectively suggest that the cue triggering this transdifferentiation may be derived from V. longisporum-colonized proximal vascular bundles (Reusche et al. 2012), transported through the transpiration stream and released into the vicinity of adaxial/xylem-associated bundle sheath cells.

The transdifferentiation of TEs from bundle sheath cells occurred without preceding cell division, and thus Reusche et al. (2012) considered this to be an example of direct transdifferentiation. However, as discussed previously, we define direct transdifferentiation as a process occurring without dedifferentiation; a process that was not investigated in the Reusche et al. (2012) study. In the Zinnia system the transdifferentiation of TEs from isolated mesophyll cells occurred via a dedifferentiation step where mesophyll cells reverted back to a pluripotent procambial-like stage before redifferentiating into TEs (Demura and Fukuda 1993, Fukuda 1997). The formation of procambial cells was also demonstrated as an intervening step during the transdifferentiation of mesophyll cells into TEs in the Arabidopsis leaf-disk culture system (Kondo et al. 2015). Similar phenomenon may
occur during the conversion from bundle sheath cells into TEs, in which pathogen-induced senescence may allow cells to undergo dedifferentiation (Grafi et al. 2011a; Reusche et al. 2012). Alternatively, Reusche et al. (2012) also discussed the possibility that certain perivascular totipotent stem cells (Sugimoto et al. 2010 2011) may have a positional and developmental relationship to the bundle sheath cells which transdifferentiated into TEs (Reusche et al. 2012).

**Conclusions**

Transdifferentiation is the remarkable ability of both plant and animal cells to undergo an irreversible switch from one cell type into another. Due to the long recognised capacity for developmental plasticity in plants, transdifferentiation has not received as much attention as that afforded in animal systems. Indeed, the first use of the term in plant science was by the animal reproductive biologist W.A. Beresford (1990), describing the transdifferentiation of TEs in the *Zinnia* mesophyll cell culture system, a phenomenon first described by Kohlenbach and Schmidt in 1975. Four decades later this remains the most extensively studied example of transdifferentiation at the morphological, biochemical and molecular levels in plant biology. Plant biologists have used the term transdifferentiation somewhat glibly to describe often different phenomena. We propose that transdifferentiation is best described as either indirect, involving dedifferentiation back to a progenitor cell state, or indirect, not involving dedifferentiation, or at least not to a progenitor cell state. Both direct and indirect transdifferentiation can occur with or without cell division. Examples of transdifferentiation in the plant literature mostly involve the formation of cells of the vascular system, in particular TEs. Presumably this reflects the importance of water transport in vascular plants, and the capacity of this system for regeneration after wounding. The *in vitro* transdifferentiation of TEs in both the *Zinnia* system and more recently in *Arabidopsis*, has been extremely valuable in identifying molecular switches responsible for TE differentiation *in planta*. More emphasis is now needed to understand at the molecular level the reverse step of transdifferentiation, namely dedifferentiation and potential re-entry into the cell cycle. Given the new ideas now
emerging regarding dedifferentiation associated with in vitro reprogramming (Rose 2016 Chapter 18), it will be interesting to compare at the molecular level partial dedifferentiation associated with reversion to a pluripotent state (transdifferentiation) (Srivastava 2002), compared to complete dedifferentiation into callus tissue as can occur during a process such as somatic embryogenesis.

References


Dalessandro, G. and L.W. Roberts. 1971. Induction of xylogenesis in pith parenchyma explants


Appendix I


Appendix III


Appendix III
