REGULATED PROTEIN TRAFFIC ACROSS TISSUE BOUNDARIES THROUGH PLASMODESMATA

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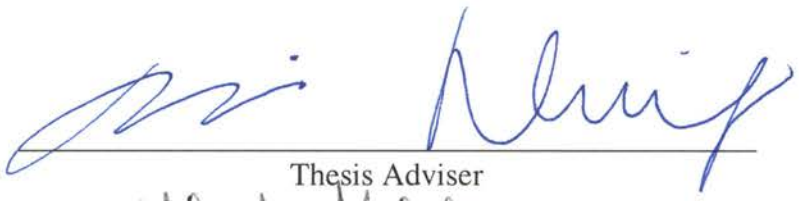

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TABLE OF CONTENTS

Chapter Page

LIST OF FIGURES

Figure Page

Chapter IV

ABBREVIATIONS

AtSUC2	Arabidopsis sucrose transporter 2
CC	Companion cell
CLSM	Confocal laser scanning microscope
CMV	Cucumber mosaic virus
CoYMV	Commelina yellow mottle virus
EP	Epidermis
GFP	Green fluorescent protein
GUS	β-Glucuronidase
MC	Mesophyll cell
MP	Movement protein
MS salts	Murashige & Skoog salts
PD	Plasmodesmata/Plasmodesma
PP	Phloem parenchyma
RBCS	RuBisco small subunit
RCNMV	Red clover necrotic mosaic virus
TMV	Tobacco mosaic virus
SE	Sieve element
SEL	Size exclusion limit

CHAPTER I

INTRODUCTION

Intercellular communication in plants

Plants are multi-cellular organisms and intercellular communication is important for growth and development. Plant cells possess two different kinds of systems for intercellular communication, either apoplastically or symplastically. The apoplastic communication has been studied in detail on auxin transport (Muday and Delong, 2001) and CLAVATA signaling pathway (Clark, 2001). Small molecules or proteins are produced in a cell and secreted to the apoplast through the cell walls and the plasma membrane. The signal molecules, the ligands, move through the apoplast and bind receptors on the plasma membrane of target cells. Upon binding, the signal will be transmitted to the nuclei of the target cells to regulate gene expression.

The other way of communication is symplasmic communication, which occurs through cytoplasmic connections called plasmodesmata (PD) (Ding 1998; Citovsky and Zambryski, 2000). PD transport various molecules for growth, differentiation and development. These molecules include H_20, amino acids, sugars, and hormones (Anderson, 1976; Goodwin, 1976; Gunning, 1976; Robards and Clarkson, 1976; van Steveninck, 1976; Drake and Carr, 1979; Sauter and Kloth, 1986; Kwiatkowska, 1991). Studies in the past decade have provided evidence that selected proteins and RNAs may also traffic intercellularly through PD to play roles in regulating development and plant-pathogen interactions (Reviewed in Ding 1998, Citovsky and Zambryski, 2000). This dissertation is aimed at understanding the regulation of macromolecular traffic through PD in plants.

Formation of plasmodesmata

Microscopic analysis provided insights about how PD are formed between plant cells. Basically, PD can be formed during cytokinesis and after cytokinesis. PD formed cytokinetically or post-cytokinetically are called primary PD and secondary PD, respectively.

During cytokinesis, at the newly developing cell plate, endoplasmic reticulum (ER) are entrapped and transformed into an appressed ER, which leads to the formation of primary PD (Hepler, 1982). The primary PD consists of the appressed ER in the center and spoke-like extensions that connect the appressed ER and plasma membrane to create microchannels (Ding et al., 1992). The primary PD are single-stranded and linear shape in appearance.

Secondary PD form post-cytokinetically across the established cell walls and can be simple or highly branched (Ding, 1998; Ehlers and Kollmann, 2001). Secondary PD can form from simple primary PD by changing into branched forms as a tissue matures (Ding and Lucas, 1996).

Regulation of PD transport

The size of microchannels in the primary PD is about 2.5 nm in diameter in tobacco (Ding et al., 1992). It is possible that certain transported materials with a diameter smaller than 2.5 nm pass through the microchannels by simple diffusion. The capacity of PD for intercellular trafficking as passive diffusion is referred to as the size exclusion limit (SEL). Utilizing microinjection of fluorescent probes of a range of sizes, the SEL of PD in several plant species is estimated to be about 0.8-1 kDa (Tucker, 1982; Barclay

and Peterson, 1982; Goodwin, 1983; Terry and Robards, 1987; Wolf et al., 1989), which may have a calculated diameter of 3 nm (Terry and Robards, 1987). The 27 kDa green fluorescent protein (GFP), which has a diameter of 2.4 nm and 4.2 nm in length (Ormo et al., 1996), can diffuse intercellularly in immature leaves and also in mature leaves of some plant species (Imlau et al., 1999; Oparka et al., 1999; Itaya et al., 2000, Crawford and Zambryski, 2000, 2001), indicating that SEL is higher in younger leaves and is also different among plant species. It should be noted that some lipids may pass through the appressed ER membranes instead of passing through the microchannels (Grabski et al., 1993).

Intercellular transport through PD, however, does not always occur by simple diffusion. Up-regulation/down-regulation of SEL of PD is often observed during physiological and developmental changes (Ding, 1998; Zambryski and Crawford, 2000). The traffic of macromolecules through PD is also regulated, which is discussed later.

Symplasmic domains

Dye-coupling experiment showed that a young plant body is a symplast, which means that all cells are connected by PD that allow free diffusion of small molecules (Lucas et al., 1993, McLean et al., 1997). Changes in the frequencies and structures of PD can lead to formation of symplasmic domains (Tucker, 1982; Erwee and Goodwin, 1983; Palevitz and Hepler, 1985). Small molecules can move within a symplasmic domain, but not between the domains. The shoot apical meristems in *Birch (Betula pubescence)* (Rinne and van der Schoot, 1998), shoot apex (Gisel et al., 1999) and root hair cells in Arabidopsis (Duckett et al., 1994), stomatal guard cells in onion (*Allium cepa*) (Palevitz

and Hepler, 1985), cotton fiber cells (Ruan et al., 2001) and developing floral meristems (Bergmans et al., 1993) are examples of symplasmic domains in plants. Sieve element (SE)-companion cell (CC) complex in the phloem is also a well-documented symplasmic domain, which was investigated in this dissertation.

The symplasmic domains are likely to play important roles in intercellular communication by changing the flow of signal molecules and nutrient transport (Lucas et al., 1993; Pflunger and Zambryski, 2001). Therefore, physiology and development of a plant are under the influence of symplasmic domains. Although formation of symplasmic domains is important, communication between the domains is also important for coordinated growth and/or development. How symplasmic domains communicate with each other is not known.

Transport of macromolecules

Studies on viral protein and nucleic acid transport initially opened the field of intercellular macromolecular traffic through PD in plants (Fujiwara et al., 1993; Noueiry et al., 1994; Ding et al., 1995). Recently, endogenous proteins including transcription factors have been reported to have the ability to move intercellularly and may possess important functions for development (Jackson et al., 1994; Lucas et al., 1995; Carpenter and Coen, 1995; Perbal et al., 1996; Sessions et al., 2000; Hake, 2001; Nakajima et al., 2001; Kim et al., 2002).

Viral movement proteins

Most plant viruses encode movement proteins (MPs) that facilitate intercellular movement of the viral genomes or viral particles. The 30 kDa MP of *Tobacco mosaic virus* (TMV) has been shown to increase the SEL of PD from 1 kDa to 10 kDa in mesophyll cells (MCs) of transgenic tobacco expressing the MP (Wolf et al., 1989) and was shown to be localized to PD (Tomenius et al., 1987; Moore et al., 1992; Ding et al., 1992). Microinjection of fluorescently labeled 35 kDa MP of *Red clover necrotic mosaic virus* (RCNMV) into a cowpea MC showed that the protein moved cell-to-cell within 3 minutes, which was the first direct evidence of intercellular protein traffic through PD (Fujiwara et al., 1993). The MP itself not only moved intercellularly but also helped the genomic RNA of the virus traffic through PD (Fujiwara et al., 1993). MPs of *Bean dwarf mosaic virus* (BDMV; Noueiry et al., 1994), *Cucumber mosaic virus* (CMV; Ding et al., 1995), and TMV (Waigmann et al., 1994; Waigmann and Zambryski, 1995; Nguyen et al., 1996) also traffic intercellularly. Because of the unique features of MPs with the ability of intercellular movement, viral MPs have been useful to study protein traffic in plants as will be described in this dissertation.

Plant transcription factors

Studies on intercellular trafficking of viral MPs have led to the discovery that endogenous plant proteins also traffic intercellularly. The first plant protein that was shown to traffic intercellularly was the 45 kDa homeobox protein called KNOTTED1 (KN1) from maize. Studies with *in situ* hybridization and immunocytochemistry suggest that KN1 traffics from inner layers to the outmost layer in the shoot apical meristem

(SAM; Jackson et al., 1994). Microinjection of KN1 into tobacco MCs directly demonstrated its ability to move intercellularly (Lucas et al., 1995). Transcription factor FLORICAULA (FLO) functions non-cell-autonomously for flower development in *Antirrhinum majas* (Carpenter and Coen, 1995). PISTILLATA (PI) has been shown to function similarly in Arabidopsis (Bouhidel and Irish, 1996). DEFICIENS (DEF) and GLOBOSA (GLO) are transcription factors that control petal and stamen organ identity, and they also function non-cell-autonomously in *A. majas* (Schwarz-Sommer et al., 1992; Sommer et al., 1990; Tröbner et al., 1992). Furthermore, DEF moves unidirectionally from inner layers to the outmost layer in the floral meristem, suggesting that PD between these layers facilitate polar protein traffic (Perbal et al., 1996). Transcription factor LEAFY (LFY) from Arabidopsis traffics cell-to-cell and rescues the *lfy* mutants (Sessions et al., 2000). Because this trasncroption factor is expressed in all cell layers in a wild type plant, the function of its traffic is not clear.

Recent report on the traffic of SHORT-ROOT (SHR), a putative transcription factor, by Nakajima et al. (2001) is of great significance as it is the first demonstration that regulated traffic of a specific protein is essential for proper development for normal plants. SHR is expressed in the stele and moves from the stele to a single layer of adjacent cells to specify formation of the endodermis (Nakajima et al., 2001). Ectopic expression of SHR causes supernumerary cell division and abnormal cell specification (Benfey et al., 1993; Nakajima et al., 2001).

Mechanism and regulation of macromolecular traffic

It has been shown that mutation in viral MPs (Fujiwara et al., 1993; Ding et al., 1995) or plant transcription factors (Lucas et al., 1995) abolishes the intercellular traffic function of these proteins. Therefore, specific interactions between the trafficking molecule and cellular factor(s) are required for traffic of these proteins. However, neither the motif(s) in the trafficking protein nor cellular factors that are involved in such interaction are well understood.

Plant development regulates traffic of proteins. CMV 3a MP:green fluorescent protein (GFP) fusion is able to target to and traffic through PD in tobacco source leaves, but not in sink leaves (Itaya et al., 1998, 2000). A source leaf is defined as the one that is photosynthetically active, and a sink leaf is the one that is not photosynthetically active and imports photoassimilates from source leaves (Turgeon, 1989). A similar pattern of PD targeting is reported for TMV MP:GFP (Ding et al., 1992; Roberts et al., 2001). CMV 3a MP:GFP expressed in companion cells of tobacco can traffic out of vascular tissues in mature stems and petioles, but not in young stems and petioles (Itaya et al., 2002).

Cellular boundaries also regulate traffic of macromolecules as revealed by studies on viral systemic movement. Cellular boundaries such as the interface between bundle sheath and phloem parenchyma (Goodrick et al., 1991; Ding et al., 1995; Wintermantel et al., 1997; Thompson and García-Arenal, 1998) and that between phloem parenchyma and the SE-CC complex (Ding et al., 1998; Wang et al., 1998) restrict the movement of viruses. MP of TMV cannot increase SEL of PD between bundle sheath and phloem parenchyma cells in tobacco leaves while it does so between MCs (Ding et al., 1992).

The molecular mechanisms of developmental and cellular regulation of protein traffic is not known. Structures and/or biochemical compositions of PD, and/or different cellular factors, either negative or positive factors, are likely to be involved in such regulation and are subject to further studies.

Research projects in this dissertation

Upon organogenesis, cells undergo coordinated division and differentiation to develop into distinct tissues. Communication between cells is critical for the coordinated division and differentiation. Research on viral MPs shed light on intercellular protein movement in plants. Studies on plant transcription factors revealed that some of the transcription factors can act non-cell-autonomously to regulate development of a plant. We propose that PD at different tissue boundaries at different developmental stages regulate protein traffic based on molecular interactions. A systematic and detailed analysis of protein traffic in plants is a prerequisite for understanding such regulation and for setting the basis for further biochemical or genetic analysis to dissect the mechanisms. This dissertation addresses the following issues: 1) Can an MP move between symplasmic domains? 2) Can an MP move across tissue boundaries such as phloem-mesophyll and mesophyll-epidermis interfaces? 3) Is the movement of MP regulated by plant development? 4) Is the movement of MP regulated in the same or different ways in different organs?

Microinjection of MPs or transcription factors has provided direct evidence of intercellular protein traffic. However, the method has some limitations. Most importantly,

microinjection is limited to MCs or EPs. Therefore, it is useful mainly to study traffic between the same cell types but not to study traffic between different tissues.

As an alternative to microinjection, biolistic bombardment was developed by Itaya et al. (1997) to study protein traffic. A gene construct encoding CMV 3a MP:GFP fusion protein is delivered biolistically to leaf epidermal cells. The fusion protein produced in single cells is targeted to PD and traffics into adjacent cells (Itaya et al., 1997). This method is also limited because proteins can be expressed usually only in EPs.

Recently, transgenic approaches have been used to express GFP and GFP fusions under the control of tissue-specific promoters (Oparka et al, 1999; Imlau et al., 1999; Kim et al., 2002; Nakajima et al., 2001; Itaya et al., 2002). With this method, the protein of interest can be produced in specific tissues. Using a combination of the transgenic approach and confocal laser scanning microscopy (CLSM), protein traffic between tissues can be studied.

This dissertation is devoted to studies on how CMV 3a MP mediates traffic between the phloem and mesophyll, and between the mesophyll and epidermis in tobacco and Arabidopsis. In chapter II, we describe transgenic tobacco and Arabidopsis expressing β-glucuronidase (GUS) under four different tissue-specific promoters and their tissue-specificity. In chapter III, we describe traffic of 3a MP fusion protein produced in CCs under the control of CC-specific promoters in transgenic tobacco and Arabidospsis. In chapter IV, we describe traffic of 3a MP fusion protein produced in MCs under the control of MC-specific promoters in transgenic tobacco. The results are presented and discussed. Future prospects are discussed in chapter V.

CHAPTER II

IDENTIFICATION OF COMPANION CELL- AND MESOPHYLL CELL-SPECIFIC PROMOTERS[1]

[1] Data presented here will be published as: Matsuda Y., Liang G., Zhu Y., Ma F., Nelson, R., and Ding, B. (2002) The *Commelina yellow mottle virus* promoter drives companion cell-specific gene in multiple organs of transgenic tobacco. (Protoplasma, in press). The data are presented here with modifications with permission from Springer-Verlag Wien)

INTRODUCTION

The question of how plasmodesmata (PD) mediate protein traffic across different tissue boundaries is of great importance to understanding how intercellular communication controls plant growth and development and how plants and pathogens interact with each other. Microinjection and biolistic bombardment methods have provided important insights about protein traffic. These methods, however, have limitations in studying protein traffic between differentiated tissues. Basically, they are often limited to studying protein traffic between same cell types. Microinjection is useful for studying protein traffic between mesophyll cells (MCs) or epidermis (EPs). Biolistic bombardment is useful but limited to EPs.

To study protein traffic across different tissue boundaries, transgenic expression of a protein in specific cells/tissues will be an ideal approach. For such experiments tissue-specific promoters are necessary. We are interested in understanding how a protein traffics between the phloem and neighboring cells and between mesophyll and epidermis. Within the phloem, we are specifically interested in traffic between the companion cells (CCs) and surrounding cells.

Several CC-specific promoters have been reported. *AtSUC2* promoter is active in CCs of mature and photosynthetically active leaves, which are called source leaves of photoassimilates (Truernit and Sauer, 1995; Stadler and Sauer, 1996). It is not active in immature and photoassimilate sink leaves. We verified the CC-specificity of this promoter in this study. The *Commelina Yellow Mottle Virus* (CoYMV) promoter was reported to be strong and predominantly active in phloem tissues of different organs such

as stem, sink and source leaves, and flowers in tobacco (Medberry et al., 1992). However, the specific cells in which the CoYMV promoter is active were not clear. In this study, we determined that the CoYMV promoter is active specifically in CCs of all organs. The CoYMV promoter and the *AtSUC2* promoter are used to study protein traffic between CCs and neighboring cells as reported in chapter III.

The tomato ribulose bisphosphate carboxylase/oxygenase (RuBisco) small subunit 3C (*RBCS3C*) promoter has shown to be active in MCs in apple (Gittins et al., 2000). *RBCS2* promoter is one of five different *RBCS* promoters in tomato (Sugita and Gruissem, 1987) and a candidate for MC-specific promoter. In this study, we determined that they are active in MCs. We use the *RBCS2* promoter to study protein traffic between MCs and EPs, as reported in chapter IV.

MATERIALS AND METHODS

Plant material and growth conditions

Tobacco (*Nicotiana tabacum* cv. Samsun NN and Xanthi NN) was grown in a growth chamber controlled at 14 hours light (28°C)/10 hours dark (22°C) cycles. *Arabidopsis thaliana* (Columbia, Col-O) was grown at 23°C under 40 W cool white fluorescent light kept on a 16 hours light/8 hour dark cycle.

Construction of binary vectors

To test tissue specificity of promoters, they were inserted upstream of a *GUS* reporter gene in binary vectors. Binary vector construction for each promoter is described below.

A plasmid named pCOI, a pGPTV-kan-based binary vector containing the CoYMV promoter (Medberry et al., 1992) between *Sal*I site and *Sst*I site, was kindly provided by Dr. Neil Olszewski at University of Minnesota. The pCOI was digested with *Sma*I and *Eco*RI. The DNA fragment containing *GUS:NOS*-terminator excised from pBI221 (Clontech) with *Sma*I and *Eco*RI was inserted into the *Sma*I-*Eco*RI digested pCOI. The resulting plasmid was named pCOI-GUS.

