# MACROMOLECULAR TRAFFICKING IN PLANTS

By

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**IN PLANTS**

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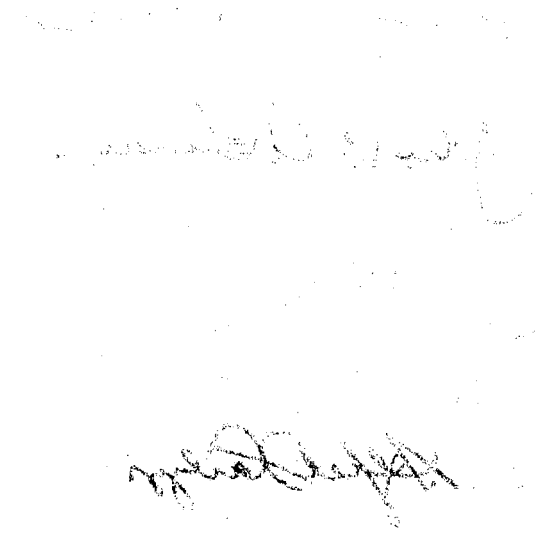

いつも心の故郷である

祖母へ捧ぐ

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[1] Medawar P.B. (1967), in "The Art of the Soluble", Methuen, London

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## ABBREVIATIONS

| | |
|---|---|
| CMV | Cucumber mosaic virus |
| CF | 5(6)-Carboxyfluorescein |
| ddi$H_2O$ | Deionized and distilled $H_2O$ |
| DEPC | Diethyl pryocarbonate |
| dpi | Days post inoculation |
| ER | Endoplasmic reticulum |
| GFP | Green fluorescent protein |
| MP | Movement protein |
| MS salts | Murashige & Skoog salts |
| PME | Pectin methylesterase |
| PD | Plasmodesmata/Plasmodema |
| RCNMV | Red clover necrotic mosaic virus |
| RDA | Representational difference analysis |
| TMV | Tobacco mosaic virus |
| SDS | Sodium dodecyl sulfate |
| SEL | Size exclusion limit |
| SSPE | Sodium chloride, sodium phosphate, EDTA buffer |

# CHAPTER I

# INTRODUCTION

*"General physiological considerations on the establishment and maintenance of correlative harmony, in short for the chain of stimulus transmission, render a continuity of the living substance so essential that it would be necessary to propose it, even if it were not already discovered. However, it is quite likely that the connections would also be utilized in the transport of substances and even, in particular cases, principally or solely for this purpose."*[1]

*Pfeffer, W. (1897)*

Intercellular transport of ions, small molecules and macromolecules occurs extensively in a plant body. Small molecules include $H_2O$, amino acids, simple carbohydrates, (Anderson, 1976; Goodwin, 1976; Gunning, 1976; Robards and Clarkson, 1976; van Steveninck, 1976; Sauter and Kloth, 1986) and other organic compounds such as hormones (Drake and Carr, 1979; Kwiatkowska, 1991). Intercellular trafficking of macromolecules such as proteins and nucleic acids was discovered only a decade ago, however examples of such trafficking have increased in recent years (Robards and Lucas, 1990; Ding 1998; Citovsky and Zambryski, 2000). A fundamental question is: what are the mechanisms and functions of macromolecular trafficking in a plant? Although we are still seeking the final answers to this question, it can be speculated that such trafficking facilitates the cell-to-cell communication that coordinates plant growth, differentiation, and development. Intercellular communication is especially important for plants since they are composed of immobile cells with rigid walls.

In spite of physical constraint, plant cells have great flexibility and adaptability for survival, partly because of their ability to redifferentiate into different types of cells in response to environmental or positional changes. It is well established that positional

[1] Translation by Carr, D. J. (1976), in "Intercellular communication in plants: studies on plasmodesmata" pp. xv. Springer-Verlag Berlin Heidelberg, New York.

information is more important than lineage in determining the developmental fate of a cell. For example, when meristematic cells that produce specific files of cells in an *Arabidopsis thaliana* root are laser-ablated, they can be replaced by neighboring cells of a different lineage (van den Berg et al., 1995). Therefore, clonally different cells can change their developmental fate according to their position within a plant. It is speculated that a yet-to-be identified signal is produced in old cells in a file and transported apoplastically or symplasmically to less differentiated cells in the same file to program their differentiation (van den Berg et al., 1995). Another example of cell-to-cell communication in development comes from trichome patterning. Trichomes are distributed non-randomly with a minimum distance from each other (Larkin et al., 1997). It is believed that formation of a trichome is not clonally determined (Larkin et al., 1996), rather a cell determined to differentiate into a trichome produces a protein called TRIPTYCHON (TRY) which functions non-cell-autonomously to inhibit trichome formation in neighboring cells (Schnittger et al., 1999).

Macromolecular trafficking is also important for plant-pathogen interactions. For example, systemic activation of plant defense responses under pathogen invasion is facilitated by trafficking of macromolecules such as systemin (Narváez-Vásquez, 1995) and probably nucleic acids in gene silencing (Matzke and Matzke, 1995; Covey et al., 1997; Ratcliff et al., 1997;Voinnet and Baulcombe, 1997).

***Dynamic symplasm established by plasmodesmata***

The cytoplasmic channels called plasmodesmata (PD) play an important role in mediating intercellular trafficking. PD interconnect most cells in a plant to establish cytoplasmic continuity (symplast). This communication network is rarely static, and can be reorganized by changes in PD frequencies, structure and functions. A group of cells can become symplasmically isolated during plant development (Tucker, 1982; Erwee and

Goodwin, 1983; Palevitz and Hepler, 1985). Such a group of cells form a symplasmic domain. Very often cells within such a domain follow a common developmental fate and differentiate into a specified tissue or organ. Symplasmic domains are formed in shoot apical meristems (Rinne and Van der Schoot, 1998), developing floral meristems (Bergmans et al., 1993), and developing roots (Duckett et al., 1994; Oparka et al., 1994). These domains may be established by closure of PD (Rinne and van der Schoot, 1998), functional changes of PD and/or a decrease in the number of PD (Bergmans et al., 1997) at specific cellular boundaries.