AtSUC2 promoter was kindly provided by Dr. Norbert Sauer at Universität Erlangen-Nürberg, Germany. *AtSUC2* promoter was amplified by polymerase chain reaction (PCR) using a plasmid named pAF (transcriptional fusion of *AtSUC2* promoter and *GFP* in pUC19) as a template, forward primer *Hind*III-*Sph*I-AtSUC2pro (5'-TGCCCAAGCTT GCATGCAAAATAGC-3'; *Hind*III-*Sph*I sites underlined) and reverse primer AtSUC2pro-*Xba*I (5'-TGCTCTAGATTTGACAAACCAAGAAAG-3'; *Xba*I site underlined). The PCR product was double-digested with *Hind*III and *Xba*I and inserted into appropriate sites of pGPTV-Kan. The resulting plasmid was named pGPTV-AtSUC2:GUS.

A binary vector named pCV1.6RBCS3CP, which contains ribulose-1,5-biphosphate carboxylase small subunit 3C (*RBCS3C*) promoter upstream of a *GUS* sequence, was kindly provided by Dr. David J. James from Horticulture Research International, Kent, UK.

A plasmid named RBCS2-LUC was kindly provided by Dr. Iris Meier from The Ohio State University, Columbus, OH. Ribulose-1,5-biphosphate carboxylase small subunit 2 (*RBCS2*) promoter was PCR amplified with *Pfu* polymerase using primers *Hind*III-RBCS2 (5'-CCCAAGCTTGATCCGACACAAACAAT-3'; *Hind*III site

underlined), and RBCS2-*Xba*I (5'-GCTCTAGAATTGCTTCTTCTCTC-3'; *Xba*I site underlined), and RBCS2-LUC as a template. The PCR product was digested with *Hind*III and *Xba*I. The double-digested fragment was inserted into pGPTV-Kan. The resulting plasmid was named pGPTV-RBCS2:GUS.

Transformation of Agrobacterium tumefaciens

Binary vectors pCOI-GUS and pCV1.6RBCS3CP (Fig. 1) were used to transform *Agrobacterium tumefaciens* GV3101. Binary vectors pGPTV-AtSUC2:GUS and pGPTV-RBCS2:GUS (Fig. 1) were used to transform *A. tumefaciens* LBA4404.

Transformation of tobacco and Arabidopsis

A standard *Agrobacterium*-mediated leaf-disc transformation method (Horsch et al., 1985) was performed to transform tobacco plants with binary vectors above. A standard floral dip method (Clough and Bent, 1998) was conducted to transform Arabidopsis plants for pCOI-GUS. Transformants were selected by kanamycin resistance (100 μg/mL).

Histochemical GUS Assay

Seeds from transgenic plants were sterilized with 70% ethanol for 2 min and 5% bleach-1% SDS solution for 15 min, followed by washing with sterilized H_20 several times. The sterilized seeds were plated on selection media (MS media +1% sucrose) with 100 μg/mL kanamycin. Three-day-old seedlings were processed for GUS assay as described below.

Whole seedlings or freshly detached leaf, petiole, flower and stem samples from soil grown adult plants were submerged in a fixation mixture (100 mM sodium phosphate/2% paraformaldehyde/1mM EDTA, pH 7.0) for 30 min on ice. Following washes with 100 mM sodium phosphate buffer/1 mM EDTA (pH 7.0), the samples were vacuum-infiltrated for 30 min in the histochemical reaction mixture (100 mM sodium phosphate buffer of pH 7.0, 2 mM X-glucuronide, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM EDTA and 0.5 % Triton X-100). After 12-18 hours of incubation, the reaction was stopped by rinsing with H_20 and samples were cleared with 70% ethanol for 2 hours and with 95% ethanol overnight to remove chlorophyll. Samples were put in 70% ethanol for 2 hours then sections were prepared by hand sectioning.

For leaf samples of RBCS3C:GUS- and RBCS2:GUS-trangenic tobacco, and stem samples of CoYMV:GUS-transgenic Arabidopsis, samples were embedded into O.C.T. compound after GUS reaction and removing chlorophyll. The cryosectioning to obtain thin sections was conducted. The procedure of cryosectioning is detailed below.

Microscopy

Histochemical blue staining of freehand sections and cryosections as well as whole samples were examined under bright-field conditions with a Nikon Eclipse-600 light microscope (Nikon Corp., Tokyo, Japan). In some cases, Nomarski optics were used to enhance contrast image of sections. All images were captured via an RT SPOT 2 Slider CCD camera and associated software (Diagnostics Instruments Inc., Sterling Heights, Michigan, USA).

In situ hybridization

For riboprobe production, *GUS-Nos* terminator was excised from pBI221 with *Xba*I and *Eco*RI and inserted in the appropriate sites of pGEM-4Z resulting in pGEM-GUS. The pGEM-GUS was linearized with *Hind*III or *Eco*RI to be used as templates for *in vitro* transcription to produce antisense and sense probes, respectively. Digoxygenin (DIG) –labeled antisense probe was generated with SP6 RNA polymerase and sense probe was generated with T7 RNA polymerase respectively, using the Ambion MEGAScript kit (Ambion Inc., Austin Texas, USA) according to the manufacturer's instructions.

For *in situ* hybridization, cryosections were prepared. Leaves and stems of transgenic plants expressing GUS were dissected into 2 x 2 mm segments and fixed in a mixture of 3.7% paraformaldehyde, 0.1% glutaraldehyde, 0.2% picric acid, 50 mM potassium phosphate, and 5 mM EGTA for 2 hours. After rinsing with 50 mM potassium phosphate/5 mM EGTA, fixed samples were infiltrated sequentially with 3:7 (v/v), 5:5 (v:v), and 7:3 (v/v), embedding mixture (2 parts of 20% sucrose and one part of O.C.T. compound):potassium phosphate-EGTA buffer at room temperature for 2 hours, respectively. Finally, the fixed samples were infiltrated with absolute O.C.T. compound for 2 hours and frozen at –20 °C. The frozen samples were sectioned to 12 μm thickness using a Microm HM500 cryostat (Walldorf, Germany). Sections were collected onto microscope slides coated with 1% gelatin and 0.1% chromium alum. The slides with sections were placed on a warming plate at 42 °C overnight to enhance section attachment and kept at 4 °C before further processing. *In situ* hybridization was performed as described in Zhu et al. (2001).

RESULTS

CoYMV promoter is active specifically in companion cells of transgenic tobacco

To test cell-specificity of the CoYMV promoter in tobacco, transgenic plants expressing GUS under the promoter were generated. A total of seven different transgenic tobacco lines expressing GUS were obtained. Since two of them showed stronger GUS activity in the phloem, these two plants were studied extensively for the promoter specificity.

The GUS expression pattern in young seedlings showed that the CoYMV promoter was active in vascular systems (Fig. 2A). In the stems of mature plants, internal (InP) and external phloem (ExP) showed GUS expression (Fig. 2B). High resolution microscopy revealed GUS activity in CCs but not in other cell types such as sieve elements (SEs) or phloem parenchyma cells (PPs) (Fig. 2C). Outside the phloem, xylem (Xy) and cortex (Cx) did not show any GUS activity. Further evidence was obtained by longitudinal sections. Only CCs showed the blue color while SEs and PPs remained clear (Fig. 2D). The cell types can be easily distinguished in the longitudinal view by the size and the morphology. SEs have sieve plates and P-protein whereas CCs do not. Phloem parenchyma cells are relatively shorter and wider than SEs and CCs. To verify the CC-specificity of the promoter activity, *in situ* hybridization to detect mRNA of GUS was performed. With anti-sense probe, only CCs were labeled intensively (Fig. 3A).

Leaves have a complex vein network and we followed the vein classification scheme of Ding et al. (1988). Briefly, class I are the primary (midrib) veins. Class II are mostly secondary branches. Class III consists of tertiary branches and terminal ends of secondary branches. Class IV and V veins (minor veins) consist of subsequent branches

from Class III veins. In all classes of veins, the CoYMV promoter drives GUS expression in CCs in source leaves (Fig. 4A-E). In sink leaves, GUS activity was also detected only in CCs (Fig. 4F). GUS activity was not detected in bundle sheath, mesophyll, or epidermis. In petioles, GUS activity was also detected only in CCs (data not shown). *In situ* hybridization showed localization of *GUS* mRNA only in CCs in leaves, confirming the CC-specificity of the CoYMV promoter (Fig. 3B-C).

In roots, GUS activity was detected only in the vascular cylinder above the meristematic region (Fig. 5A). The transverse section showed that most probably GUS is active in CCs (Fig. 5B).

In flowers, the vein network in sepals showed GUS activity (Fig. 5C). A weak GUS signal was detected in veins of petals, anthers and carpels (data not shown).

CoYMV promoter is specifically active in companion cells of transgenic Arabidopsis

In order to examine the CoYMV promoter activity in Arabidopsis, eight CoYMV:GUS-transgenic plants were obtained and examined for GUS activity. In young and mature leaves, GUS activity was detected in the vasculature (Fig 6A-B). In inflorescence stems, GUS activity was detected in the phloem (Fig. 6C). High magnification image showed that GUS expression was observed in CCs and not in other cells (Fig. 6D).

Interestingly, the root cap of three-day-old seedlings also showed GUS expression (Fig. 7A). GUS activity was also detected in the vascular system in roots (Fig. 7B).

The vein network of sepals showed GUS signal (Fig. 7C). A weak GUS signal was detected in veins in petals, anthers and carpels (data not shown).

AtSUC2 promoter is active in companion cells of transgenic tobacco

To confirm CC-specificity of the *AtSUC2* promoter in tobacco, a total of 4 lines of transgenic plants expressing GUS under the promoter were generated. GUS activity was detected in the CCs in the petioles (Fig. 8) and all veins in source leaves (data not shown). However, in sink leaves, GUS activity was not detected (data not shown), as reported previously in Arabidopsis (Truernit and Sauer, 1995; Stadler and Sauer, 1996).

Tomato *RBCS3C* and *RBCS2* promoters are active in mesophyll and phloem of transgenic tobacco

To examine cell-specificity of the *RBCS3C* and *RBCS2* promoters, transgenic tobacco expressing GUS under *RBCS3C* and *RBCS2* promoters, respectively, were generated. A total of four RBCS3C:GUS-transgenic tobacco and six RBCS2:GUS-transgenic tobacco were obtained. The *RBCS3C* promoter was active in palisade (PMCs) and spongy mesophyll cells (SMCs) in mature leaves (Fig. 9A). The vascular cells showed stronger activity than MCs (Fig. 9A). Epidermis (EPs) remained clear, which indicated no GUS activity in EPs (Fig. 9B).

In RBCS2:GUS-transgenic plants, GUS activity was detected in PMCs and SMCs, indicating that the *RBCS2* promoter was active in these cells (Fig. 9C). The phloem also showed GUS activity (Fig. 9C). It is notable that EPs did not show any GUS activity (Fig. 9D).

Neither RBCS2:GUS- nor RBCS3C:GUS-transgenic plants showed GUS activity in sink leaves (data not shown).

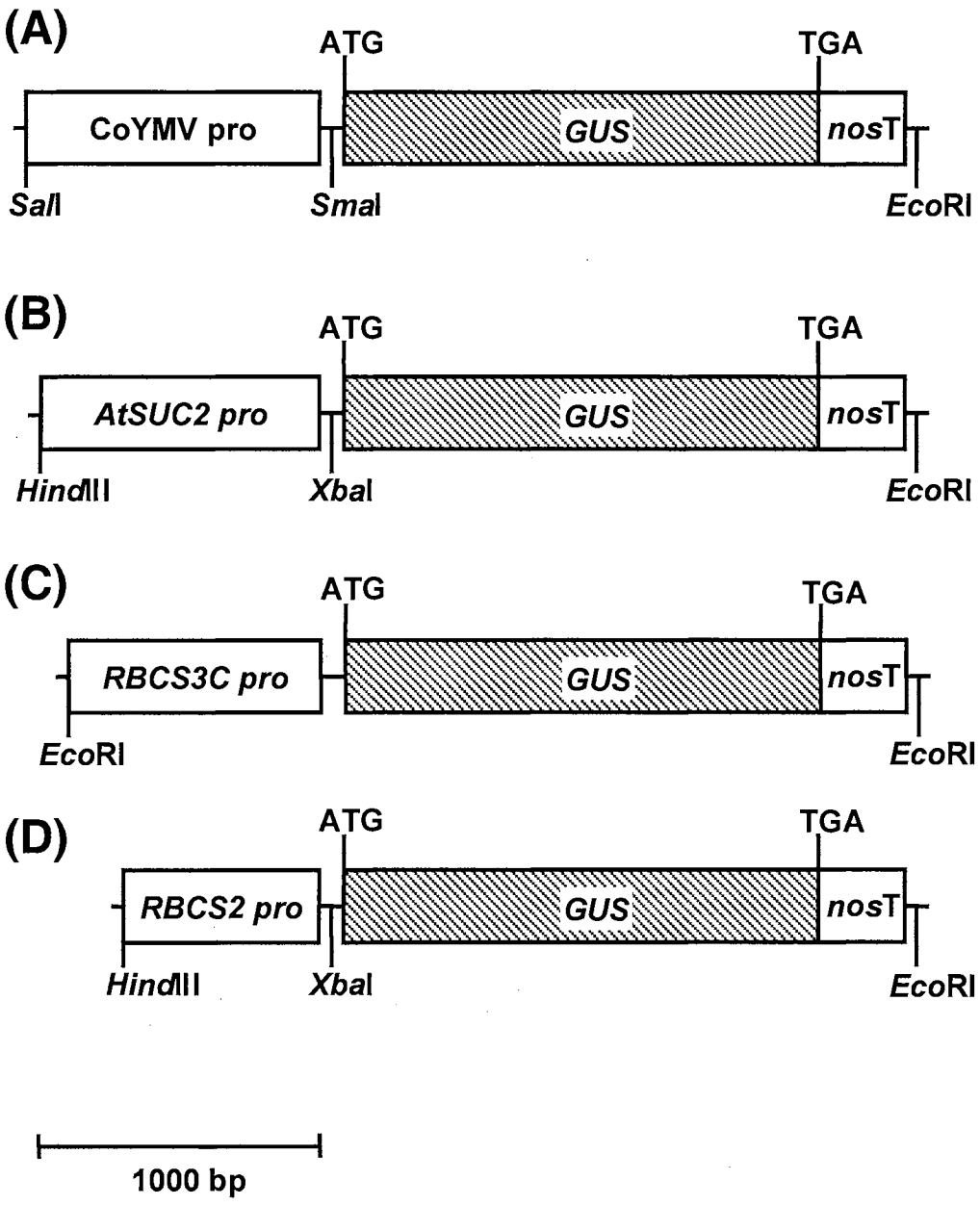

Figure 1. Transcriptional fusions used for transformation via *Agrobacterium tumefaciens*.
(**A**) CoYMV promoter:*GUS* fusion
(**B**) *AtSUC2* promoter:*GUS* fusion
(**C**) *RBCS3C* promoter:*GUS* fusion
(**D**) *RBCS2* promoter:*GUS* fusion

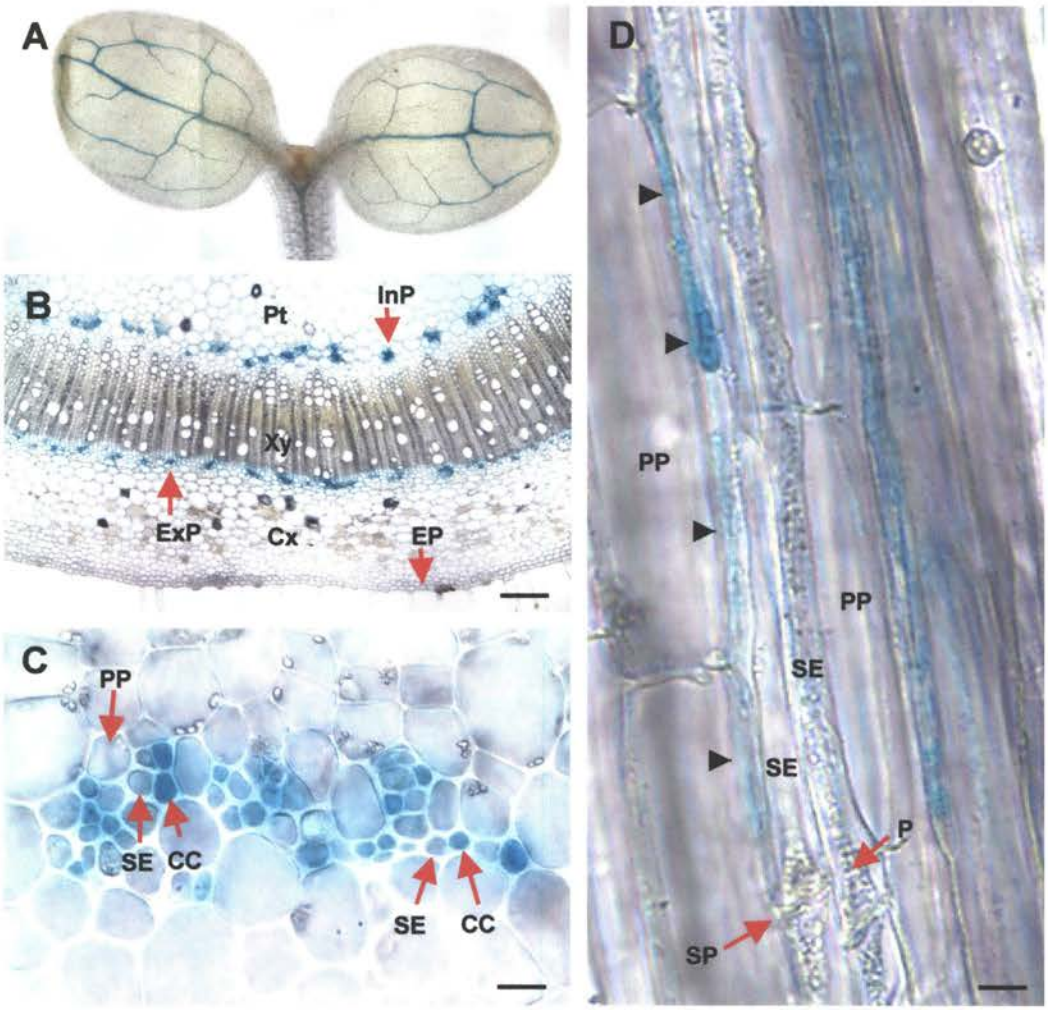

Fig. 2

Figure 2. CoYMV promoter-driven GUS expression in companion cells of transgenic tobacco cotyledons (**A**) and stems (**B-D**).
(A) A 3-day-old seedling of transgenic tobacco, showing GUS staining (blue) in the vasculature.
(**B**) Transverse sectional view of part of a stem. GUS activity is detected only in the internal phloem (InP) and external phloem (ExP), but not in the epidermis (EP), cortex (Cx), pith (Pt) or xylem (Xy). Bar:150 μm.
(C) High magnification view of internal phloem showing GUS staining exclusively in companion cells (CC). PP, phloem parenchyma cell; SE, sieve element. Bar:10 μm.
(D) Longitudinal view of stem internal phloem, showing further confirmation of phloem cell types. Sieve element (SE) is identified by the presence of sieve plate (SP) and accumulation of phloem protein (P-protein; P). Companion cell, which contains GUS staining and is indicated by arrowheads, has one transverse wall coterminal with the sieve plate. Both the sieve elements and phloem parenchyma cells (PP) contain no GUS activity. Bar:5 μm.

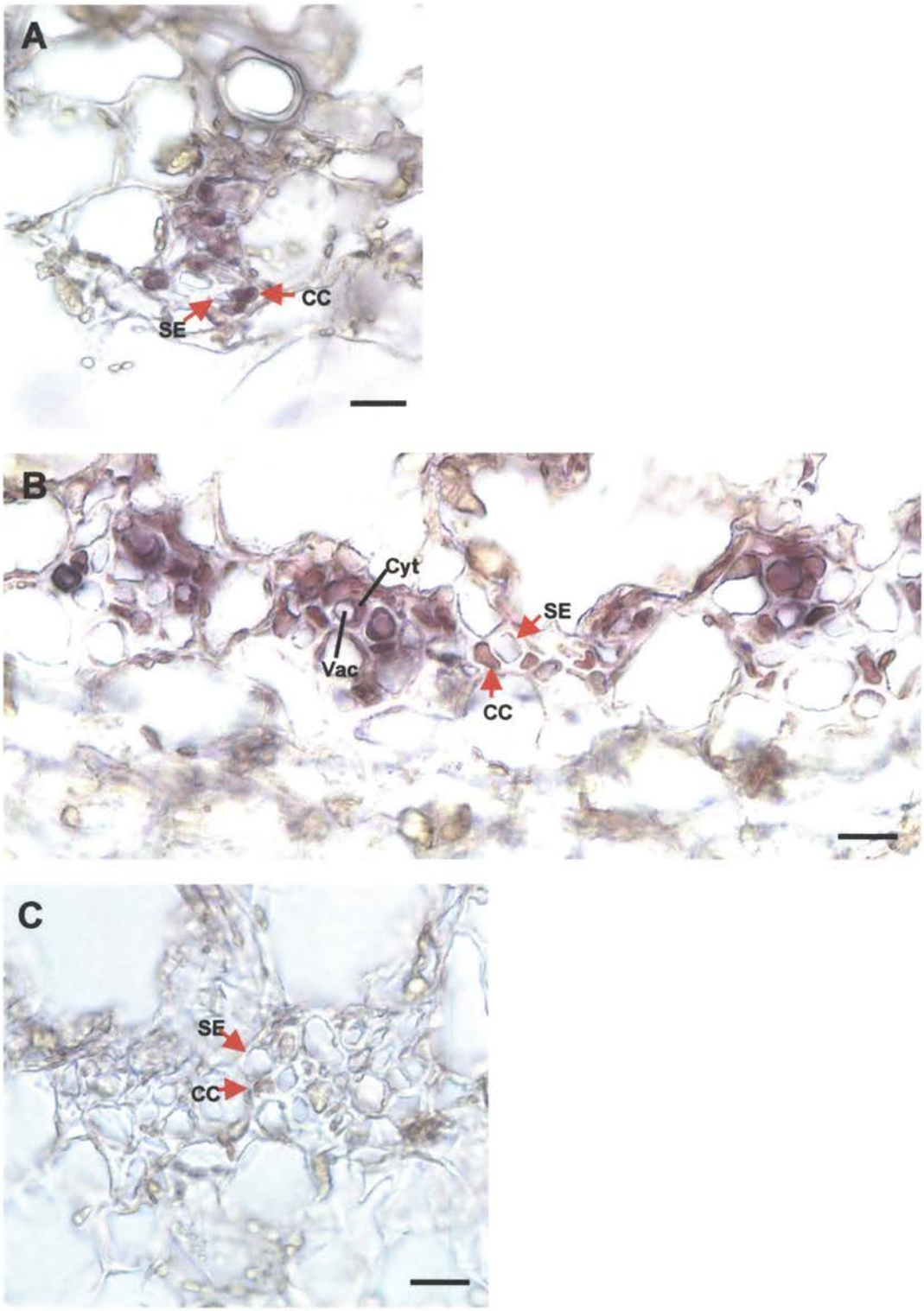

Fig. 3

Figure 3. *In situ* localization of *GUS* mRNA in transgenic tobacco.
(A) Transverse section of stem probed with antisense *GUS* riboprobe. *GUS* mRNA is localized only in the companion cells (CC). The hybridization signal (purple stain) is only evident in companion cells (CC). Bar:10 μm.
(B) Transverse section of a class I vein probed with antisense *GUS* riboprobe. At the subcellular level, the hybridization signal is predominantly detected in the cytoplasm (Cyt), but not in the vacuole (Vac), demonstrating the high precision of mRNA localization on cryosections. SE, sieve element. Bar:10 μm.
(C) Transverse section of a class I vein probed with sense *GUS* riboprobe. There is no hybridization signal in any cells. CC, companion cell; SE, sieve element. Bar:10 μm.

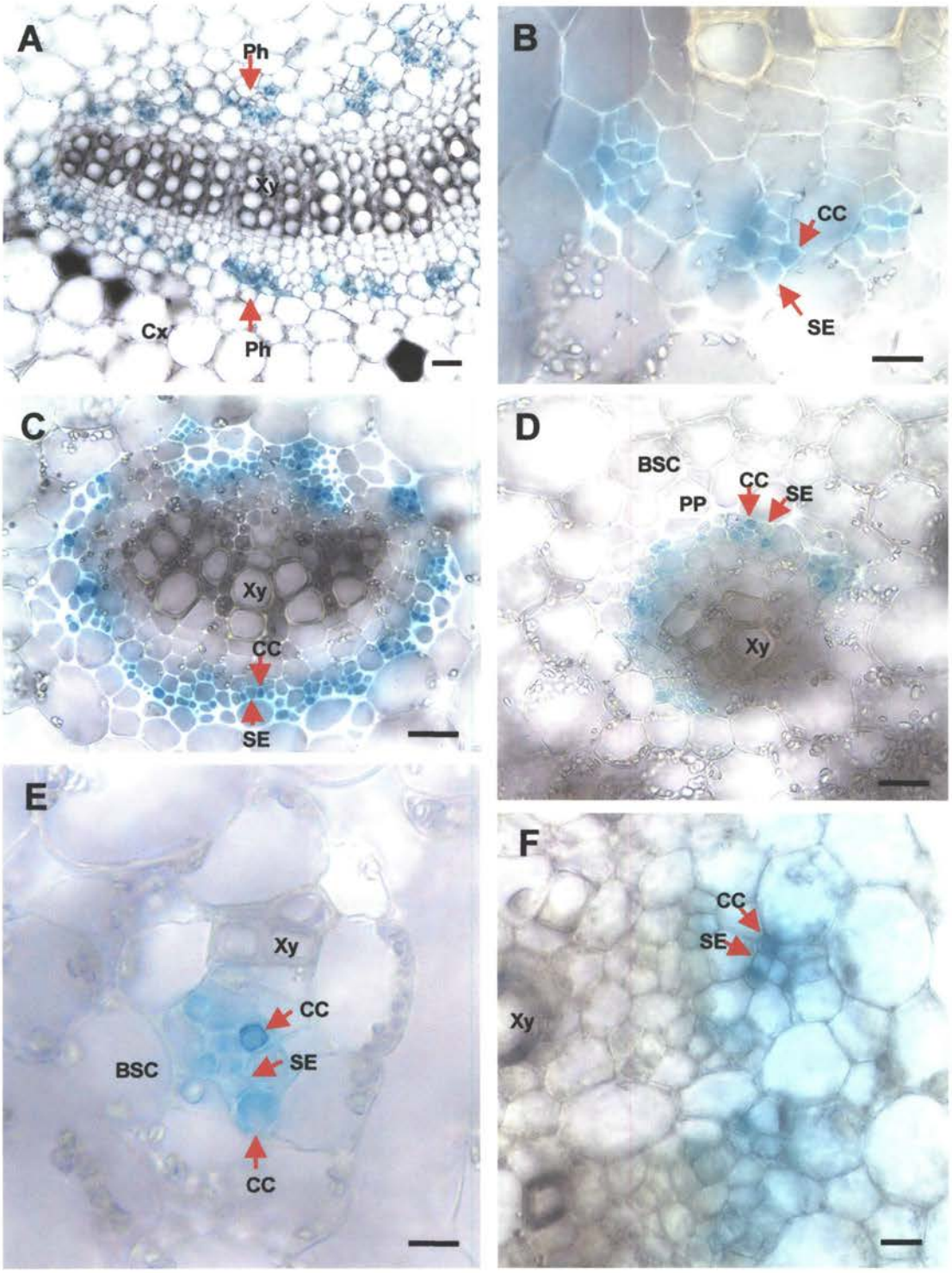
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Fig. 4

Figure 4. CoYMV promoter-driven GUS expression in companion cells of transgenic tobacco leaves.
(**A-E**) are from mature source leaves and (**F**) is from an immature sink leaf.
(A) Bright field image of a class I vein. Bar:30 μm.
(B) High magnification Nomarski image of a class I vein. Bar:30 μm.
(**C**) Class II vein. Bar:30 μm.
(D) Class III vein. Bar:30 μm.
(E) Minor vein. Bar:10 μm.
(F) Class I vein of a sink leaf. The arrow points to a sieve element (SE) that is immature based on its thin walls and rectangular shape. Bar:10 μm. BSC, bundle sheath; CC, companion cell; Cx, cortex; PP, phloem parenchyma; SE, sieve element; Xy, xylem.

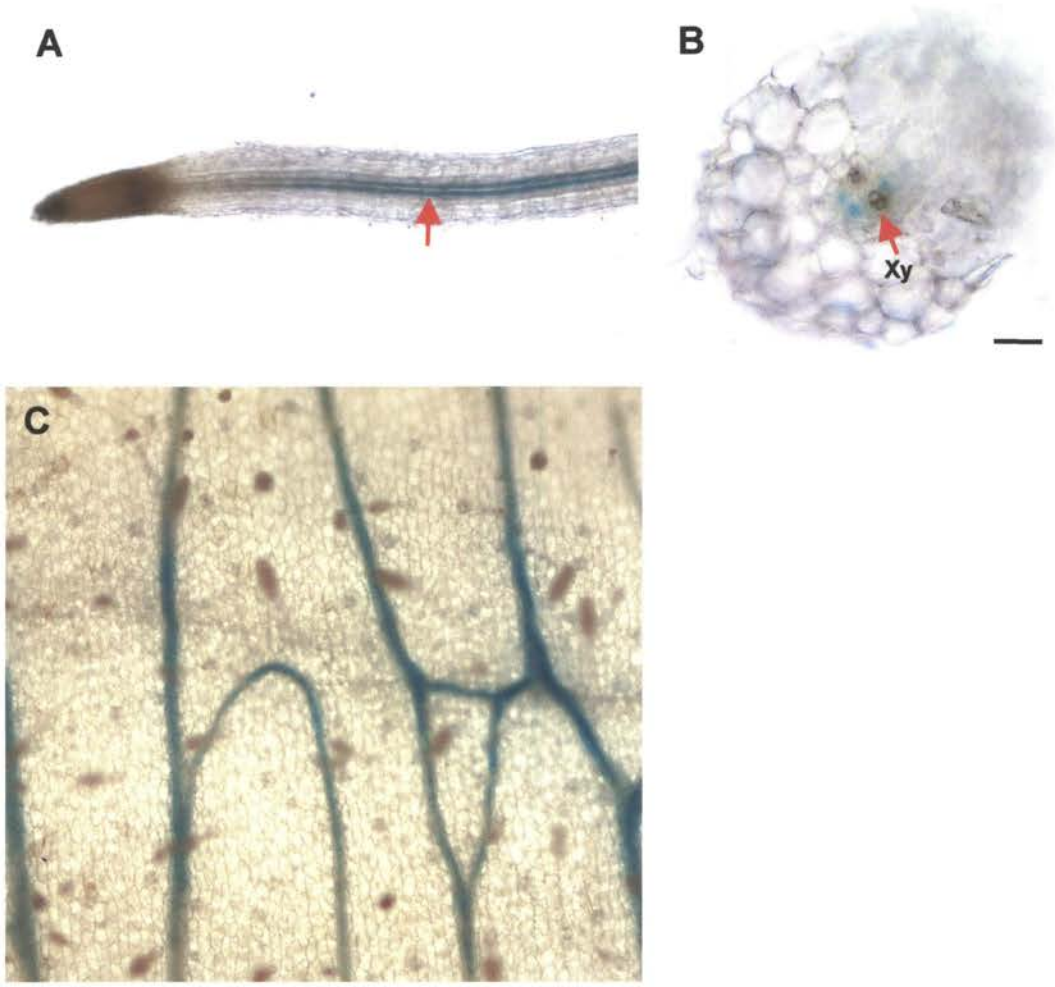


Figure 5. CoYMV promoter-driven GUS expression in roots (**A-B**) and flowers (**C**) of transgenic tobacco.
(A) Longitudinal view of a root showing GUS staining only in the vascular cylinder (arrow).
(B) Transverse view of a root, showing GUS activity in the phloem (blue stain). The specific cell type containing GUS cannot be resolved but it is most likely companion cell. Xy, xylem. Bar:50 μm.
(C) Surface view of sepal of transgenic tobacco. The vascular system in sepal shows GUS activity.

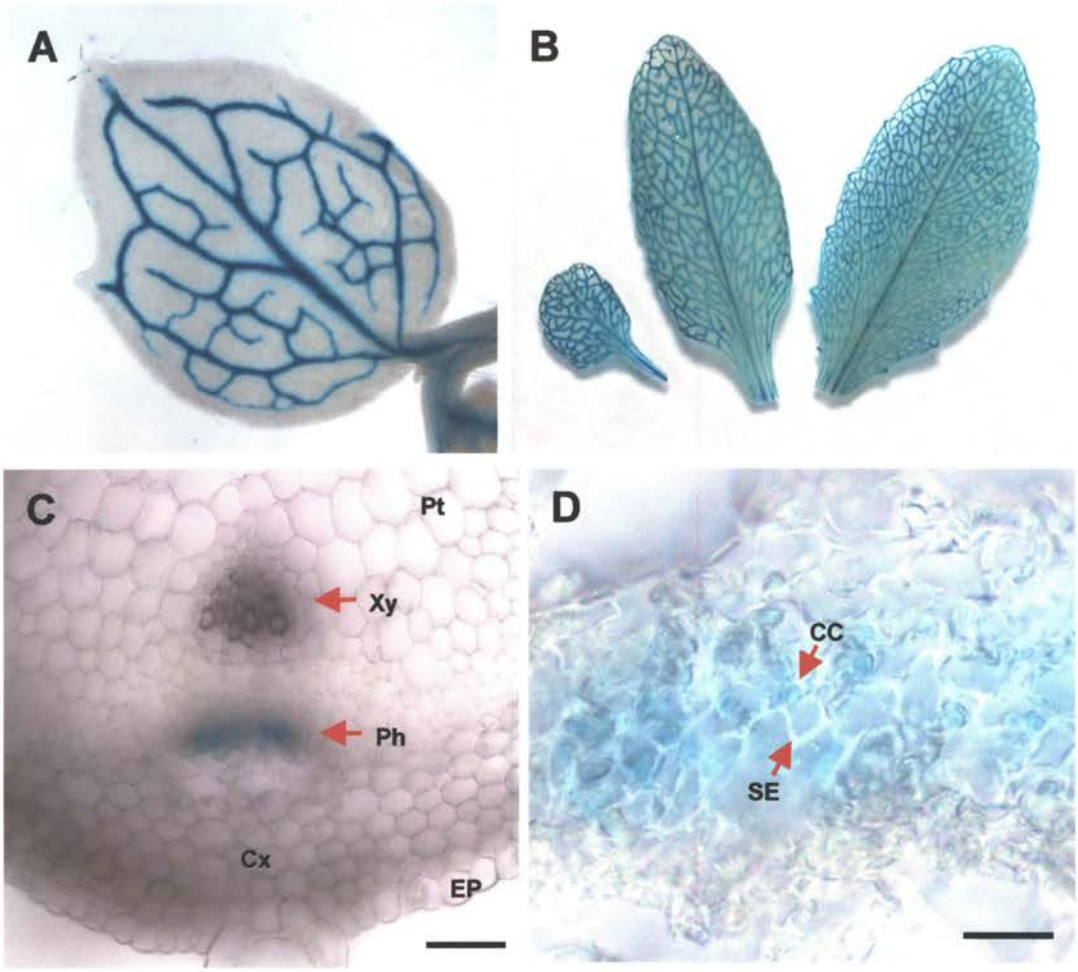


Figure 6. CoYMV promoter-driven GUS expression in leaves and stems of transgenic Arabidopsis.
(A) Young leaf of transgenic Arabidopsis showing GUS staining (blue) in the vasculature.
(B) Mature leaves of CoYMV:GUS-transgenic Arabidopsis showing GUS staining in the vascular system.
(C) Transverse section of stem of CoYMV:GUS-transgenic Arabidopsis. The GUS activity is detected only in the phloem (Ph), but not in the epidermis (EP), cortex (Cx), pith (Pt) or xylem (Xy). Bar:50 μm.
(D) High magnification view of transverse section of stem of CoYMV:GUS-transgenic Arabidopsis showing localization of GUS activity in companion cells (CCs). SE, sieve element. Bar:10 μm.