Symplasmic domain formation by isolation of groups of cells reflects one aspect of the dynamic nature of the intercellular communication network. Another aspect is establishment of new symplasmic connections. For example, secondary PD (see the definition below) are formed between separate carpels during floral development (van der Schoot et al., 1995), during maturation of vascular tissue (Turgeon, 1996; Sjölund, 1997), and between developing lateral roots and the main root (Oparka et al., 1995). Thus, the formation, modification, and differential distribution of PD are important in the establishment and reorganization of the intercellular communication network that could have direct consequences on plant development and physiology (Robards and Lucas, 1990; Lucas et al., 1993; Ding and Lucas, 1996; McLean et al., 1997; Ding et al., 1999).

### *Formation of plasmodesmata*

#### *Primary plasmodesmata*

PD can be formed in two basic ways: cytokinetically or post-cytokinetically. PD formed during cytokinesis are called primary PD. The process of primary PD formation has been well characterized (Hepler, 1982; Robards and Lucas, 1990; Lucas et al., 1993). During cytokinesis, endoplasmic reticulum (ER) strands are entrapped within the newly developing cell plate. The highly compressed ER strands, together with the plasma

membrane, form primary PD. These PD usually have a single strand of ER and therefore appear linear morphologically.

*Secondary plasmodesmata*

Secondary PD are formed post-cytokinetically across existing cell walls (Lucas et al., 1993). Observations on fusing protoplasts suggest that branched half PD are formed in regenerating cell walls by entrapment of ER strands as in primary PD formation. When two cells are fused, the half PD from each cell become connected (Monzer, 1991; Ehlers and Kollmann, 1996). Studies on graft unions suggest that formation of secondary PD at the fusing walls starts with anchoring of ER strands to local wall regions. The cell walls then start thinning locally and allow ER strands from adjoining cells to unite. Rebuilding of the cell walls entraps the ER strands resulting in formation of secondary PD (Kollmann et al., 1985; Kollmann and Glockmann, 1985; 1991). These secondary PD are structurally characterized as multi-branches interconnected in the middle lamella region of the cell walls.

Secondary PD are also formed during normal plant development. In the shoot apical meristem, cells in the $L_1$ layer (the outermost layer) divide anticlinally (the new cell wall plate is perpendicular to organ surface) to produce epidermis (Sussex, 1989). There are no cell division walls between $L_1$ and $L_2$ (the layer beneath the $L_1$). Therefore, PD connecting $L_1$ and $L_2$ cells are secondary PD. The mechanism of secondary PD formation at this boundary is currently unknown. Formation of secondary PD is also found between tissues of different origins as discussed above (van der Schoot et al., 1995; Oparka et al., 1995; Turgeon, 1996; Sjolund, 1997).

*Structural modification of existing plasmodesmata*

Primary PD formed during cytokinesis can be modified and become branched during cell wall thickening by fusion of Golgi vesicles (Ehlers and Kollmann, 1996). Specifically, cytoplasmic ER strands associated with primary PD are entrapped in developing cell wall adding new branches to the existing primary PD. This type of PD is called "branched primary PD" (Ding et al., 1999).

In tobacco leaves, highly branched PD with central cavity appear during leaf development (Ding et al., 1992b, 1993; Itaya et al., 1998; Oparka et al., 1999). They are formed by lateral fusion of primary PD at the middle lamella region followed by addition of new cytoplasmic strands across the existing cell walls. The initial step in the formation can be seen as 'H- or Y-shaped' PD (Ding et al., 1992b, 1993; Itaya et al., 1998). Since *de novo* addition of cytoplasmic strands through existing cell walls is involved in the formation of this type of PD, they are called "complex secondary PD" (Ding, 1998). However, it should be noted that some researchers prefer to call them "modified" or "branched" PD as they originated from primary PD (Ehlers and Kollmann 1996). Functional differences between primary and complex secondary PD are discussed in Chapter III.

***Substructure and biochemistry of plasmodesmata***

*Primary PD*

Primary PD consist of the plasma membrane that is continuous from one cell to another and the appressed ER cylinder in the center. Protein-like particles are embedded in both the plasma membrane and the appressed ER membrane (Ding et al., 1992a; Botha et al., 1993). Spoke-like extensions connect the protein-like particles in the plasma membrane and the appressed ER (Ding et al., 1992a; Botha et al., 1993). Actin and myosin have been suggested to be components of PD (White et al., 1994; Radford and White, 1998).

The spaces between the plasma membrane and appressed ER membrane are called microchannels and are about 2.5 nm in diameter (Ding et al., 1992a; Botha et al., 1993). Most transporting molecules appear to go through microchannels (Ding et al., 1992a; Schulz, 1995). Some lipids may move through the ER membrane (Grabski et al., 1993). Extracellular sphincters surrounding the orifices of PD have been observed and speculated to regulate PD transport (reviewed in Overall and Blackman, 1996). Callose could be one of the components of the sphincters (Radford et al., 1998; Rinne and van der Schoot, 1998). Existence of these structures, however, is not universal. It should be noted that although the membranes are physically continuous from one cell to another through PD, some chemical components of the cytoplasmic ER and plasma membrane may be different from those of the PD (Ding, 1998).

*Secondary PD*

Detailed information on the substructure of secondary PD is currently not available. However, electron microscopic analysis indicates continuous ER between adjacent cells through secondary PD (reviewed in Lucas et al., 1993; Ding and Lucas, 1996). Citovsky et al. (1993) speculated that a putative protein kinase in tobacco mesophyll cell walls could be one of the specific components of secondary PD. Several other putative PD proteins have been reported to be localized to PD (Monzer and Kloth, 1991; Kotlizky et al., 1992; Epel et al., 1996; Blackman et al., 1998; Yahalom et al., 1998). However, their functions remain to be identified.