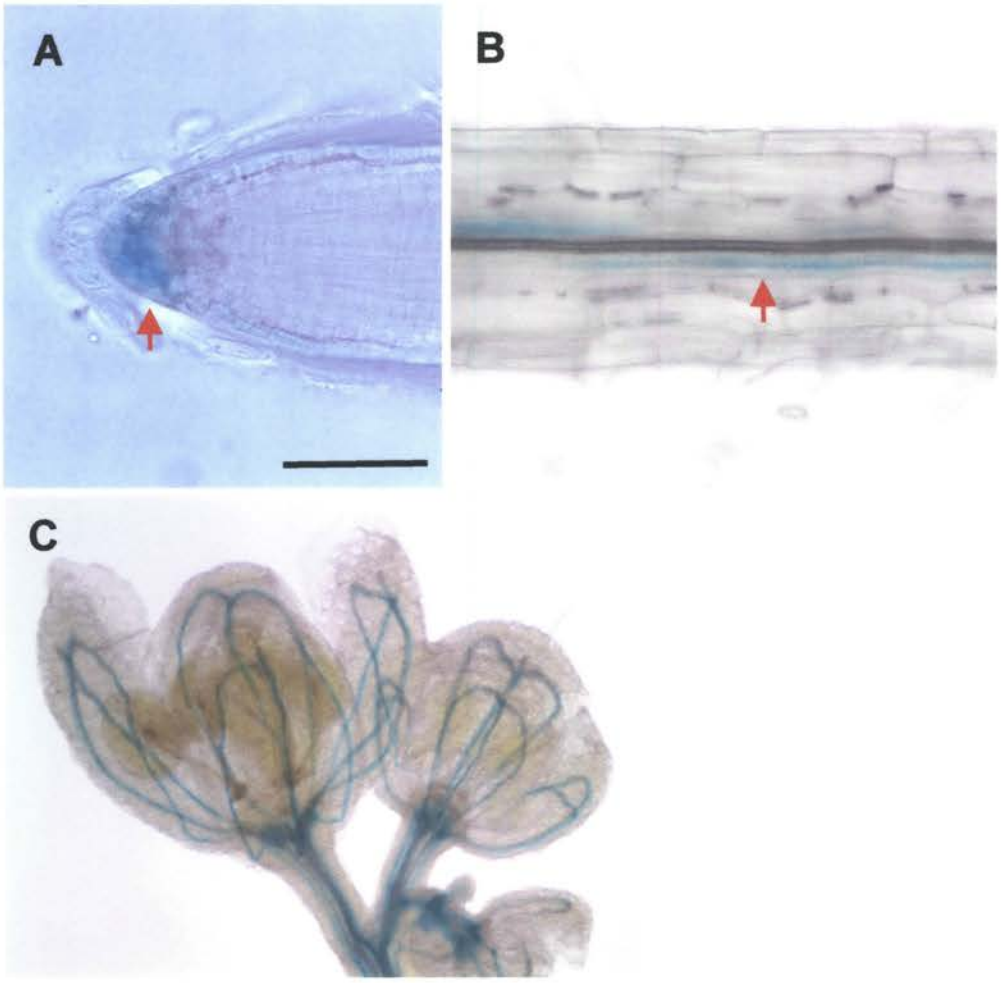


Figure 7. CoYMV promoter-driven GUS expression in roots and flowers of transgenic Arabidospsis.
(A) Longitudinal view around root tip of CoYMV:GUS-transgenic Arabidopsis. The GUS activity is detected in root cap (arrow). Bar:50 μ m.
(B) Longitudinal view of mature region of root showing GUS staining only in vascular cylinder (arrow).
(C) Flowers of CoYMV:GUS-transgenic Arabidopsis showing GUS staining in vasculature.

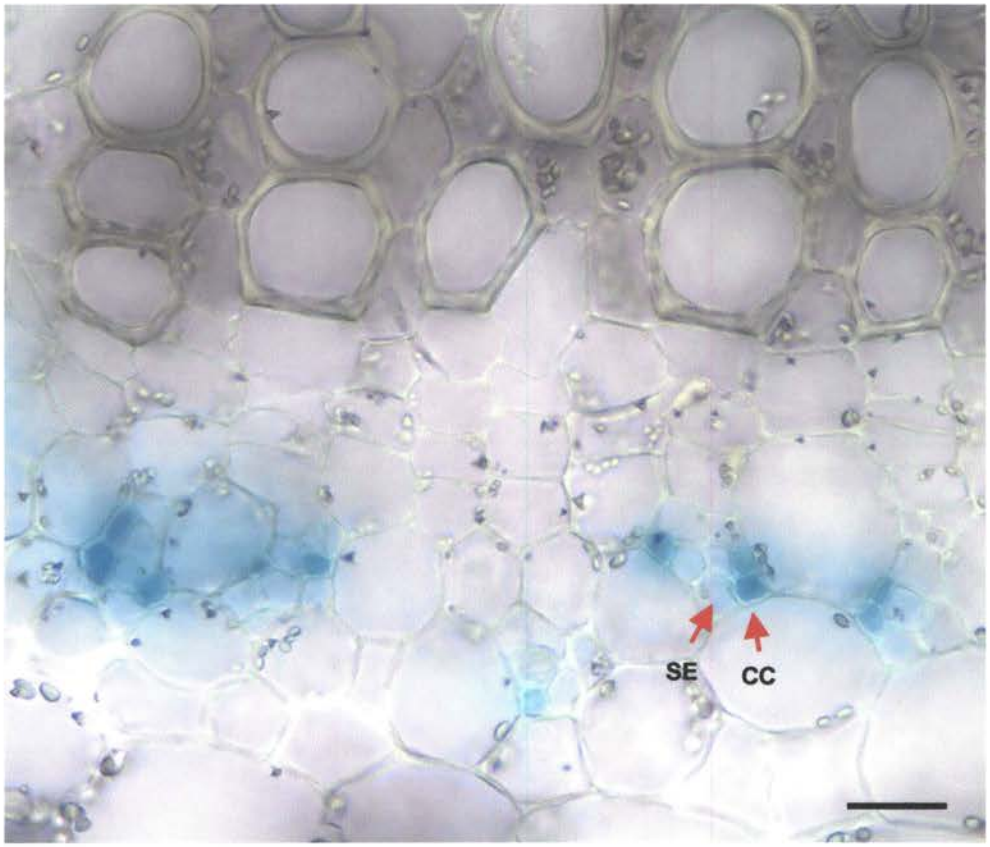


Figure 8. *AtSUC2* promoter-driven GUS expression in transgenic tobacco.
The petiole in source leaf showing GUS signal in CCs. CC, companion cell. SE, sieve element. Bar:50 μ m.

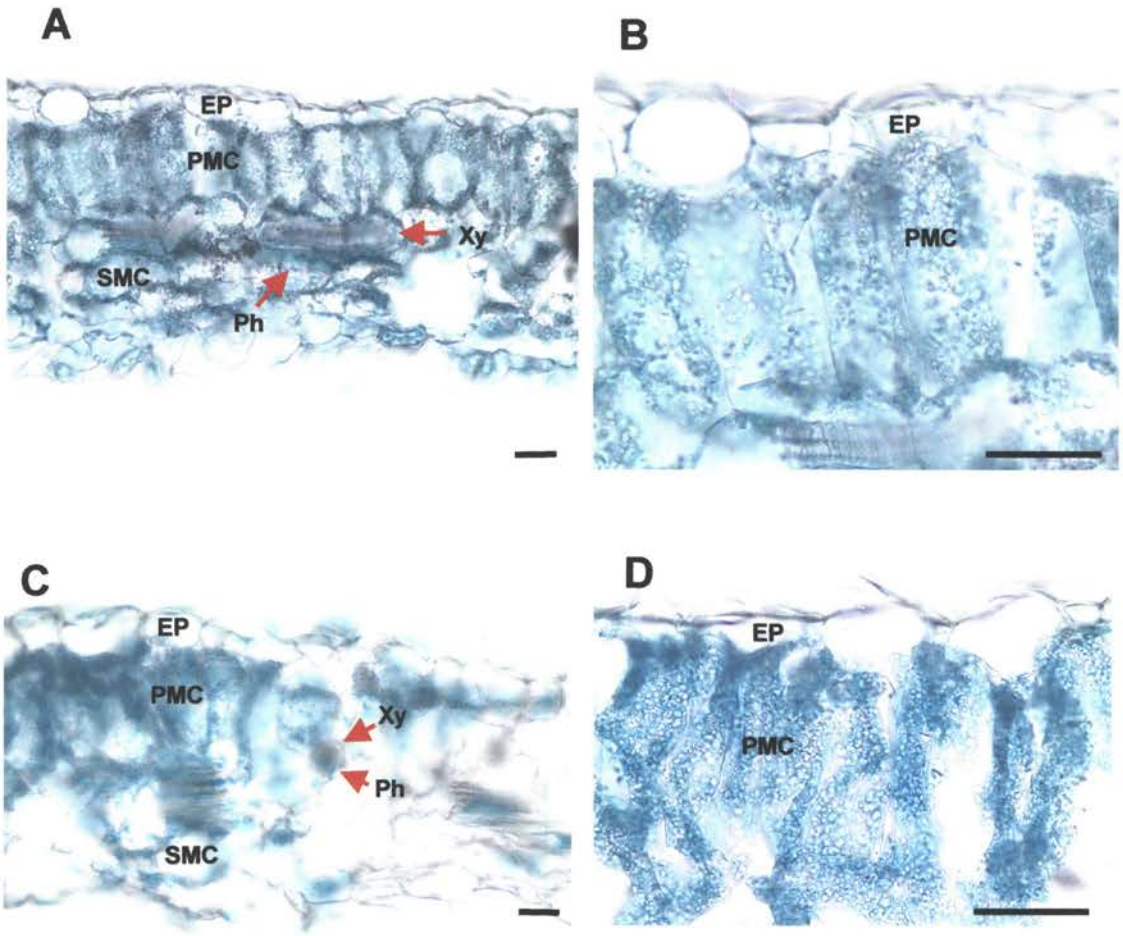


Figure 9. *RBCS3C* and *RBCS2* promoter-driven GUS expression in mature leaves of transgenic tobacco.

(A) Transverse section of RBCS3C:GUS-transgenic tobacco. GUS activity is detected in palisade mesophyll cell (PMC), spongy mesophyll cell (SMC) and also in phloem (Ph) cells. Xy,xylem. Bar:20 μ m.

(B) High magnification view of transverse section of RBCS3C:GUS-transgenic tobacco. GUS activity is not detected in epidermis (EP). Bar:20 μ m.

(C) Transverse section of RBCS2:GUS-transgenic tobacco. GUS activity is detected in palisade mesophyll cell (PMC), spongy mesophyll cell (SMC) and also in phloem (Ph) cells. Bar:20 μ m.

(D) High magnification view of transverse section of RBCS2:GUS-transgenic tobacco. GUS activity is absent from epidermis (EP). Bar:20 μ m.

DISCUSSION

In general, we confirmed the vascular specificity of CoYMV promoter reported previously (Medberry et al. 1992). However, the results in this chapter showed that the CoYMV promoter is active specifically in CCs of all organs examined in tobacco and Arabidopsis (i.e., stem, root, leaf, sepal and petal). The data came from both GUS staining and *in situ* localization of *GUS* mRNA experiments. The reason for Medberry et al. (1992) to show general vascular specificity, but not to the CC level, of the CoYMV promoter could be due to the histochemical GUS protocol used in that study. They did not include oxidative agents, potassium ferri- and ferrocyanide in the reaction mixture. Therefore, GUS reaction products likely diffused out of CCs, making it difficult to determine the cell-specificity. It was reported that 5 mM potassium ferri-and ferrocyanide in the reaction mixture prevented the diffusion of color substance produced by GUS (Stomp 1992; De Block and Debrouwer, 1992). Thus, the inclusion of these chemicals in our GUS histochemical staining allowed us to achieve highly precise cellular localization of promoter activity.

The CC-specificity in multiple organs distinguishes the CoYMV promoter from other CC-specific promoters reported. *AtSUC2* promoter is active in CCs of stem, root and mature leaves in Arabidopsis (Truernit and Sauer, 1995; Stadler and Sauer, 1996) and in tobacco (Imlau et al., 1999; Oparka et al., 1999). However, in sink leaves *AtSUC2* promoter does not show the promoter activity. The *Cucumis melo* galactinal synthase (*CmGAS1*) promoter is only active in the minor veins of mature leaves in Arabidopsis and tobacco (Haritatos et al., 2000). Thus, among CC-specific promoters the CoYMV

promoter has a unique activity and can be a good tool to express genes of interest in CCs in different organ types. This may be due to its viral origin. The viral promoter may have evolved to utilize endogenous gene expression systems that are present in all organs throughout development, and/or escape developmental regulation so that the virus can invade a plant effectively.

We have provided the first demonstration of mesophyll-specificity of the *RBCS2* promoter, and showed that the *RBCS3C* promoter is also specific to mesophyll in tobacco. Clear absence of the activity of these promoters in EPs indicates that the promoters can be useful in expressing a GFP fusion protein in the mesophyll to study its traffic into epidermis.

CHAPTER III

TRAFFIC OF VIRAL MOVEMENT PROTEIN:GREEN FLUORESCENT PROTEIN FUSION OUT OF SYMPLASMICALLY-ISOLATED SIEVE ELEMENT-COMPANION CELL COMPLEX

INTRODUCTION

In a young plant embryo, all cells are connected by plasmodesmata (PD) as demonstrated by structural (Schultz and Jensen, 1968; Mansfield and Briarty, 1991) and dye-coupling studies (McLean et al., 1997; Pfluger and Zambryski, 2001; Kim et al., 2002). During organogenesis, the differentiation of cells leads to the formation of specialized tissues with distinct functions. Dramatic changes of PD connections in structure and functions result in the formation of symplasmic domains (Erwee and Goodwin, 1983; van der Schoot and van Bel, 1990). Symplasmic isolation may allow groups of cells to follow distinct developmental pathways (McLean et al., 1997, Ding et al., 1999; Lucas et al., 1993). On the other hand, coordinated differentiation of cells is essential for a plant body to function properly. How symplasmically-isolated cells communicate with their neighboring cells is virtually unknown.

The sieve element (SE)-companion cell (CC) complex in the phloem is one of the most important symplasmic domains. The SE-CC complex plays a pivotal role in long distance phloem transport of photoassimilates (Patrick, 1997; Oparka and Turgeon, 1999; Oparka and Santa Cruz, 2000). In addition, macromolecules such as proteins including CmPP16 (Xoconostle-Cazares et al., 1999; Ruiz-Medrano et al., 1999) and RNAs including mRNA of LeT6, a tomato KNOTTED1-like homeobox gene (Kim et al., 2001) and viroids, circular single-stranded RNA plant pathogens (Palukaitis, 1987; Zhu et al., 2001), move through the SE-CC complex. The flowering signals (Bernier et al., 1993) and defense signals such as systemin, an 18-amino acid peptide wound signal (Pearce et al., 1991; Narvaez-Vasquez, 1995), also move through the SE-CC complex to organs

located distantly from sites where the signals are generated. While these molecules move through the SE-CC complex, the complex does not work as a simple conduit for any transport. Phloem entry and exit of transported materials must be regulated to control for transport.

PD frequencies between the SE-CC complex and adjacent cells in minor veins decrease dramatically upon leaf development in many plants (Gamalei 1989; Grusak et al., 1996; Turgeon et al., 2001). The remaining PD at the interface do not appear to be functional for photoassimilate transport. Instead of symplasmic transport, the membrane-associated sugar transporters in the SE-CC complex take up photoassimilates apoplastically to maintain flow of photoassimilates from mesophyll cells (MCs) to the SE-CC complex (Grusak et al., 1996; Bush, 1993; von Schaewen et al., 1990). Dye-coupling studies showed symplasmic isolation of the SE-CC complex in the transport phloem of stems, petioles, hypocotyls and roots in many species (van der Schoot and van Bel, 1989, 1990; van Bel and Kempers, 1991; Knoblauch and van Bel, 1998), which could explain why the high turgor pressure is maintained in the SE-CC complex for long-distance transport in plants (Oparka and Turgeon, 1999).

The symplasmic isolation of the SE-CC complex raises questions of how the complex communicates with its neighboring cells and also how viruses can enter and exit the complex to spread systemically. Viruses utilize their movement proteins (MPs) to traffic intercellularly. Many MPs move themselves and viral genomes through PD (Ding 1998, Oparka and Santa Cruz, 2000; Carrington et al., 1996; Ghoshroy et al., 1997; Lazarowitz and Beachy, 1999). Thus, viral MP traffic into and out of the SE-CC complex may be a key factor for viral systemic movement (Oparka and Turgeon, 1999). However,

there was no experimental evidence that an MP can traffic between the SE-CC complex and neighboring cells through the PD.

To test protein traffic between the SE-CC complex and neighboring cells, we generated transgenic tobacco and Arabidopsis plants that express *Cucumber Mosaic Virus* (CMV) 3a MP fused to green fluorescent protein (GFP) in CCs under the control of CC-specific CoYMV and *AtSUC2* promoters, respectively. Confocal microscopic examination of samples from these plants demonstrated traffic of the fusion protein out of the SE-CC complex. 3a MP:GFP traffic in CoYMV:3a MP:GFP-transgenic tobacco was primarily the work of Dr. Asuka Itaya and others, which will be published in The Plant Cell in September, 2002 (Itaya et al., 2002). Data from the CoYMV:3a MP:GFP-transgenic tobacco will be presented briefly in this chapter for comparison purposes with permission from Dr. A. Itaya.

I have made substantial contributions to fully establish that protein traffic occurs between the SE-CC complex and its neighboring cells. Dr. A. Itaya generated CoYMV:3a MP:GFP-transgenic Arabidospsis. I performed most of the microscopic work to examine the traffic pattern of 3a MP:GFP in these plants. The data are presented in this chapter. In addition, I generated CoYMV:GFP-transgenic Arabidopsis, and AtSUC2:3a MP:GFP- and AtSUC2:GFP-transgenic tobacco. Data from these plants are also presented in this chapter.

MATERIALS AND METHODS

Plant material and growth conditions

The procedures were as described in chapter II.

Construction of binary vectors

To test protein traffic between tissue boundaries, CC-specific CoYMV and *AtSUC2* promoters were inserted upstream of *3a MP:GFP* and *GFP* in binary vectors. Binary vector construction for each promoter is described below.

The construction of plasmids named pRTL2-3a MP:GFP and pRTL2-GFP which contain *3a MP:GFP* and *GFP* sequence, respectively, was previously described by Itaya et al. (1997). The fusion of *Tobacco Etch Virus* (TEV) leader sequence and *3a MP:GFP*, *TEV:3a MP:GFP* was amplified by *Pfu* polymerase with primers, *Ava*I-TEV leader and GFP-*Sac*I and pRTL2-3a MP:GFP as a template. The fusion of TEV leader sequence and *GFP*, *TEV:GFP,* was amplified by *Pfu* polymerase with primers *Ava*I-TEV leader (5'-CCCCCGGGCTCAACACAACATATA-3'; *Ava*I site underlined) and GFP-*Sac*I (5'-TCCGAGCTCTTACTTGTACAGCTCGTCCA-3'; *Sac*I site underlined), and pRTL2-GFP as a template. Each PCR product was double-digested with *Sma*I and *Sac*I, and inserted into binary vector pGPTV-Kan at the appropriate sites to replace *GUS*, resulting in pGPTV-3a MP:GFP and pGPTV-GFP, respectively.

TEV:GFP was inserted at the *Sma*I site of pCOI (chapter II) by conventional blunt end ligation. The resulting binary vector was named pCOI-GFP.

AtSUC2 promoter described in chapter II was inserted at the appropriate sites of pGPTV-3a MP:GFP and pGPTV-GFP. The resulting binary vectors were named pGPTV-AtSUC2:3a MP:GFP and pGPTV-AtSUC2:GFP, respectively.

Transformation of Agrobacterium tumefaciens

Binary vectors pCOI-GFP, pGPTV-AtSUC2:3a MP:GFP and pGPTV-AtSUC2:GFP (Fig. 1) were used to transform *A. tumefaciens* LBA4404.

Transformation of tobacco and Arabidopsis

The procedures for transformation of Arabidopsis with pCOI-GFP and for transformation of tobacco with pGPTV-AtSUC2:3a MP:GFP and pGPTV-AtSUC2:GFP were as described in chapter II. Transgenic Arabidopsis plants expressing 3a MP:GFP under the CoYMV promoter were provided by Dr. Asuka Itaya.

Confocal laser scanning microscopy

Free-hand sections of transgenic leaves and stems were examined for the presence of fluorescent signals from 3a MP:GFP or GFP under a PCM-2000 confocal laser scanning microscope (CLSM) equipped with Argon and green HeNe Lasers (Nikon Corp., Tokyo, Japan).

RESULTS

3a MP:GFP traffics out of symplasmically isolated SE-CC complex in transgenic tobacco

Two CC-specific promoters, the CoYMV promoter and the *AtSUC2* promoter, were used to drive expression of 3a MP:GFP in the CCs of transgenic tobacco. Results from the CoYMV:3a MP:GFP-transgenic tobacco have been described in detail by Itaya et al. (2002). The highlights of results will be presented here for comparison. Briefly, 3a MP:GFP was found to traffic from SE-CC complex into neighboring cells in all leaf and stem samples examined by CLSM. Its traffic was most extensive in mature stems, reaching all pith cells (Fig. 2A-B). In leaves, traffic of 3a MP:GFP to xylem parenchyma was extensive, but to nonvascular tissues such as bundle sheath cells or MCs it was limited (Fig. 2C-F). Traffic of 3a MP:GFP out of the SE-CC complex through PD was confirmed by *in situ* localization of *3a MP:GFP* mRNA in CCs, immunolocalization of 3a MP:GFP protein to PD at the interfaces between CCs and other cells, and movement of the fusion protein from a transgenic stock to a non-transgenic scion and further out of the phloem in the scion (Itaya et al., 2002).

To provide independent verification of the ability of 3a MP:GFP to traffic out of the SE-CC complex, I generated transgenic tobacco expressing 3a MP:GFP and GFP, respectively, under the control of the *AtSUC2* promoter. Although GFP was confined to CCs (Fig. 3A), 3a MP:GFP was present in CCs and neighboring cells in veins (Fig. 3B-C). Thus, 3a MP:GFP trafficked out of the SE-CC complex in veins. However,

3a MP:GFP was detected only in the SE-CC complex of the petioles in source leaves (Fig. 3D) after approximately 500 sections were examined. This is presumably due to the weak activity of the *AtSUC2* promoter in the petioles (chapter II).

3a MP:GFP traffics out of SE-CC complex in transgenic Arabidopsis

To test whether traffic of 3a MP:GFP out of the symplasmically-isolated SE-CC complex is a general phenomenon, and to develop materials for future genetic studies on the regulation of this traffic, we investigated the ability of 3a MP:GFP to traffic out of the SE-CC complex in CoYMV 3a MP:GFP-transgenic Arabidopsis. A total of four lines of transgenic Arabidopsis plants expressing 3a MP:GFP were obtained. Vein classification of Arabidopsis leaves is summarized in Fig 4A.

3a MP:GFP trafficked out of the SE-CC complex and into xylem parenchyma in young (data not shown) and mature inflorescence stems (Fig. 5A-C, See Fig. 4B for sample location). In both young and mature stems, 3a MP:GFP was not detected in pith, which is different from the situation in transgenic tobacco plants. In order to test whether traffic of 3a MP:GFP out of the SE-CC complex is due to the diffusion, transgenic Arabidopsis plants expressing only GFP under the CoYMV promoter were generated. In all of the 10 independent lines of transgenic plants, GFP was confined to the SE-CC complex in the stem (Fig. 5D). Thus, 3a MP mediates specific traffic of the fusion protein.

In leaves, 3a MP:GFP trafficked out of the SE-CC complex in all veins examined (Fig. 6A-D) whereas GFP remained in the SE-CC complex (Fig. 6E). The traffic of

3a MP:GFP was more extensive as an organ matured but not as significant as observed in tobacco (Fig. 2D-F).

In the mature part of Arabidopsis roots (see Fig. 4B for the sample location), 3a MP:GFP trafficked out of the SE-CC complex (Fig. 7A) whereas GFP remained in the SE-CC complex in transgenic plants expressing only GFP (Fig. 7B). In the immature part of roots where the root apical meristem is located, 3a MP:GFP was almost undetectable and only a few fluorescent dots were observed in the SE-CC complex (Fig. 8A). 3a MP:GFP fusion produced in the root cap was also localized to PD as fluorescent dots (Fig. 8A). The fusion protein did not traffic into other cells (Fig. 8A). Significantly, 3a MP:GFP expression was transient, first visible at 3 days post germination (dpg). It became undetectable after 5 dpg. In contrast, GFP diffused out of the SE-CC complex into surrounding cells in the region (Fig. 8B-D). GFP diffusion in the immature part of roots is consistent with previous findings with AtSUC2:GFP-transgenic Arabidopsis (Imlau et al., 1999) and is reminiscent of fluorescent dye unloading into the root tip (Oparka et al., 1994).

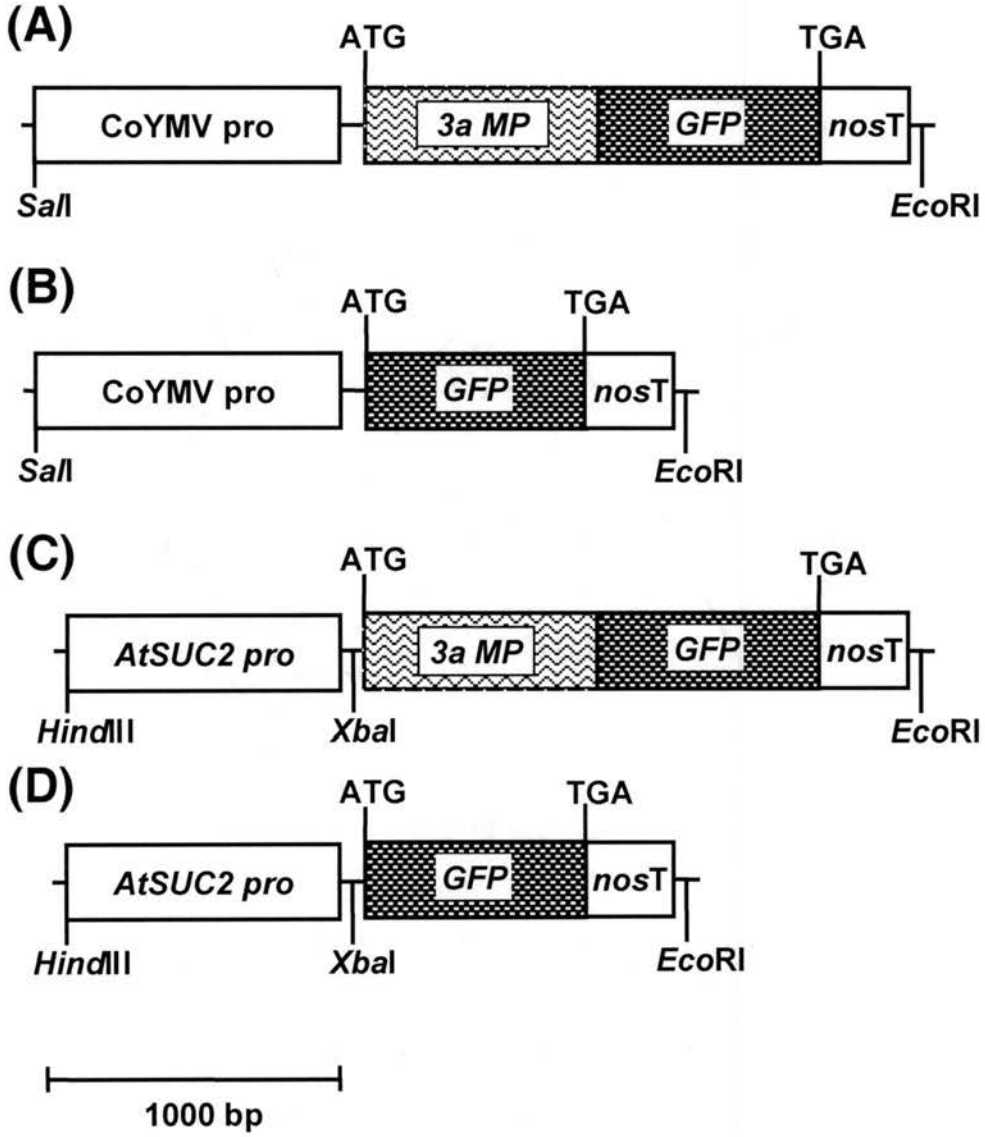
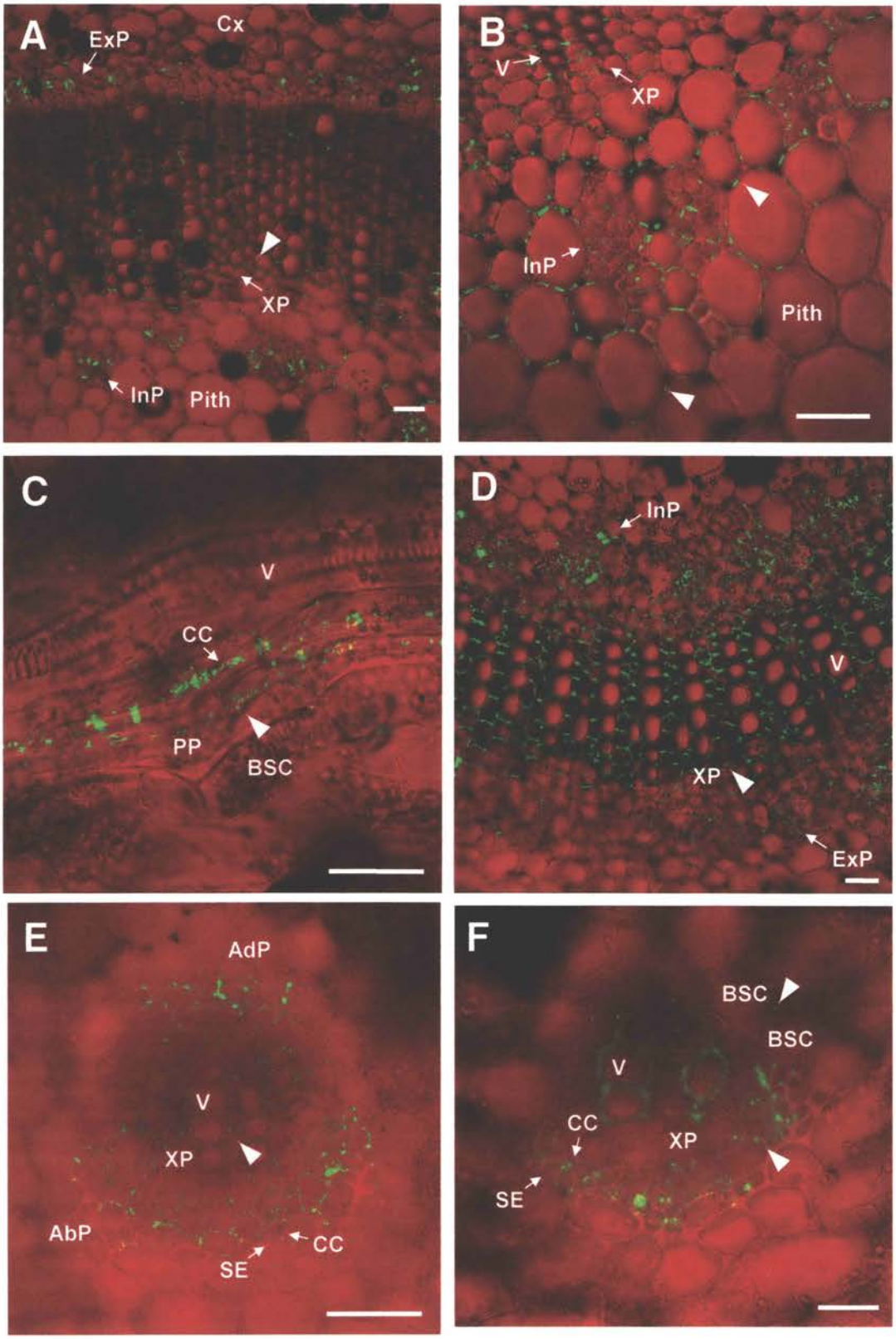


Figure 1. Chimeric constructs used for transformation via *Agrobacterium tumefaciens*
(**A**) CoYMV promoter:*3a MP:GFP* fusion
(**B**) CoYMV promoter:*GFP* fusion
(**C**) *AtSUC2* promoter:*3a MP*:*GFP* fusion
(**D**) *AtSUC2* promoter:*GFP* fusion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V
XP
AbP
CC
SE
F
BSC
BSC
V
CC
XP
SE

Fig. 2

Figure 2. Traffic of 3a MP:GFP out of symplasmically-isolated sieve element (SE)-companion cell (CC) complex in stems and leaves of CoYMV:3a MP:GFP-transgenic tobacco. Bright-field images were taken in grayscale and pseudocolored in red for better cell identification and signal recognition for green fluorescence. All pictures are kindly provided by A. Itaya.
(**A-B**) Confocal images of stem sections from CoYMV:3a MP:GFP-transgenic tobacco. The punctate green dots in the cell walls (arrowheads) represent the presence of 3a MP:GFP in PD.
(**A**) Low magnification transverse view. 3a MP:GFP produced in internal (InP) and external phloem (ExP) traffics into neighboring cells such as xylem parenchyma (XP) and pith. Cx, cortex; V, vessel. Bar:50 μm.
(**B**) High magnification transverse view. 3a MP:GFP is detected in the cell walls of pith. Bar:50 μm.
(**C-F**) Confocal images of leaf sections from CoYMV:3a MP:GFP- transgenic tobacco.
(**C**) Longitudinal view of minor vein. 3a MP:GFP traffics out of SE-CC complex and into bundle sheath cells (BSC). V, vessel; CC, companion cell; PP, phloem parenchyma. Bar:20 μm.
(**D**) Transverse view of class I vein. XP, xylem parenchyma; InP, internal phloem; ExP, external phloem. Bar:50 μm.
(**E**) Tranverse view of class II vein. V, vessel; SE, sieve element; CC, companion cell; XP, xylem parenchyma; AdP, adaxial phloem; AbP, abaxial phloem Bar:50 μm.
(**F**) Transverse view of class III vein. 3a MP:GFP trafficked out of SE-CC complex and into bundle sheath cells (BSC). V, vessel; SE, sieve element; CC, companion cell; XP, xylem parenchyma. Bar:20 μm.

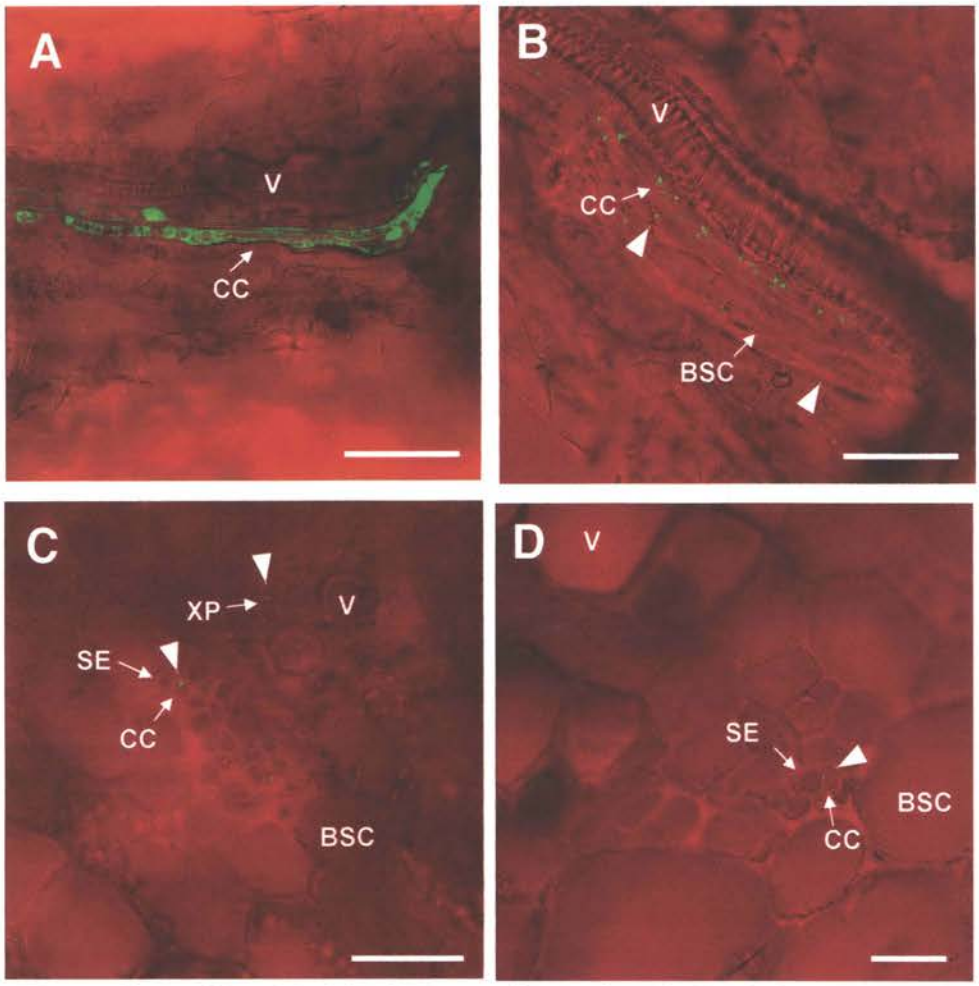


Figure 3. Traffic of 3a MP:GFP out of symplasmically-isolated sieve element (SE)-companion cell (CC) complex in leaves of AtSUC2:3a MP:GFP-transgenic tobacco. Bright-field images were taken in grayscale and pseudocolored in red for better cell identification and signal recognition for green fluorescence.

(**A**) Confocal image of longitudinal view of minor vein of AtSUC2:GFP-transgenic tobacco. GFP is confined to companion cells (CC). V, vessel. Bar:20 μ m.

(**B-D**) Confocal images of leaf sections from AtSUC2:3a MP:GFP-transgenic tobacco. The punctate green dots in the cell walls (arrowheads) represent the presence of 3a MP:GFP in PD.

(**B**) Longitudinal view of minor vein. 3a MP:GFP traffics out of SE-CC complex and into bundle sheath cells (BSC). V, vessel. Bar:20 μ m.