***Physiological control of plasmodesmal gating***

As discussed above, prominent changes in the structure and number of PD during plant development establish dynamic symplasmic domains. Transient changes in the size

exclusion limit (SEL) of PD, on the other hand, can be modulated by physiological or environmental stimuli.

*Down-regulation of PD SEL*

SEL of PD can be down-regulated in following conditions. A pressure differential of 200 kPa or more can block intercellular transport in trichome cells (Oparka and Prior, 1992). Changes in light quality alter the rates of lateral symplasmic transport of carboxyfluorescein in maize seedlings (Epel and Erlanger, 1991). Plasmolysis greatly down-regulates PD transport in *Egeria densa* leaves (Erwee and Goodwin, 1984) and in pea root tips (Schulz, 1995).

Inhibition of intercellular transport by divalent cations, especially $Ca^{2+}$, has been well documented (Erwee and Goodwin, 1983; Baron-Epel et al., 1988; Holdaway-Clarke et al., 2000). Similar results were obtained by microinjection of inositol trisphosphates ($IP_3$), the second messenger that stimulates release of $Ca^{2+}$ from intracellular storage sites, or mastoparan (wasp poison) that increases cytoplasmic concentration of $IP_3$ and $Ca^{2+}$ (Tucker, 1990; Tucker and Boss, 1996). $Ca^{2+}$ effects on PD may represent a defense mechanism, as $Ca^{2+}$ is released under various stress conditions in some cases (Erwee and Goodwin, 1993). $Ca^{2+}$ may induce some kinase activity, leading to phosphorylation of PD components that down-regulates transport functions (Tucker and Boss, 1996).

Diffusion through PD is not only governed by the SEL. The chemical or structural nature of a molecule can be important. For example, the aromatic amino acids tyrosine (Tyr), phenylalanine (Phe) and tryptophan (Try) cannot diffuse between cells in *Egeria* leaves whereas glycine (Gly) and glutamic acid (Glu) can (Erwee and Goodwin, 1984). Diffusion of Phe, Try, methionine (Met), and histidine (His) are also inhibited in *Setcreasea* (Tucker and Tucker, 1993). Therefore, the biochemical properties of a small molecule are important for intercellular transport. This may explain why transport of

auxin (IAA, derivative of Try) is inhibited (Drake and Carr, 1978), whereas transport of gibberellins is not (Drake and Carr, 1979; Kwiatkowska, 1991). Giberellins have a similar structure to fluorescein which can readily diffuse between cells (Tucker, 1993).

*Up-regulation of PD SEL*

SEL of PD can be also up-regulated. Deplasmolysis can revoke the inhibitory effect of $Ca^{2+}$ on PD transport. Furthermore, deplasmolysis leads to an increase in SEL and loss of selectivity for amino acids (Erwee and Goodwin, 1984). Symplasmic phloem unloading is enhanced by osmotic stress in the root tip of pea seedlings (Schultz, 1994) and in mature phloem of *Phaseolus vulgaris* (Offler and Patrick 1996). These results suggest a role of PD in osmoregulatory mechanisms.

SEL of PD is increased by azide treatment in trichomes of *Setcreasea* (Tucker 1993) and in wheat root (Cleland et al., 1994). Azide reduces the cellular ATP content creating a condition similar to anaerobiosis. In fact, inducing anaerobiosis by $N_2$ gas treatment also increases SEL of PD. Dilation of PD under anaerobiosis may enhance sugar delivery to the cells to compensate for ATP reduction (Cleland et al., 1994). Based on these observations, ATP-dependent phosphorylation of PD components is speculated to be involved in maintaining the basal SEL of PD (Cleland et al., 1994).

Microinjection of cytochalasin D or profilin that depolymerize actin filaments induces an increase in SEL of PD from 1 kDa to greater than 20 kDa in tobacco mesophyll cells (Ding et al., 1996). This result implies involvement of actin filaments in the regulation of PD gating (Ding et al., 1996; Blackman and Overall, 1998).

***Transport of macromolecules***

Various proteins and nucleic acids of both viral and plant origins traffic intercellularly. Viral protein and nucleic acid trafficking is apparently key to spread of the viral

infection. The specific function of endogenous macromolecular trafficking remains largely unknown. Nevertheless, it is considered a potentially important area of modern plant biology.

*Intercellular trafficking of pathogens*

Many plant viruses encode one or more nonstructural proteins called movement proteins (MPs) that facilitate intercellular movement of viral particles or genomes (Carrington et al., 1996; Gilbertson and Lucas, 1996; Nelson and van Bel, 1998; Lazarowitz and Beachy, 1999). For example, tobacco mosaic virus (TMV) encodes a 30 kDa MP to facilitate TMV movement (Deom et al., 1987; Meshi et al., 1987). This protein is specifically localized to PD (Tomenius et al., 1987; Atkins et al., 1991; Ding et al., 1992b; Moore et al., 1992) and has the ability to increase SEL of PD from 1 kDa to 10 kDa in tobacco mesophyll cells (Wolf et al, 1989). Fluorescently labeled MP from red clover necrotic mosaic virus (RCNMV) moved from cell to cell when it was microinjected into a tobacco mesophyll cell. This was the first direct evidence of intercellular protein trafficking in a plant (Fujiwara et al., 1993). Furthermore, this MP could facilitate trafficking of the viral RNA genome (Fujiwara et al., 1993). These functions have been found for MPs from other viruses including bean dwarf mosaic virus (Noueiry et al., 1994), cucumber mosaic virus (CMV; Ding et al., 1995) and TMV (Nguyen et al., 1996), and for coat protein (CP) and helper component-proteinase (Hc-Pro) of bean common mosaic necrosis potyvirus and lettuce mosaic potyvirus (Rojas et al., 1997).