(**C**) Transverse view of class III vein. 3a MP:GFP traffics out of SE-CC complex and into xylem parenchyma (XP). V, vessel; BSC, bundle sheath cell. Bar:20 μ m.

(**D**) Transverse view of petioles. 3a MP:GFP is detected only in the cell walls of SE-CC complex. BSC, bundle sheath cell. Bar:20 μ m.

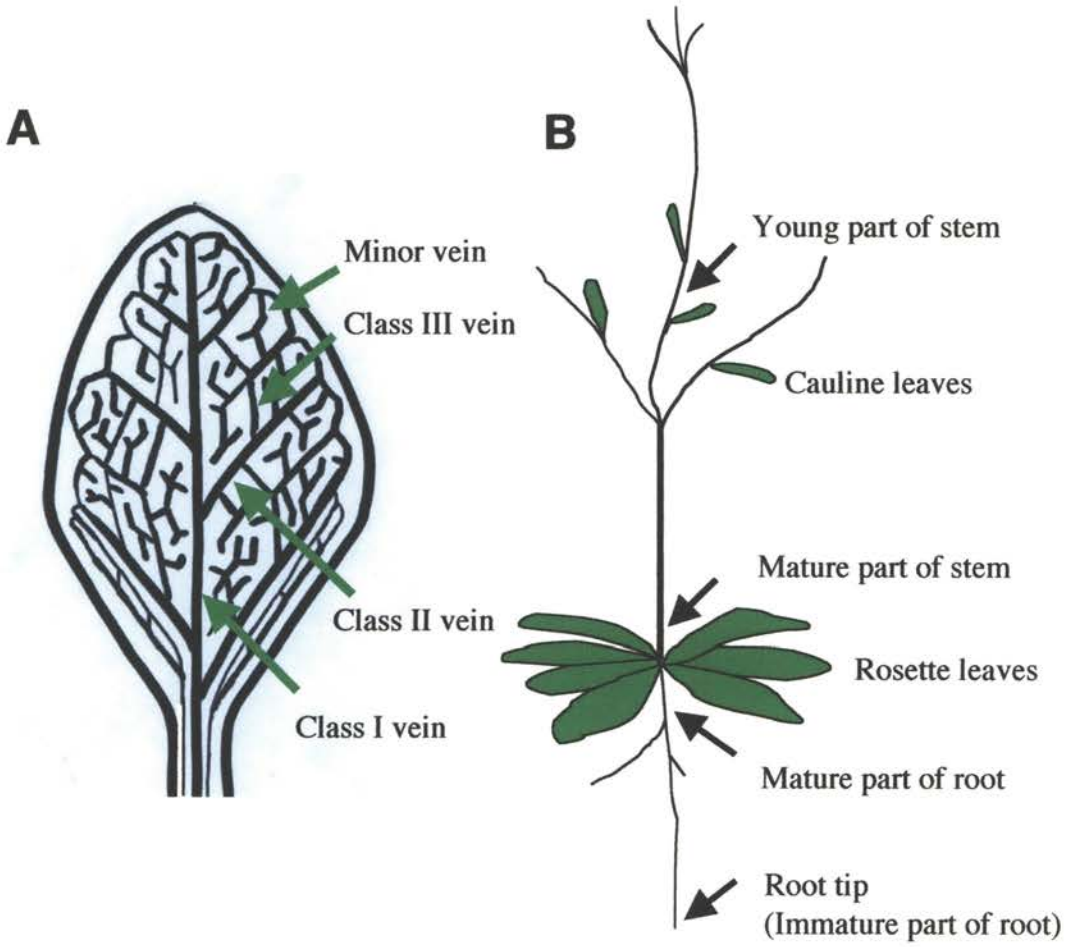


Figure 4. Vein classification and diagramic representation of Arabidopsis.
(**A**) Vein classification of a mature leaf. The class I vein (midrib) gives rise at regular intervals to class II veins. Class III comes from class II. Minor veins are from class III vein.
(**B**) An adult Arabidopsis plant. The arrows indicate the locations where samples were used for microscopic analysis.

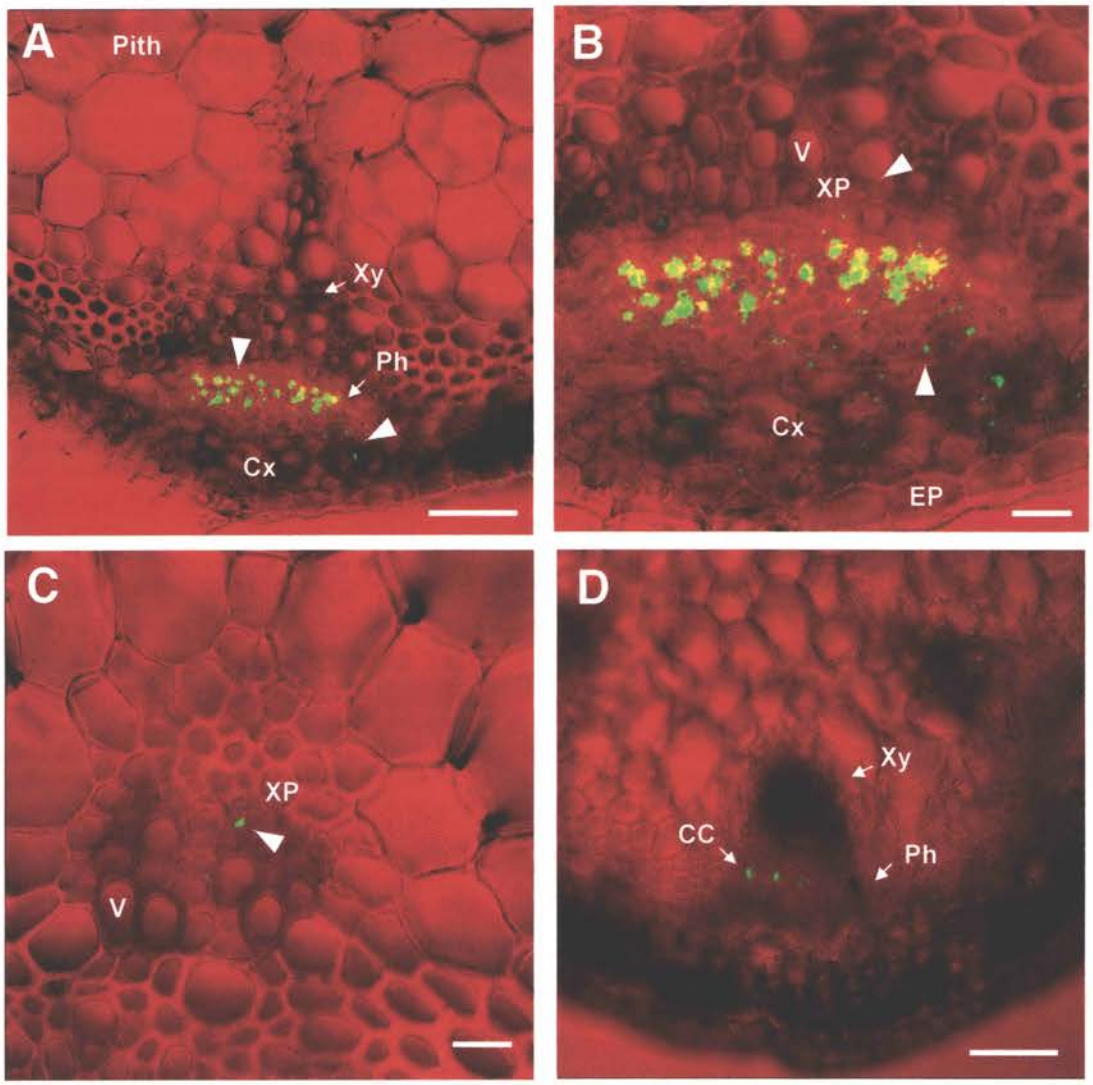


Figure 5. Traffic of 3a MP:GFP out of symplasmically-isolated sieve element (SE)-companion cell (CC) complex in CoYMV:3a MP:GFP-transgenic Arabidopsis. Bright-field images were taken in grayscale and pseudocolored in red for better cell identification and signal recognition for green fluorescence.

(**A-C**) Stem sections from CoYMV:3a MP:GFP-transgenic Arabidopsis.

(**A**) Low magnification transverse view. The punctate green dots in the cell walls (arrowheads) represent the presence of 3a MP:GFP in PD. 3a MP:GFP produced in the phloem (Ph) traffics into neighboring cells such as xylem parenchyma (XP) and cortex (Cx). Xy, xylem; V, vessel. Bar: 50 μm.

(**B**) High magnification transverse view of Fig. 5A including phloem. Bar: 10 μm.

(**C**) High magnification transverse view of xylem. 3a MP:GFP appears in the cell walls of xylem parenchyma (XP). Bar: 10 μm.

(**D**) Transverse view of stem section of CoYMV:GFP-transgenic Arabidopsis. GFP is confined to companion cells (CC) of the phloem (Ph). Xy, xylem. Bar: 50 μm.

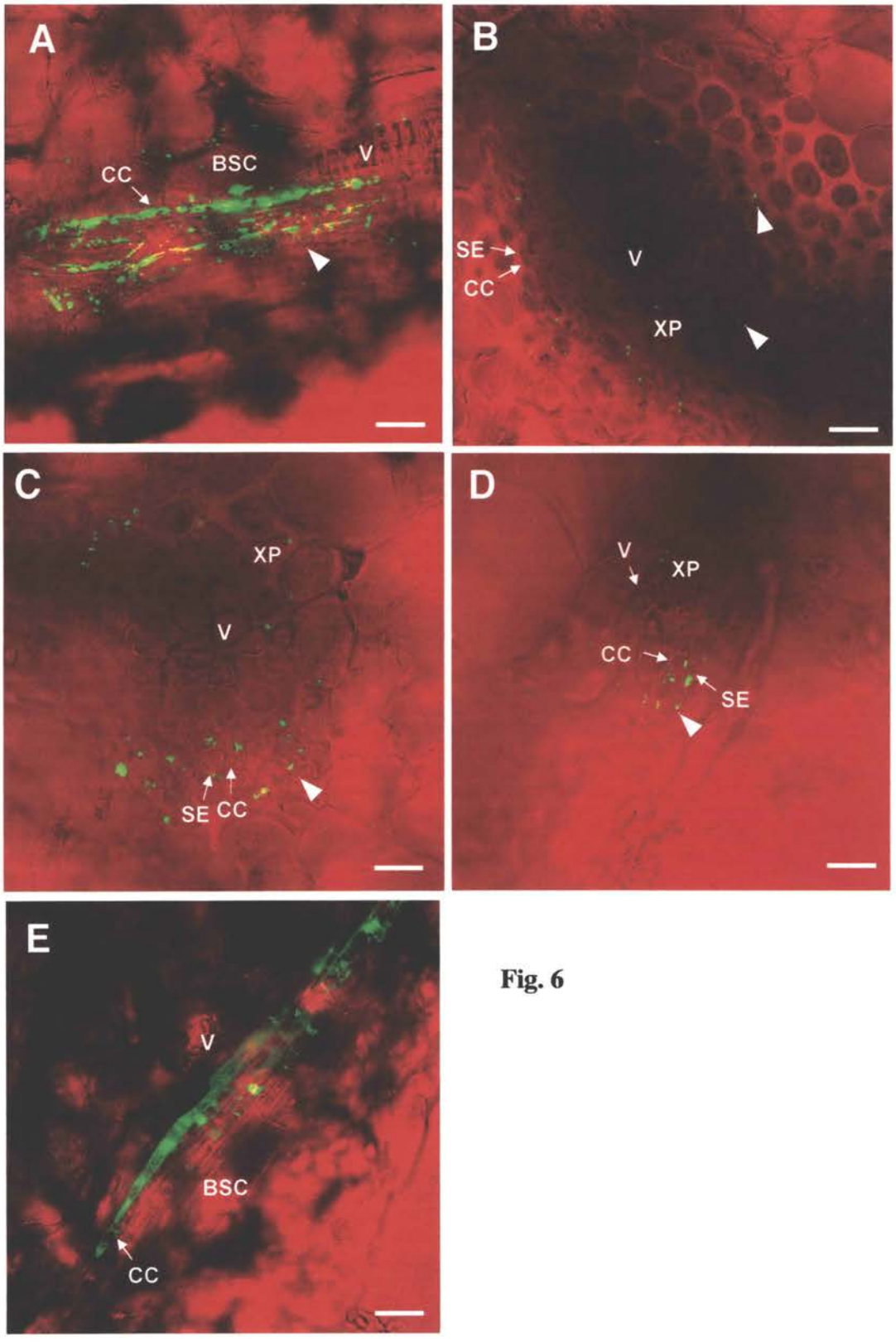


Fig. 6

Figure 6. Traffic of 3a MP:GFP out of symplasmically-isolated sieve element (SE)-companion cell (CC) complex in leaves of CoYMV:3a MP:GFP-transgenic Arabidopsis. Bright-field images were taken in grayscale and pseudocolored in red for better cell identification and signal recognition for green fluorescence.

(**A-D**) Confocal images of leaf sections from CoYMV:3a MP:GFP-transgenic Arabidopsis. The punctate green dots in the cell walls (arrowheads) represent the presence of 3a MP:GFP in PD.

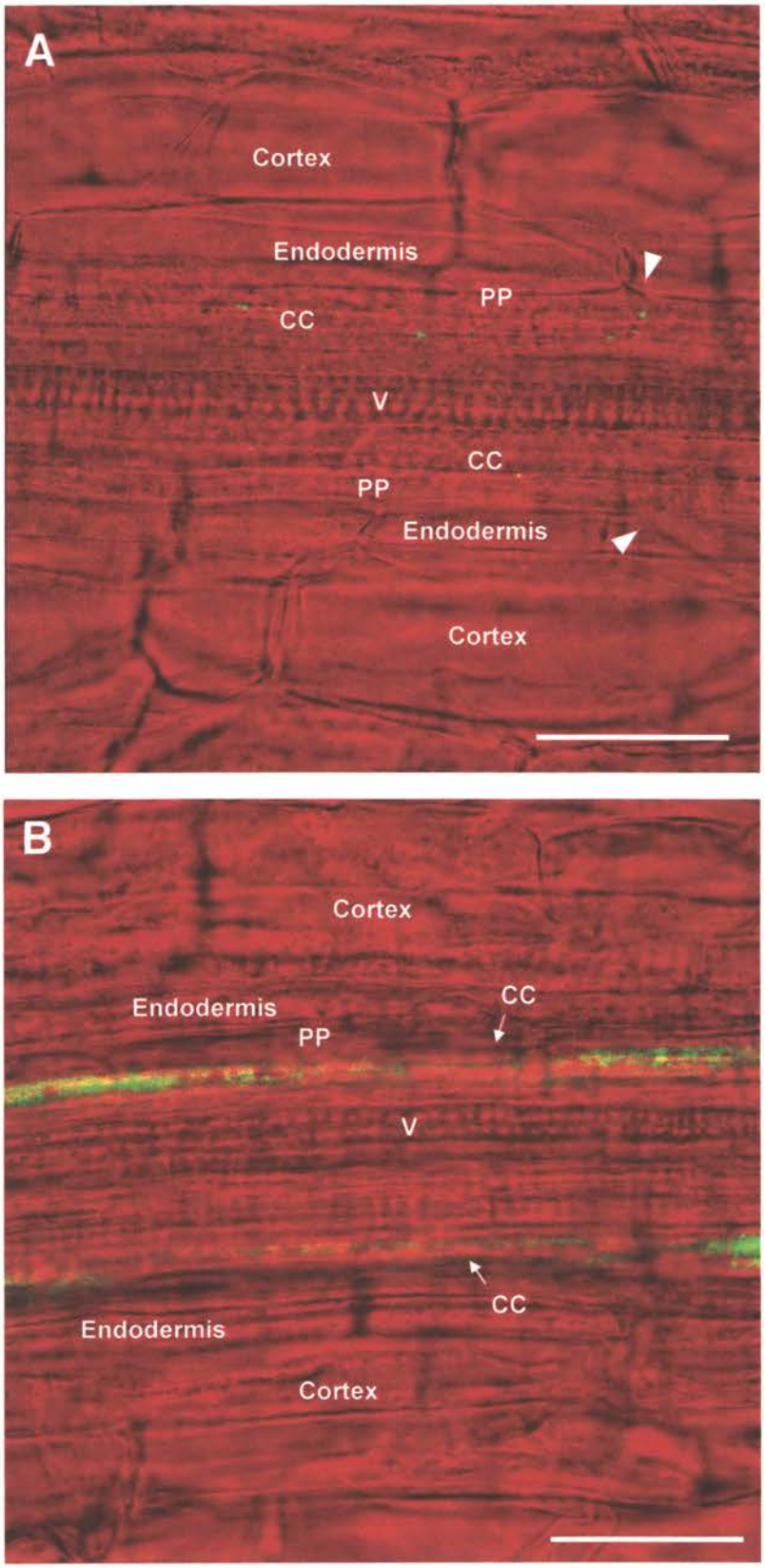
(**A**) Longitudinal view of class III vein. 3a MP:GFP traffics out of SE-CC complex into bundle sheath cells (BSC). V, vessel. Bar:10 μ m.

(**B**) Transverse view of class I vein. 3a MP:GFP traffics from SE-CC complex to xylem parenchyma (XP). V, vessel. Bar:10 μ m.

(**C**) Transverse view of class II vein. 3a MP:GFP traffics from SE-CC complex to xylem parenchyma (XP). V, vessel. Bar:10 μ m.

(**D**) Transverse view of class III vein. 3a MP:GFP traffics out of SE-CC complex and into xylem parenchyma (XP). V, vessel. Bar:10 μ m.

(**E**) Longitudinal view of minor vein of CoYMV:GFP-transgenic Arabidopsis. GFP is confined to companion cells (CC). V, vessel. Bar:10 μ m.


A
Cortex
Endodermis
PP
CC
V
CC
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B
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Endodermis
CC
PP
V
CC
Endodermis
Cortex

Fig. 7

Figure 7. Traffic of 3a MP:GFP out of symplasmically-isolated sieve element (SE)-companion cell (CC) complex in roots of CoYMV:3a MP:GFP-transgenic Arabidopsis. Bright-field images were taken in grayscale and pseudocolored in red for better cell identification and signal recognition for green fluorescence.
(**A**) Confocal image of longitudinal view of mature part of a root from CoYMV:3a MP:GFP- transgenic Arabidopsis. The punctate green dots in the cell walls (arrowheads) represent the presence of 3a MP:GFP in PD. 3a MP:GFP traffics out of SE-CC complex. V, vessel. PP, phloem parenchyma. Bar:10 μ m.
(**B**) Conforcal image of longitudinal view of the mature part of a root from CoYMV:GFP-transgenic Arabidopsis. GFP is confined to SE-CC complex. PP, phloem parenchyma. Bar:10 μ m.