*Potato spindle tuber viroid* (PSTVd) is a small plant RNA pathogen. Evidence to date suggests that it does not encode any proteins. Nevertheless, it traffics from cell to cell through PD (Ding et al., 1997) and long distance through the phloem (Palkaitis, 1987; Zhu et al., 2001). The ability of a viroid to traffic through the phloem suggests that

plants possess endogenous mechanisms for long distance RNA trafficking. Interestingly, PSTVd could traffic into the sepals, but not into the ovary, petals or stamens despite vascular continuity among all floral parts in infected tomato and *Nicotiana benthamiana* (Zhu et al., 2001). These findings suggest that the phloem can mediate selective RNA trafficking to different organs.

*Intercellular trafficking of plant transcription factors and mRNAs*

Studies on viral and viroid trafficking led to the assumption that there exist cellular mechanisms for intercellular trafficking of plant endogenous proteins and RNAs. This assumption has now been confirmed. The maize transcription factor KNOTTED1 (KN1) controls meristem identity and its ectopic expression results in abnormal cell division and knot formation (Hake and Freeling, 1986). Chimeric plants with mutant (*KN*) in mesophyll cells (derived from $L_2$ or inner layer) in a wild-type (*kn*) background still exhibit abnormal cell division of epidermis (derived from $L_1$) and form knots in the developing leaf (Hake and Freeling, 1986). This observation suggests that KN1 protein functions non-cell-automonously from mesophyll to epidermis. Immunolabeling and *in situ* hybridization further demonstrated that KN1 is present in all layers of the shoot apical meristem, but *KN1* mRNA is absent in $L_1$ (Jackson et al., 1994). This result suggests that KN1 traffics from inner layers ($L_2$ or inner) to the $L_1$. Direct trafficking of KN1 has been demonstrated by microinjection in tobacco mesophyll cells (Lucas et al., 1995). Furthermore, KN1 increases the SEL of PD and facilitates trafficking of its own mRNA (Lucas et al., 1995).

The transcription factor FLORICAULA (FLO) which is involved in flower development in *Antirrhinum majas* functions non-cell-automonously (Carpenter and Coen, 1995; Hantke et al., 1995). In a mutant *flo* plant, restoration of FLO in $L_1$ (periclinal chimera) recovered the wild-type phenotype by interdermal activation of

downstream genes (Carpenter and Coen, 1995; Hantke et al., 1995). On the other hand, chimeric plants with only a sector in a layer comprised of revertant cells (mericlinal chimera) do not show the wild-type phenotype throughout the entire layer (Carpenter and Coen, 1995). These data indicate that FLO could facilitate interdermal, but not intradermal communication (Carpenter and Coen, 1995). Similar results are obtained with PISTILLATA (PI), a transcription factor for flower formation in *A. thaliana*. When this protein is expressed in $L_1$ of the *pi* mutant plant, the normal flowers are produced (Bouhidel and Irish, 1996). Microinjection has shown that FLO traffics intercellularly (reviewed in Mezitt and Lucas, 1996).

DEFFICIENS (DEF) and GLOBOSA (GLO) are MADS-box transcription factors that control petal and stamen organ identity in *A. majas* (Schwarz-Sommer et al., 1992; Sommer et al., 1990; Tröbner et al., 1992). *DEF* belongs to the B genes (such as *APETALA3* in *A. thaliana*) in 'ABC' model of flower development. DEF and GLO forms a heterodimer and bind to a specific promoter to control specific gene expression (Schwarz-Sommer et al., 1992; Tröbner et al., 1992). GEF and GLO are shown to function non-cell-automonously between the layers, but not within the layer in the floral meristem (Perbal et al., 1996). Interestingly, DEF appears to traffic 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 rescue the mutant phenotype. Thus, PD between $L_1$ and $L_2$ appear to facilitate polar trafficking of some proteins. This is perhaps one of the mechanisms that regulate cell communication in developing meristems (Perbal et al., 1996).

A recent report by Sessions et al. (2000) shows that transcription factor LEAFY (LFY) from *A. thaliana* moves from cell to cell and rescues the *lfy* mutants. The chimeric plants expressing LFY in *lfy* mutant background, created by FLP recombinase, exhibited the normal flower phenotype. Non-cell-autonomy is manifested across layers and within a layer. When LFY is specifically expressed in $L_1$ using a $L_1$-specific promoter, the

protein is detected in $L_1$ and other layers even though the mRNA is found only in $L_1$. These results indicate that LFY moves from $L_1$ to inner layers and is biologically functional in the inner layers. APETALA1 (AP1), however, acts cell-autonomously and does not move intercellularly (Sessions et al., 2000). These results indicate that intercellular trafficking of selective transcription factors can be important for plant development.

*Trafficking of phloem proteins and mRNAs*

During phloem development, some cells differentiate into sieve elements that facilitate long-distance transport of nutrients and signals throughout the entire plant (Sjölund, 1997). Sieve elements are living cells, but do not have nuclei or ribosomes (Cronshaw, 1981). Therefore, it has been speculated that proteins in sieve elements are synthesized in companion cells and transported into sieve elements. This hypothesis is supported by the rapid incorporation of $^{35}$S-labeled methionine supplied from the cut end of a leaf into soluble proteins in the phloem sap in wheat (Fisher et al., 1992) and *Ricinus communis* (Sakuth et al., 1993). The phloem sap from *Oryza sativa* L. contains more than 150 polypeptides (Nakamura et al., 1993). The protein composition in the phloem sap is stable over a 6-day period suggesting that the proteins are synthesized and transported continuously (Nakamura et al., 1993). Furthermore, *in situ* hybridization demonstrated that mRNAs encoding a number of phloem proteins such as PP1 and lectin PP2 from *Cucurbita maxima* (Clark et al., 1997; Dannenhoffer et al., 1997) and RPP13-1 from *O. sativa* (Ishiwatari et al., 1998) are expressed in companion cells, suggesting that these proteins are produced in companion cells and then traffic to sieve elements. PP1 and PP2 also traffic long distance through the phloem (Golecki et al., 1999). The sucrose transporter *SUT1* mRNA and possibly also the protein traffic from companion cells to sieve elements in tobacco, potato, and tomato (Kühn et al., 1997). Direct evidence of cell-

to-cell movement of phloem proteins is obtained by microinjection (Balachandran et al., 1997; Ishiwatari et al., 1998).