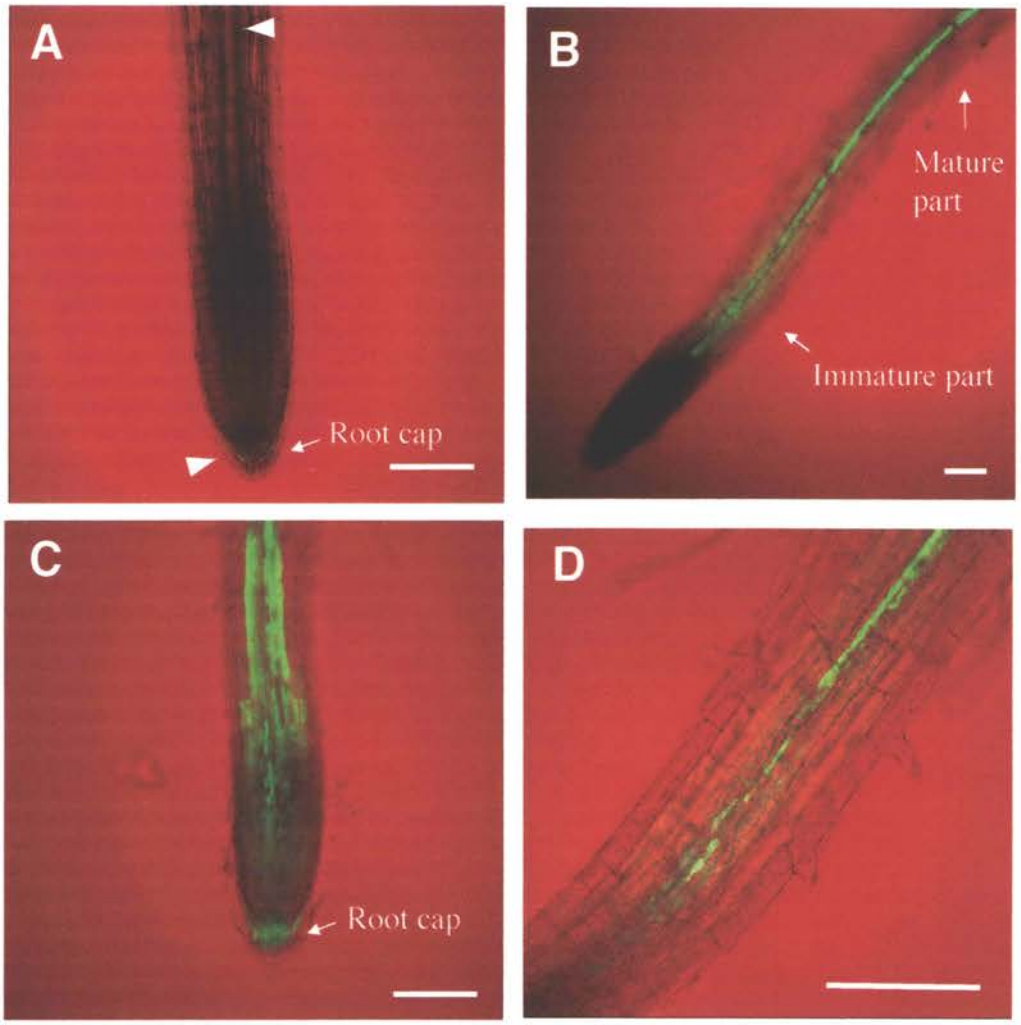

Figure 8. Diffusion of GFP out of sieve element (SE)-companion cell (CC) complex in young part of roots of CoYMV:GFP-transgenic Arabidopsis. Bright-field images were taken in grayscale and pseudocolored in red for better cell identification and signal recognition for green fluorescence.
(**A**) Confocal image of longitudinal view around root tip from CoYMV:3a MP:GFP-transgenic Arabidopsis. The punctate green dots in the cell walls (arrowheads) represent the presence of 3a MP:GFP in PD. 3a MP:GFP appears in root cap. 3a MP:GFP is almost undetected in immature part of a root. Bar:50 μ m.
(**B-D**) Confocal image of longitudinal view around root tip from CoYMV:GFP-transgenic Arabidopsis. Bar:50 μ m.
(**B**) Longitudinal view around root tip. GFP diffuses in the immature part of root but is confined to SE-CC complex in mature part of a root. Bar:50 μ m.
(**C**) High magnification view of root tip. GFP appears in root cap. Bar:50 μ m.
(**D**) High magnification view of the immature region of a root of Fig. 8B. Bar:50 μ m.

DISCUSSION

We have demonstrated that CMV 3a MP mediates traffic of a fusion protein out of the SE-CC complex via PD in transgenic tobacco and Arabidopsis. In general, 3a MP:GFP showed more extensive traffic in CoYMV:3a MP:GFP-transgenic tobacco (Itaya et al., 2002; Fig. 2A-F) than CoYMV:3a MP:GFP-transgenic Arabidopsis (Fig. 5A-C and 6A-D). The observed traffic of 3a MP:GFP out of the SE-CC complex is in contrast to the restriction of small molecules such as fluorescent dyes in the SE-CC complex revealed by dye-coupling experiments (van der Schoot and van Bel, 1989, 1990; van Bel and Kempers, 1991; Knoblauch and van Bel, 1998). The 3a MP:GFP traffic is also in contrast to the restriction of some plant proteins within SE-CC complex. These plant proteins include RTM1 and RTM2 (Chisholm et al., 2001), phloem protein PP1 and PP2 (Dannenhoffer et al., 1997; Golecki et al., 1999), sucrose transporter SUT1 (Kühn et al., 1997), thioredoxin h (Ishiwatari et al., 1998), and CmPP16 (Xoconostle-Cazares et al., 1999). Thus, PD at the interface between the SE-CC complex and neighboring cells seem to possess the ability to restrict diffusion of small molecules and traffic of some proteins out of the SE-CC complex, while allowing traffic of selected proteins out of the complex.

The extent of movement of 3a MP:GFP depends on the developmental stages and organ types. In the mature stems of CoYMV:3a MP:GFP-transgenic tobacco plants, 3a MP:GFP trafficked more extensively to the pith than in immature stems (Itaya et al., 2002). In mature Arabidopsis stems, 3a MP:GFP trafficked to cortex and xylem parenchyma cells. However, it rarely traffics to the pith (Fig. 5A), in contrast to the situation in tobacco (Itaya et al., 2002; Fig. 2A-B). These differences may be due to a

different combination of endogenous factors involved in 3a MP:GFP traffic in different species. Investigation into the endogenous factors that are involved in the regulation of traffic between tissue boundaries and in different species should shed light on the mechanisms and functions of selective transport in plants.

Viruses can spread systemically by entering and exiting the SE-CC complex despite that the complex is symplasmically isolated from surrounding cells in terms of restriction of small molecule diffusion. MPs or other viral proteins are likely to be key players to establish systemic movement of viruses through the SE-CC complex (Carrington et al., 1996; Oparka and Turgeon, 1999). Our results showing 3a MP:GFP traffic supports the hypothesis. Upon infection of CMV, 3a MP enters SEs from CCs through PD (Blackman et al., 1998). This suggests the importance of 3a MP for CMV to move into the SE for long-distance transport. However, how a virus exits the SE-CC complex is not known. Our results suggest that CMV also utilizes 3a MP to traffic out of the SE-CC complex. Some viruses are known to be restricted to the phloem (Sanger et al., 1994; van der Heuvel et al, 1995). It is worth examining the ability of their MPs for traffic out between the SE-CC complex and surrounding cells.

It is significant that in the young region of the Arabidopsis roots, GFP can diffuse out of the SE-CC complex and enter all surrounding cells. On the contrary, 3a MP:GFP produced in the SE-CC traffics into other cells in the mature part of the roots, however, 3a MP:GFP produced in root cap remained in this tissue. SHR produced in the stele traffics only one cell layer to specify formation of endodermis (Nakajima et al., 2001). These data, collectively, support the hypothesis that the signal-directed protein traffic, presumably also the case for the SHR, is highly regulated by cell- or tissue-specific

factors. Elucidating the mechanisms of such regulation should provide significant insights into the role of intercellular protein traffic in plant development.

CHAPTER IV

TRAFFIC OF VIRAL MOVEMENT PROTEIN:GREEN FLUORESCENT PROTEIN FUSION FROM MESOPHYLL TO EPIDERMIS

INTRODUCTION

Organogenesis during plant development is accomplished through division, growth and differentiation of groups of cells in the shoot apical meristem (SAM). In most angiosperms, the SAM consists of three cell layers of different genetic lineages (Satina et al., 1940; Sussex, 1989). In the outermost layer called layer 1 (L_1), the cells undergo anticlinal cell divisions and give rise to the epidermis (EPs). The inner L_2 cells divide anticlininally within the SAM and in all planes during organogenesis. The innermost L_3 cells divide in all planes. The L_2 produces the bulk of leaf tissues including mesophyll cells (MCs) and floral organs and L_3 mostly contributes to the stem (Satina et al., 1940; Stewart, 1978; Tilney-Bassett, 1986).

Intercellular communication between layers is important for coordinated differentiation of cells. Such intercellular communication may be accomplished by non-cell-autonomous function of certain transcription factors. In wild-type maize, the 45 kDa homeobox protein KNOTTED1(KN1) appears to function to maintain cells in a meristematic state. KN1 is normally expressed in the SAM and determines the meristem cell fate (Hake and Freeling, 1986). Ectopic expression of KN1 in MCs can cause abnormal cell division of epidermis (EPs), which is derived from L_1 of SAM (Hake and Freeling, 1986). In wild-type maize, immunolabeling showed that KN1 is localized in all the layers of nuclei in the SAM. However, *in situ* hybridization localized the *KN1* mRNA in L_2 and L_3, but not in L_1 (Jackson et al., 1994). These findings suggest that KN1 could move from inner layers to L_1. Direct evidence of intercellular movement of KN1 was provided by microinjection of KN1 in MCs, which revealed that the KN1 not only

traffics intercellularly through PD but also increases the SEL of PD and facilitates traffic of its own sense mRNA (Lucas et al., 1995). Recently, Kim et al. (2002) showed that GFP:KN1 fusion can move from L_1 to inner layers when it is expressed under the control of the L_1-specific *AtML1* promoter in Arabidopsis.

A transcription factor called FLORICAULA (FLO) functions non-cell-autonomously during transition of inflorescence stem to floral meristem in *Antirrhinum majas* (Carpenter and Coen, 1995; Hantke et al., 1995). The *flo* mutants have inflorescence branches instead of flowers. Periclinal chimeras in which the L_1 was wild type and L_2 and L_3 were mutant alleles showed normal phenotype, suggesting that FLO moves from L_1 to L_2 and L_3 to restore the wild-type phenotype (Carpenter and Coen, 1995). Microinjection of FLO into MCs showed that FLO moves intercellularly (Mezitt and Lucas, 1996). In Arabidopsis, a similar transcription factor, PISTILLATA (PI), was found to possess same functions for flower development. The normal flower formation was observed when PI is expressed in L_1 of *pi* mutant (Bouhidel and Irish, 1996).

DEFICIENS (DEF) and GLOBOSA (GLO) are MADS-box transcription factors (Schwarz-Sommer et al., 1990) in *A. majas* that control petal and stamen organ identity (Schwarz-Sommer et al., 1992; Sommer et al., 1990; Tröbner et al., 1992). DEF and GLO function non-cell-autonomously between the layers, but not within the layer in the floral meristem (Perbal et al., 1996). DEF appears to move from L_2 or inner layers to L_1, but not from L_1 to inner layers. Therefore, expression of DEF and GLO in L_1 do not restore the mutant phenotype. Thus, PD between L_1 and L_2 layers seem to facilitate unidirectional traffic of some proteins.

Transcription factor LEAFY (LFY) from Arabidopsis traffics cell-to-cell and rescues the *lfy* mutants (Sessions et al., 2000). The chimeric plants expressing LFY in *lfy* mutant background, created by FLP recombinase, showed the normal flower phenotype. When LFY is specifically expressed in L_1 using a L_1-specific promoter, *AtML1*, the transcription factor is detected in L_1 and other layers even though *in situ* hybridization revealed that the mRNA is located only in L_1. These results suggest that LFY traffics from L_1 to inner layers and is biologically functional in the inner layers. APETALA1 (AP1), however, acts cell-autonomously and does not traffic cell-to-cell (Sessions et al., 2000). These results suggest that protein traffic is selective presumably based on the function of the proteins. Furthermore, traffic of some proteins across tissue interfaces is important for plant development. However, the mechanisms that regulate protein traffic between specific tissues are not understood.

Non-specific intercellular traffic of GFP has been shown to be developmentally regulated and organ-specific. Biolistic bombardment experiments showed that GFP can diffuse between EPs in sink leaves but not in source leaves of tobacco (Itaya et al., 2000). In addition, Oparka et al. (1999) reported that GFP produced in EPs of tobacco by biolistic bombardment diffused from EPs to MCs in sink leaves. When GFP was expressed under the control of companion cell (CC)-specific *AtSUC2* promoter in transgenic tobacco, GFP was transported from source leaves into sink leaves and unloaded to all cell types including EPs in the sink leaves (Oparka et al., 1999). The question of whether GFP would diffuse between EP and MC in source leaves has not been studied.

To investigate the regulation of protein traffic between MCs to ECs, I generated transgenic tobacco plants that express GFP or CMV 3a MP:GFP, respectively, in MCs under the control of the MC-specific *RBCS3C*, *RBCS2* (see chapter II), -1.2AldP (Kagaya et al., 1995), ppcA-L-Ft (Stockhaus et al., 1994) and *CAB2* (Anderson et al., 1994) promoters. Confocal microscopic examination of samples was conducted to determine whether traffic of GFP or CMV 3a MP:GFP from MCs to EPs occurs in source leaves of transgenic tobacco.

MATERIALS AND METHODS

Plant material and growth conditions

Tobacco (*Nicotiana tabacum* cv. Samsun NN) was grown in a growth chamber controlled at 14 hours light (28°C)/10 hours dark (22°C) cycles.

Construction of binary vectors

To test protein traffic between MC and EP, MC-specific *RBCS2*, *RBCS3C*, -1.2AldP, ppcA-L-Ft and *CAB2* promoters were inserted upstream of *3a MP:GFP* and *GFP* in binary vectors. Binary vector construction for each promoter is described below.

The binary vector pCV1.6RBCS3CP containing the *RBCS3C* promoter (chapter II) was double-digested with *Eco*RI and *Sna*BI. The DNA fragment containing the *RBCS3C* promoter and 385 bp of *GUS* sequence was ligated into *Eco*RI-*Sma*I double-digested pBKII(+). The resulting plasmid was named pBK-RBCS3C:GUS385. The 385 bp GUS sequence was removed by sequential digestion with *Mef*I and *Spe*I. The

linearized plasmid without the 385 bp of *GUS* sequence was treated with *Pfu* polymerase to make the conhesive ends blunt. The fragment was self-ligated resulting in pBK-RBCS3C. The *RBCS3C* promoter was excised out from the pBK-RBCS3C with *Hind*III and *Xba*I and inserted into pGPTV-3a MP:GFP and pGPTV-GFP at the appropriate sites, respectively. The resulting plasmids were named pGPTV-RBCS3C:3a MP:GFP and pGPTV-RBCS3C:GFP, respectively.

The PCR-amplified *RBCS2* promoter (chapter II) was double-digested with *Hind*III and *Xba*I, and inserted into pGPTV-3a MP:GFP and pGPTV-GFP at the appropriate sites, respectively. The resulting plasmids were named pGPTV-RBCS2:3a MP:GFP and pGPTV-RBCS2:GFP, respectively.

Binary vector –1.2AldP-GUS containing -1.2AldP promoter, 1.2 kb of 5' upstream of chroloplastic Aldolase from rice (Kagaya et al, 1995), was kindly provided by Dr. Tsutsumi in Iwate University, Japan. The -1.2AldP promoter was double-digested with *Hind*III and *Xba*I, and inserted into pGPTV-3a MP:GFP and pGPTV-GFP at the appropriate sites, respectively. The resulting plasmids were named pGPTV-1.2AldP:3a MP:GFP and pGPTV-1.2AldP:GFP, respectively.

Plasmid ppcA-L-Ft (pBS) was kindly provided by Dr. Peter Westhoff at Heinrich-Heine-Universität, Germany. The ppcA-L-Ft promoter, 5' upstream sequence between position –2118 to +66 of phosphoenolpyruvate carboxylase from *Flaveria trierva* (Stockhaus et al., 1994), was excised from the plasmid with *Hind*III and *Sma*I, and inserted into appropriate sites of pGPTV-3a MP:GFP and pGPTV-GFP, resulting in pGPTV-ppcA-L-Ft:3a MP:GFP and pGPTV-ppcA-L-Ft:GFP, respectively.

Plasmid pBK-CAB2 was kindly provided by Dr. Steve Kay at Scripps Institute. *CAB2* promoter, 5' upstream sequence between position -198 to +1 of chlorophyll a/b binding protein from Arabidopsis (Anderson et al., 1994), was PCR-amplified with primers *Hind*III-CAB2 (5'-CCCAAGCTTAACTTGTGGTCACAA-3'; *Hind*III site underlined) and CAB2-*Xba*I (5'-TGCTCTAGAGATTAAAACTGGTTC-3'; *Xba*I site underlined), and pBK-CAB2 as a template. The PCR product was double-digested with *Hind*III and *Xba*I and was inserted into pGPTV-3a MP:GFP and pGPTV-GFP at the appropriate sites. The resulting plasmids were named pGPTV-CAB2:3a MP:GFP pGPTV-CAB2:GFP, respectively.

Transformation of Agrobacterium tumefaciens

Binary vectors pGPTV-RBCS2:3a MP:GFP and pGPTV-RBCS2:GFP (Fig. 1) were used to transform *A. tumefaciens* LBA4404. Other binary vectors were also used to transform *A. tumefaciens* LBA4404.

Transformation of tobacco and Arabidopsis

The procedures were as described in chapter II.

Confocal laser scanning microscopy

Free-hand sections of transgenic leaves were examined under the Nikon PCM-2000 CLSM as described in chapter III.

RESULTS

Several MC-specific promoters were used to drive 3a MP:GFP expression in transgenic tobacco. These promoters include *RBCS3C* (chapter II), *RBCS2* (chapter II), -1.2AldP (Kagaya et al., 1995), ppcA-L-Ft (Stockhaus et al., 1994) and *CAB2* (Anderson et al., 1994). Initial analyses showed that in transgenic plants expressing 3a MP:GFP under the *RBCS3C*, –1.2AldP, ppcA-L-Ft , and *CAB2* promoters, the signal of the fusion protein was very weak or absent. Presumably, these promoters were not strong enough. Alternatively, other factors such as gene silencing might have contributed to the poor expression of 3a MP:GFP. These plants were not used for further analysis.