CmPP16 from *C. maxima* appears to possess properties similar to those of viral MPs. Microinjection and grafting experiments revealed that CmPP16 moves intercellularly, mediates the transport of its sense and antisense mRNA, and facilitates systemic movement of its mRNA through sieve elements (Xoconostle-Cazares et al., 1999). A number of mRNAs from phloem sap of *C. maxima* contain a conserved NAC domain that may be involved in apical meristem development (*CmNACP* mRNA; Ruiz-Medrano et al., 1999). Interestingly, some of *CmNACP* mRNAs are selectively transported into the apical meristem (Ruiz-Medrano et al., 1999). These findings provide evidence that plants possess endogenous mechanisms for long-distance transport of RNA. Some trafficking RNAs may function as informational molecule for long-distance coordination of plant developmental and physiological processes.

Long-distance signaling is essential for systemic activation of defense mechanisms. The 18 amino acid polypeptide systemin is translocated from a local wounded or infected leaf to the whole plant through the phloem (Narváez-Vásquez et al., 1995). Systemic signals that activate homology-dependent post-transcriptional gene silencing are speculated to be nucleic acids (Palauqui et al., 1997; Voinnet and Baulcombe, 1997; Jorgensen et al., 1998; Fagard and Vaucheret, 2000).

***Outstanding issues - research projects of this thesis***

Many outstanding issues remain to be resolved regarding the mechanisms and biological significance of PD-mediated protein and RNA trafficking. This thesis addresses the following issues: 1) What are the functional differences between primary and complex secondary PD? 2) How is protein trafficking regulated cellularly and developmentally? 3)

What are the cellular factors that are involved in macromolecular trafficking? 4) What are the consequences of macromolecular trafficking?

To help resolve these issues, my research project integrated multiple approaches including molecular biology, cell biology, virology, and genetics. Major portions of work have already been published in refereed journals (the Plant Journal, Plant Physiology, and Protoplasma). The published material presented in this dissertation has been updated to incorporate information that became available after publication and modified to a thesis format. Materials and methods are described in greater detail with the aim to facilitate future work using the techniques. Chapter II describes the development of biolistic bombardment as a new approach to study intercellular protein trafficking in plants. I constructed all plasmids, and conducted biolistic bombardment and fluorescence microscopic analysis of protein localization and trafficking. This work was published in the Plant Journal (Itaya et al., 1997). Chapter III covers specific protein trafficking between various cells during plant development. I constructed all plasmids and transgenic plants, and conducted fluorescence microscopic analysis of protein localization and trafficking. Electron microscopy and immunocytochemistry were conducted by other members of our research group. Data regarding developmental regulation of specific protein trafficking were published in Plant Physiology (Itaya et al., 1998). Data regarding cellular regulation has been submitted for publication. Chapter IV is devoted to nonspecific protein trafficking in several plant species. My role in this project was to investigate nonspecific trafficking in tobacco, *A. thaliana*, and tomato plants. Other members of our lab collected data regarding tomato and cucumber plants. The work was published in Protoplasma (Itaya et al., 2000). Chapter V discusses development of a genetic system to identify cellular factors for PD trafficking. I generated transgenic *A. thaliana* plants, identified lines that contain one copy of T-DNA, selected the homozygous plants and performed pilot EMS mutagenesis experiments with the

assistance of other members of our research group. Chapter VI presents an investigation of tomato gene expression patterns during viroid infection, as part of a large project to study the mechanisms and functions of RNA trafficking. I conducted inoculation of tomato plants, tissue sampling, RNA extraction, representational difference analysis, macroarray construction, hybridization and data analysis. Chapter VII summarizes the thesis research and discusses future prospects in the field.

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# CHAPTER II

## BIOLISTIC BOMBARDMENT: A NEW APPROACH TO STUDY INTERCELLULAR PROTEIN TRAFFICKING IN PLANTS[1]

[1] Data presented here were published as: Itaya, A., Hickman, H., Bao, Y., Nelson, R., Ding, B. (1997) Cell-to-cell trafficking of cucumber mosaic virus movement protein:green fluorescent protein fusion produced by biolistic gene bombardment in tobacco. Plant J. **12,** 1223-1230. The data are presented here with modifications with permission from Blackwell Science Ltd and the Society for Experimental Biology.

# INTRODUCTION

Study of macromolecular trafficking in plants was initiated by microinjection of proteins and nucleic acids into a plant cell as discussed in Chapter I. Microinjection provided seminal information on macromolecular trafficking in a plant. However, there were some concerns regarding this technique. Foremost, the proteins used for injection are produced in *Escherichia coli*, hence they lack the post-translational modification which may be required for specific functions of some proteins. Therefore, the data obtained using proteins produced in *E. coli* may not always be relevant to the true function of a protein (Padgett at al., 1996). Over-loading a cell with a protein may have unknown effects (Sanderfoot and Lazarowitz, 1995, 1996). Changes in cell turgor pressure during injection may result in closure of PD (Oparka and Prior, 1992). Damage caused by puncturing the cell wall and the plasma membrane with a needle may have unwanted consequences in cell physiology. Finally, the technique itself is very difficult to perform, and is limited to only skilled researchers.

Other methods such as immunolocalization and *in situ* hybridization have been used to assess protein trafficking *in planta* (Jackson et al., 1994). However, the results obtained are indirect. Chimeric plants expressing a protein of interest in a sector were created to investigate non-cell-autonomy of protein function and localization (Carpenter and Coen, 1995; Hantke et al., 1995; Bouhidel and Irish, 1996; Perbal et al., 1996; Sessions et al., 2000). The results obtained are informative, but the technique itself is very difficult, time consuming and labor intensive.

We have developed an alternative approach by combining biolistic gene bombardment and green fluorescent protein (GFP) fusion. This technique is very simple, and can provide direct evidence of protein trafficking *in planta.* We developed the

method by using the cucumber mosaic virus (CMV) 3a movement protein (3a MP) which was shown to traffic intercellularly by itself when microinjected into tobacco mesophyll cells (Ding et al., 1995). Mutant 3a MP (M5) that failed to traffic in the microinjection study provided a negative control (Ding et al., 1995). We created fusion gene constructs containing *3a MP:GFP* or *M5 MP:GFP* under the control of the cauliflower mosaic virus (CaMV) 35S promoter that drives constitutive expression in many plant cells. Upon biolistic delivery of these genes into tobacco epidermal cells, we monitored *in planta* production and intercellular trafficking of the fusion protein under a fluorescence microscope. The methods and results are presented and discussed in the following sections.

## 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 gene expression vectors*

*35S:3a MP:GFP and 35S:M5 MP:GFP constructs*

A *3a MP* gene or a *M5 MP* gene cloned in pET-11a (kindly provided by Dr. Peter Palukaitis at Scottish Crop Research Institute, Dundee, Scotland) was amplified by polymerase chain reaction (PCR) using a forward primer named "*Nco*I-3a" containing the *Nco*I restriction site at the 5' end and the 5' portion of *3a MP* sequence at the 3' end (5'GGCATGCCATGG$_{NcoI}$CTTTCCAAGGTA3'; see Appendix for annotation of the primer sequence), and a reverse primer named "5'GFP-3'3a" containing the 5' portion of

a *GFP* sequence at the 5' end and the 3' portion of *3a MP* sequence at the 3' end (5'CCCTTGCTCACCATAAGACCGTTAACCAC3').

The *GFP* gene cloned in pEGFP-1 (Clontech, Cat. # 6086-1) was amplified by PCR using a forward primer named "5'3a-3'GFP" containing the 3' portion of a *3a MP* sequence at the 5' end and the 5' portion of *GFP* sequence at the 3' end (5'TGGTTAACGGTCTTATGGTGAGCAAGGGCG3'), and a reverse primer named "GFP-*Bam*HI" containing the *Bam*HI restriction site at the 5' end and the 3' portion of *GFP* sequence at the 3' end (5'GCGC<u>GGATCC</u>$_{BamHI}$TTACTTGTACAGCTCG).

It should be noted that primers containing restriction endonuclease recognition sites at the end of DNA fragments require additional nucleotides ranging from one to several in order to increase the efficiency of digestion. The information about additional nucleotides can be obtained from an appendix of the New England Biolabs catalogue.

The PCR mixture contained 100 ng of template DNA, 100 ng each of the forward and reverse primers, 2 μl of dNTPs (2.5 mM each), 2.5 units of *Taq* polymerase, 2.5 units of pfu Turbo polymerase (Stratagene, Cat. # 600250), 10 μl of 10X *pfu* buffer, and $H_2O$ up to 100 μl of total volume. The *pfu* Turbo polymerase posses a 3'to 5'exonuclease proofreading function which minimizes the errors to $10^{-9}$ during PCR. PCR was performed under the following conditions: 94°C for 5 min, (94°C for 30 s, 45°C for 30 s, 72°C for 1 min) X 30 cycles, 7°C for 7 min, and 4°C storage. The annealing temperature (45°C) was calculated based on the base composition of a primer, using the formula 2 (A+T) + 4 (G+C) - 5. The extension time was calculated based on the length of PCR products (1 min/1 kb PCR product).

The amplified *3a MP* gene (or *M5 MP* gene) and the *GFP* gene were combined (5 μl of each) and subjected to recombinant PCR (RC-PCR; Higuchi 1990) with the forward primer "*Nco*I-3a" and the reverse primer "GFP-*Bam*HI" to produce a fusion gene *3a MP:GFP*. PCR components and cycle conditions were determined as described above.

The RC-PCR products were cleaned by phenol/chloroform extraction followed by ethanol precipitation prior to digestion. Alternatively, RC-PCR products were cleaned by using the QIAquick PCR purification kit (Qiagen, Cat.# 28104).

Cleaned RC-PCR products and the vector pRTL2 (kindly provided by Dr. James Carrington, Washington State University) were digested with restriction endonucleases *Nco*I and *Bam*HI at 37°C for one hour to overnight. Digested insert DNA and vector DNA were purified by either phenol/chloroform extraction followed by ethanol precipitation, or by using the QIAEX II Gel Extraction System (Qiagen, Cat.# 20021). Insert and vector DNAs were ligated by using either Takara Ligation Kit (Panvera, Cat.# TAK6021), or T4 ligase (Life science technology, Cat# 15224-017) following manufacture's instructions. The ligation product, the *35S:3a MP:GFP* or *35S:M5 MP:GFP* construct, was transferred into E. *coli* DH5α cells, and transformants were selected by ampicillin resistance (100 μg/ml). The sequences of the cloned genes were verified by DNA sequencing at the Oklahoma State University Recombinant DNA/Protein Resource Facility.

*35S:GFP construct*

A *GFP* gene was amplified by PCR using a forward primer named "*Nco*I-GFP" containing the *Nco*I restriction site at the 5' end and the 5' portion of *GFP* sequence at the 3' end (5'GGCATGCCATGGTGAGCAAGGGCGA3'), and the reverse primer "GFP-*Bam*HI" (see above). The amplified *GFP* gene was then cloned into plasmid pRTL2 under the *35S* promoter at the *Nco*I and *Bam*HI sites as described above. The *35S:GFP* construct was used to transform E. *coli* DH5α cells and transformants were selected by ampicillin resistance (100 μg/ml). The sequences of the cloned gene were verified by DNA sequencing.