However, good expression of GFP and 3a MP:GFP fusion protein was achieved from the *RBCS2* promoter. The results from RBCS2:GFP- and RBCS2:3a MP:GFP-transgenic tobacco, respectively, are presented in this chapter.

GFP produced in MCs of source leaves moves into sink leaves

A total of six transgenic tobacco plants expressing GFP under the control of the *RBCS2* promoter were examined for the presence of GFP in source and sink leaves. As shown in Fig. 2A, GFP was detected in all MCs but not in the EPs, consistent with MC-specific activity of the *RBCS2* promoter. However, in sink leaves where the *RBCS2* promoter is not active, GFP was detected in the EPs as well as MCs (Fig. 2B). GFP was even detected in the trichomes. In all cells, GFP was concentrated in the nuclei. Therefore, GFP produced in MCs of source leaves was apparently translocated to sink leaves through the phloem. In the sink leaves, GFP was unloaded into all cell types.

Importantly, while GFP did not move from MCs into EPs in source leaves, it did move from MCs into EPs in sink leaves. Therefore, PD between MCs and EPs have a large SEL in sink leaves, which is down-regulated when the leaves become mature.

3a MP:GFP traffics from mesophyll cells to epidermis in source leaves

To test signal-directed protein traffic from MCs to EPs, 3a MP:GFP fusion protein was expressed in MCs of source leaves in tobacco under the control of the *RBCS2* promoter. A total of six transgenic lines expressing 3a MP:GFP were obtained. 3a MP:GFP was targeted to PD at the MC-EP tissue boundary (Fig. 2C) and also accumulated in PD between EPs (Fig. 2C and Fig. 3), indicating that the 3a MP:GFP trafficked from the MCs to EPs in the source leaves. This is in contrast to the non-movement of GFP from MCs to EPs in source leaves (Fig. 2A).

In sink leaves, there was no 3a MP:GFP signal (Fig. 2D). This is consistent with the observation that *RBCS2* promoter is not active in sink leaves. The data also indicate that translocation of 3a MP:GFP from source to sink leaves was very limited. During leaf maturation, 3a MP:GFP was first detected in the tip region of the leaf (Fig. 4), corresponding to maturation of the leaf from tip to base.

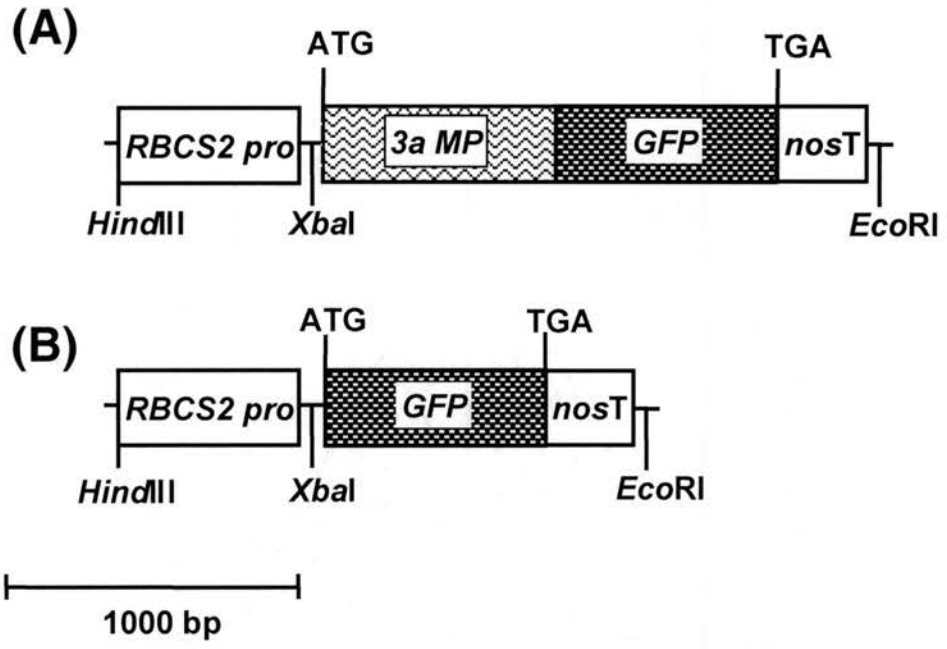

Figure 1. Chimeric constructs used for transformation via *Agrobacterium tumefaciens*
(**A**) *RBCS2* promoter:*3a MP:GFP* fusion
(**B**) *RBCS2* promoter:*GFP* fusion

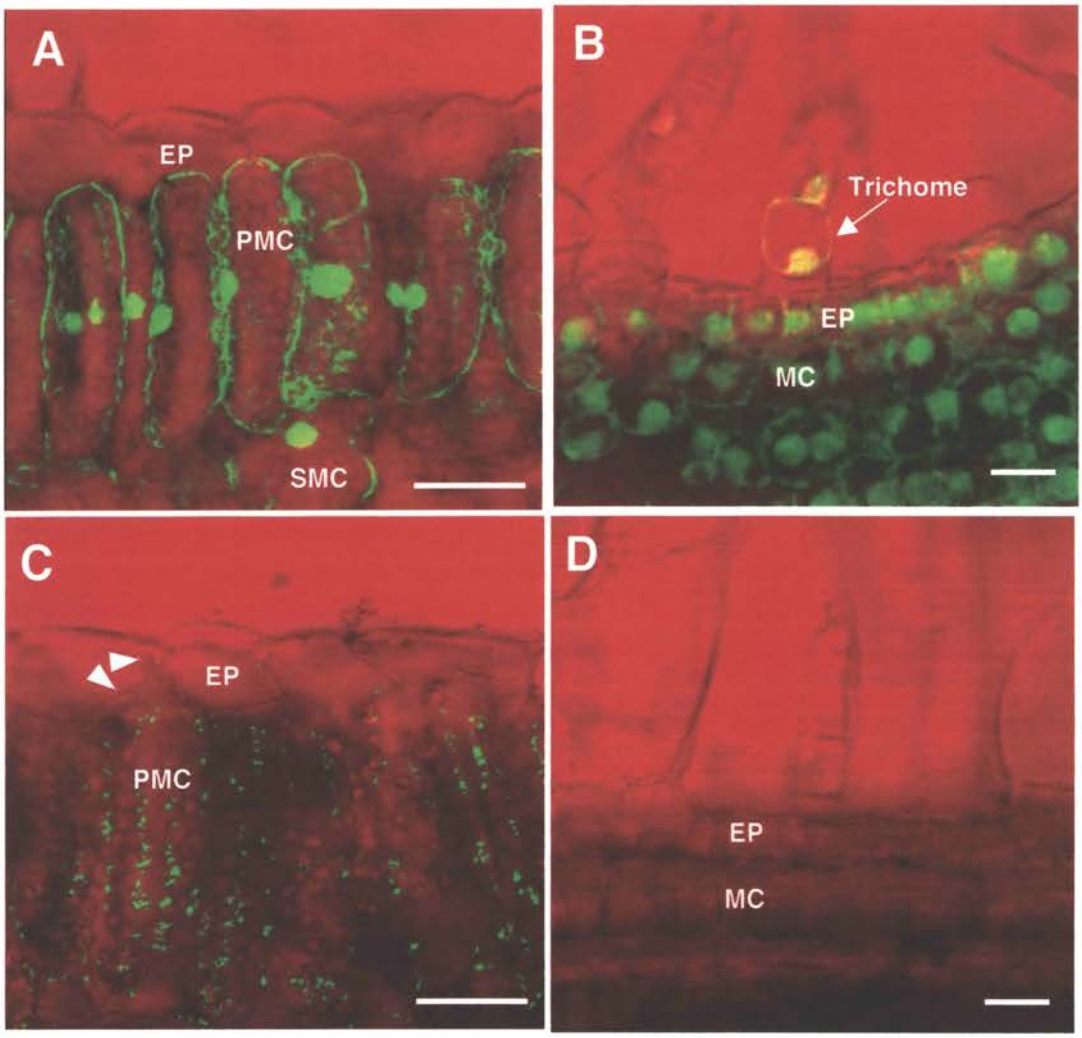

Figure 2. Traffic of 3a MP:GFP from mesophyll to epidermis in source leaves of RBCS2:3a MP:GFP-transgenic tobacco.
Bright-field images were taken in grayscale and pseudocolored in red for better cell identification and signal recognition for green fluorescence.
(**A-B**) Confocal images of leaf sections from RBCS2:GFP-transgenic tobacco.
(**A**) Transverse view of source leaf. GFP is confined to palisade mesophyll (PMC) and spongy mesophyll (SMC). Bar:20 μ m.
(**B**) Transverse section of sink leaf. GFP is present in all cell types. MC, mesophyll; EP, epidermis. Bar:10 μ m.
(**C-D**) Confocal images of leaf sections from RBCS2:3a MP:GFP-transgenic tobacco. The punctate green dots in the cell walls (arrowheads) represent the presence of 3a MP:GFP in PD.
(**C**) Transverse view of source leaf. 3a MP:GFP traffics from PMC to epidermis (EP). 3a MP: GFP is detected in the cells walls between EP and MC and between EP (arrowheads). Bar: 20 μ m.
(**D**) Transverse section of sink leaf. There is no 3a MP: GFP siganal. MC, mesophyll. EP, epidermis . Bar: 10 μ m.

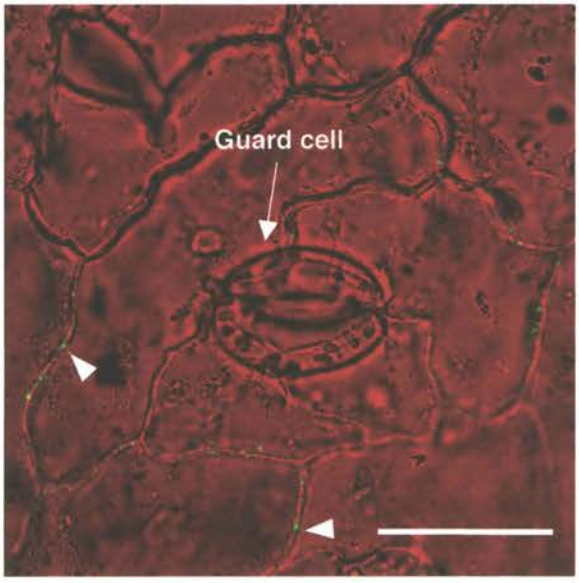

Figure 3. 3a MP:GFP traffics from mesophyll to epidermis and accumulates in PD between epidermal cells in RBCS2:3a MP:GFP-transgenic tobacco.
Bright-field image was taken in grayscale and pseudocolored in red for better cell identification and signal recognition for green fluorescence. Confocal image of the surface of a source leaf from RBCS2:3a MP:GFP-transgenic tobacco. The punctate green dots in the cell walls (arrowheads) represent the presence of 3a MP:GFP in PD. Bar: 20 μm.

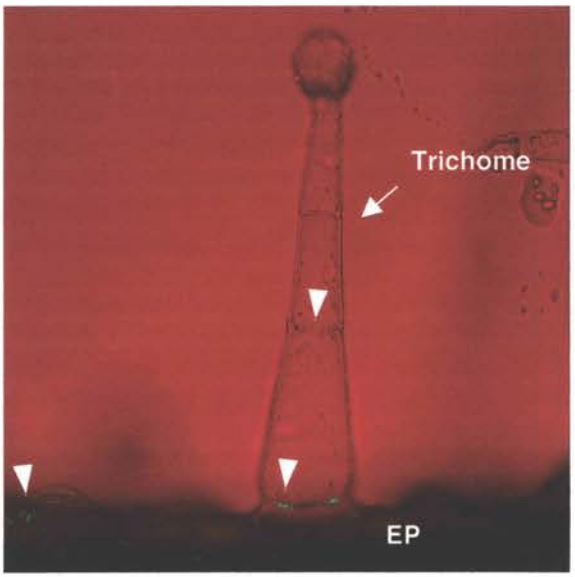

Figure 4. 3a MP:GFP traffic in the tip of a sink-to-source transition leaf in RBCS2:3a MP:GFP-transgenic tobacco. Bright-field image was taken in grayscale and pseudocolored in red for better cell identification and signal recognition for green fluorescence. The punctate green dots in the cell walls (arrowheads) represent the presence of 3a MP:GFP in PD. 3a MP:GFP appears in the cell walls between the trichomes and epidermal cells (EP).

DISCUSSION

Results from the present study have important implications in understanding the mechanisms of regulation of protein traffic and viral movement. Previous work showed that between epidermal cells in tobacco, the PD SEL in sink leaves is rather large to allow diffusion of GFP (Oparka et al., 1999; Itaya et al., 2000). During leaf development, the SEL is down-regulated so that GFP no longer diffuses intercellularly in source leaves (Oparka et al., 1999, Itaya et al, 2000). Significantly, this down-regulation of PD SEL is correlated with the onset of CMV 3a MP:GFP traffic in source leaves (Itaya et al., 1998, 2000). Here, we demonstrated that PD traffic is similarly regulated between mesophyll and epidermis. Non-traffic of GFP and traffic of 3a MP:GFP between mesophyll and epidermis in source leaves of transgenic tobacco provide the first evidence for the existence of a cellular mechanism that regulates specific protein traffic between mesophyll and epidermis. We suggest that such a mechanism may have evolved to permit selective traffic of endogenous plant proteins across this cellular interface to regulate leaf development or physiology.

Models have been proposed that, for many viruses, including CMV, their MPs bind the viral genomes (RNA and DNA) to form ribonucleoprotein complexes to move intercellularly (Gilbertson and Lucas, 1996; Carrington et al., 1996; Ghoshroy et al., 1997; Ding, 1998; Lazarowitz and Beachy, 1999). All of these models imply that the MPs move across all cellular interfaces. However, MP traffic has only been demonstrated between mesophyll cells or between trichome cells by microinjection (see review by Ding, 1998), between epidermal cells by biolistic bombardment (Itaya et al., 1997, 1998,

2000; Crawford and Zambryski, 2000, 2001), and between CCs and surrounding cells by transgenic methods (Itaya et al., 2002). Our demonstration of MP traffic from the mesophyll to epidermis fills an important knowledge gap in MP traffic between various cellular interfaces. Thus, movement of a ribonucleoprotein, as mediated by an MP, between the mesophyll and epidermis is feasible. Our data also demonstrate that testing the ability of a viral MP to traffic across all cellular boundaries is necessary to understand how a virus moves across these boundaries during systemic infection.

Since *RBCS2* promoter is not active in sink leaves, we were not able to test the ability of 3a MP:GFP to traffic between mesophyll and epidermis in such leaves. Viruses infect young leaves efficiently. Therefore, a future challenge is to develop methods to study protein traffic between various cellular interfaces in sink leaves.

Our success in expressing 3a MP:GFP in the mesophyll of transgenic tobacco suggests that expression of a GFP fusion protein in the mesophyll of Arabidopsis should make it possible to study genetically the mechanisms of protein traffic between the mesophyll and epidermis.

CHAPTER V

SUMMARY AND FUTURE PROSPECTS

Cytoplasmic network for intercellular communication established via plasmodesmata (PD) is essential for plant biology. My Ph. D. dissertation project was focused on investigating the regulation of protein traffic through PD between "symplasmically isolated" SE-CC complex and surrounding cells, and between mesophyll (MC) and epidermis (EP). We identified CC-specific and MC-specific promoters. These promoters were used to express CMV 3a MP:GFP fusion in specific cells. We found that the fusion protein trafficked out of the "symplasmically-isolated" SE-CC complex to surrounding cells, and from MC to EP.

It has been shown that small molecules do not diffuse between symplasmic domains (Lucas et al, 1993; Pfluger and Zambryski, 2001). However, how symplasmic domains communicate with each other for coordinated development, physiology or defense is not understood. Traffic of 3a MP:GFP out of "symplasmically-isolated" SE-CC complex suggests that symplasmic domains may communicate with each other through protein traffic. The deduced transport functions of PD between the SE-CC complex and surrounding cells as demonstrated by dye-coupling experiments (Lucas et al, 1993; Pfluger and Zambryski, 2001) and PD frequencies (Gamalei, 1989; Botha and van Bel, 1992) may need to be re-evaluated by using biologically relevant molecules.

The mechanisms and functions of protein traffic in plants are still poorly understood. In general, endogenous cellular factors involved in protein traffic are little known. To understand the mechanisms of protein traffic through PD, identification of the cellular factors is most needed. Fortunately, we have CoYMV:3a MP:GFP-transgenic Arabidopsis. Mutant screening using CoYMV:3a MP:GFP-transgenic Arabidopsis could lead to identification of genes involved in the protein traffic between the SE-CC complex

and surrounding cells. Studying the functions of the gene products would give new insights of how the SE-CC complex communicates with surrounding cells.

Similarly, mutant screening using RBCS2:3a MP:GFP-transgenic Arabidopsis could lead to isolation of genes involved in the protein traffic between MC and EP. Comparison of the genes products isolated from this system with those from mutant screening with CoYMV:3a MP:GFP-transgenic Arabidopsis would shed light on tissue-specific protein transport systems at different tissue boundaries.

We have studied protein traffic in plants using a viral movement protein, 3a MP, as a probe. Recent discoveries of plant transcription factors with the ability of cell-to-cell movement (Lucas et al., 1995; Mezitt and Lucas, 1996; Perbal et al., 1996; Sessions et al., 2000; Hake, 2001; Nakajima et al., 2001) imply that plants have endogenous movement proteins. To identify more endogenous proteins with distinct functions could help understanding the mechanism of protein traffic in plants in detail.

While CMV infects a wide range of plants and invades plants systemically, there are viruses that infect only the phloem and not other cells (Sanger et al., 1994; van der Heuvel et al., 1995). Mechanisms for the phloem-limitation of such viruses are not known. Functions of viral MPs may play important roles for viral exit from the phloem. Testing traffic of MPs from the phloem-limited viruses out of the SE-CC complex to surrounding cells and beyond the phloem could provide clues whether the functions of MPs is involved in the exit of viruses out of the phloem. Similarly, investigating the ability of MPs to move out of the SE-CC complex and its relationship with viral systemic infection in different plant species may help understand the role of MPs in host specificity.

CMV utilizes 3a MP to move cell-to-cell. In addition, coat protein (CP) (Suzuki et al., 1991; Canto et al., 1997; Kaplan et al., 1998; Schmitz and Rao, 1998; Nagano et al., 2001) and 2b protein (Ding et al., 1995) have been shown to be involved in the movement of CMV. Studying how 3a MP, CP, and 2b interact with each other to promote CMV to move cell-to-cell and long-distance should be a major focus of CMV research.

My thesis work provides the basis for some possible future studies discussed above to advance the field. I hope my research in the past and the future can contribute to plant biology and virus research.

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VITA 2

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