***Biolistic bombardment by a vacuum gun (Biolistic PDS 1000/He system, BioRad)***

*Gold particle preparation and DNA coating*

Thirty mg of 1 μm gold particles (BioRad, Hercules, California, USA) was washed with 500 μl of absolute ethanol for three times, then resuspended in 500 μl of $ddiH_2O$, and stored at 4°C. Gold particles in 50 μl of $ddiH_2O$ (for 4 shots) was transferred into a new tube. DNA (2-8 μg) was added to the gold solution. The amount of DNA can vary depending on promoter activity or protein stability. For example, 2 μg of *35S:GFP* (for 4 shots) was enough for GFP expression, whereas 4 - 8 μg of *35S:3a MP:GFP* or *35S:M5 MP:GFP* (for 4 shots) was needed for 3a MP:GFP and M5 MP:GFP expression. Volume wise, the DNA solution should be kept below 10 μl. Twenty μl of 0.1 M spermidine and 50 μl of 2.5 M $CaCl_2$ were added to the gold and DNA solution, mixed gently, and incubated on ice for 8 min. The gold and DNA mixture was then collected by brief spin, washed once with 200 μl of absolute ethanol, and then resuspended in 100 μl (25 μl X 4 shots) of absolute ethanol.

*Sample preparation and bombardment*

Leaves were detached from a plant, and placed in a petri dish with wet filter papers. The petiole of a detached leaf was covered with a wet cotton ball to keep moist. Lower epidermis was bombarded following the manufacture's instructions (Biolistic PDS 1000/He system, BioRad, Hercules, California, USA). Leaf samples were placed on the second stage from the bottom of the PDS system during bombardment. Different pressures 1,300 psi, 1,100 psi, or 900 psi were used depending on the types of leaf samples. For example, 1,300 psi was used for mature tobacco leaves, 1,100 psi for younger and thinner tobacco leaves, and 900 psi for young tobacco leaves or thin leaves such as the leaves of *A. thaliana*.

*Fluorescence microscopy*

GFP fluorescent signals were detected under a Nikon Optiphot-2 epifluorescence microscope with a filter set consisting of a blue excitation filter (420 nm-490 nm), a dichroic mirror (510 nm), and a green barrier filter (520 nm-560 nm) (Nikon Corp., Tokyo, Japan). Fluorescent images were acquired and processed by either a Hamamatsu C2400 CCD camera and a Hamamatsu Argus 20 image-processing system (Hamamatsu Photonics KK, Hamamatsu-City, Japan), or a SPOT-2 CCD camera and associated software (Diagnostics Instruments, Inc., Sterling Heights, USA).

## RESULTS

***In planta expression and intercellular trafficking of 3a MP:GFP fusion protein***

The DNA constructs used for biolistic bombardment experiments are shown in Figure 1. Fully expanded mature leaves of tobacco were bombarded with either *35S:GFP, 35S:3a MP:GFP* or *35S:M5 MP:GFP*. GFP fluorescence could be detected as early as three hours post bombardment. With our DNA constructs, the number of cells expressing GFP signals reached maximum by 12 h post bombardment. The number of expressing cells in a bombarded leaf ranged from a few to several hundred.

The 3a MP:GFP fusion protein trafficked out of the transformed cell in approximately 50% of the cases (Table 1, Fig. 2A, also see Chapter IV). The fluorescent signal could be observed one to three cells away from the expressing cell. This observation agrees with the results obtained by microinjection studies showing that 3a MP itself is capable of intercellular trafficking (Ding et al., 1995). The most prominent feature of the 3a MP:GFP was its localization in the cell walls as distinct bright dots (Fig. 2B), which was not detected in microinjection experiments (Ding et al., 1995). We also observed aggregations or filamentous structures of 3a MP:GFP in the cytoplasm (see

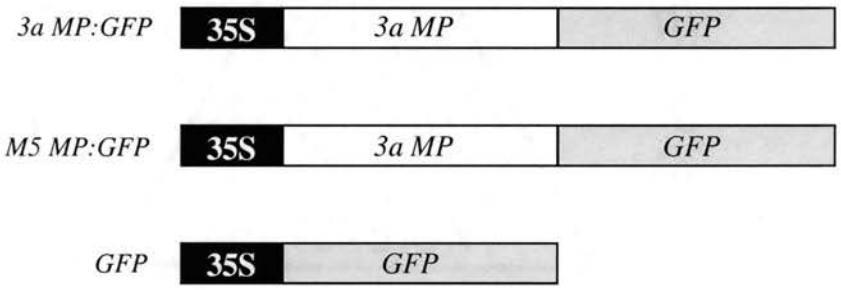


**Figure 1.** Fusion gene constructs used in biolistic bombardment

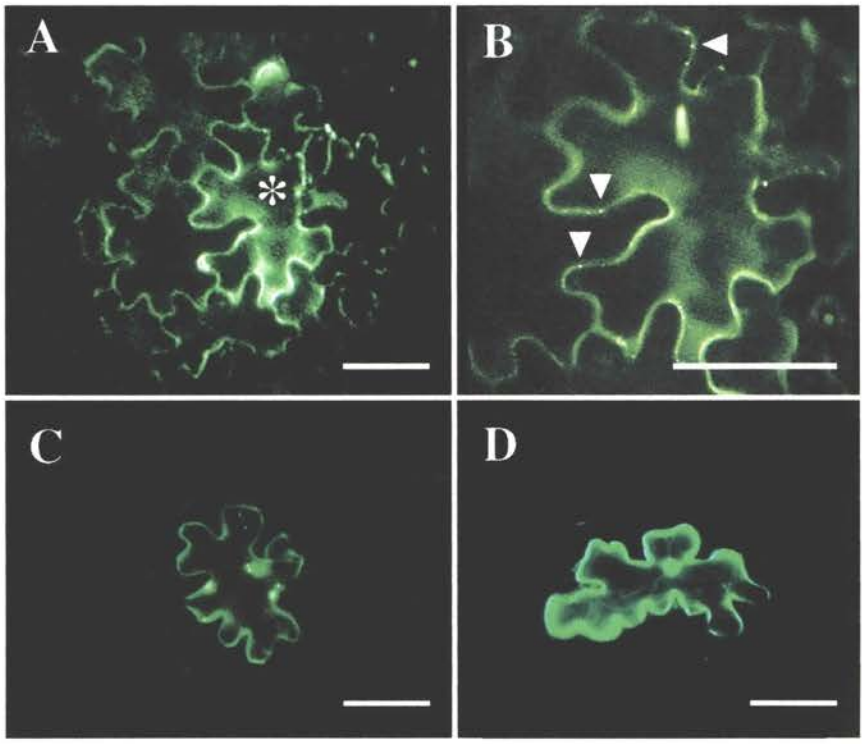


**Figure 2.** Cell-to-cell movement of 3a MP:GFP fusion protein in leaf epidermis of tobacco plant (*N. tabacum* cv. Samsun NN).

**(A)** 3a MP:GFP fusion protein was produced in the cell marked by an asterisk. Movement of this fusion protein into neighboring cells is indicated by the appearance of green fluorescence in these cells. Image was taken 12 h after bombardment.
**(B)** Green fluorescent dots in the walls of the cell producing 3a MP:GFP (arrowheads). Note that these dots are absent from cells producing M5 MP:GFP **(C)** or GFP **(D)**.
**(C)** M5 MP:GFP fusion protein does not traffic into neighboring cells. Image was taken 29 h after bombardment.
**(D)** Free GFP did not traffic from a single cell 30 h after bombardment.
Scale bars = 40 $\mu$m

**Table 1.** Intercellular trafficking capabilities of different fusions between 3a MP and GFP in leaf epidermis of *N. tabacum* cv. Samsum NN.

| Bombarded DNA | Total cells observed | Cells permitting movement |
|---|---|---|
| 3a MP:GFP | 43 | 19 (44%) |
| M5 MP:GFP | 31 | 0 |
| GFP | 56 | 1 (1.8%) |

Percentage of cells permitting movement are given in parentheses. Data for each DNA construct were collected from five leaves of five different plants that were bombarded and expressed the appropriate protein.

Fig. 9 in Chapter III). The fluorescent signal from the 3a MP:GFP usually faded out within 30 h after bombardment, except for the fluorescent dots in cell walls and the filamentous structure that could stay for several days. Disappearance of the fluorescence signal was probably due to degradation of the 3a MP:GFP rather than the dilution effect of intercellular trafficking, because the fluorescence signal from the M5 MP:GFP which was incapable of intercellular trafficking also disappeared within 30 h.

The M5 MP:GFP showed weaker fluorescence in comparison with 3a MP:GFP (Fig. 2C). It is unclear whether this is due to low expression level, different conformation, or a low stability of the M5 MP:GFP fusion protein. The M5 MP:GFP did not show fluorescent dots in the cell walls, nor did it traffic into adjacent cells (Table 1 and Fig. 2C). This non-trafficking of M5 MP:GFP is consistent with observation from microinjection (Ding et al., 1995).

Free GFP expressed in a plant cell was very bright and distinct, highlighting the nucleus and peripheral region of the cell (Fig 2D). GFP does not contain any nuclear localization signals. Therefore, GFP apparently enters the nucleus by diffusion (Chiu et al., 1996; Hanson and Köhler, 2001). In mature tobacco leaves, GFP expressed in single cells did not traffic into adjacent cells. GFP expressed in plant cells was very stable, and was not degraded for 5-7 days.

## DISCUSSION

We demonstrated that using biolistic gene bombardment to express GFP fusion protein in a plant cell is a valid approach to study protein trafficking. The procedure is very simple and does not require elaborate techniques. Furthermore, to study protein trafficking in a plant, biolistic bombardment provides several advantages over other methods such as microinjection. The protein is produced directly in plant cells and therefore it undergoes

any post-translational modifications necessary for functions. Direct production of the protein in a plant cell is essential to understand the interaction between the trafficking protein and the cellular factors. In fact, we have observed aggregations, filamentous structures or specific localization of 3a MP:GFP to cell walls that were not detected in microinjection studies. The convenience and usefulness of biolistic bombardment is further demonstrated by a large number of papers using this method which appeared following our initial publication (Reichel et al., 1999; Oparka et al., 1999; Huang and Zhang, 1999; Yang et al., 2000; Crawford and Zambryski, 2000; 2001; Tamai and Meshi, 2001).

It should be noted that biolistic bombardment also has disadvantages in some aspects. For example, the tissue to be studied is limited to epidermis as gold particles cannot reach inner tissues. In this regard, transgenic plants expressing the protein of interest in desired tissues or organs, and chimeric plants expressing the protein in specific layers or sectors will be the choice of methods. Transgenic plants constitutively expressing the protein are very useful for immunolocalization studies as they readily provide sufficient cells to study.

GFP fusion protein can be useful as shown in this study. However, it may not always reflect the true function of the protein because GFP can block the functional site(s) of the protein. Furthermore, the transport rate of the protein is difficult to assess because maturation of GFP takes approximately 2-4 hours at 22°C (Heim et al., 1994; Tsien, 1998). Therefore, it is difficult to address the kinetics of protein trafficking.

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# CHAPTER III

# DEVELOPMENTAL AND CELLULAR REGULATION OF SPECIFIC PROTEIN TRAFFICKING[1]

[1] Data regarding developmental regulation of protein trafficking were published as: Itaya, A., Woo, Y.-M., Masuta, C., Bao, Y., Nelson, R. S., Ding, B. (1998) Developmental regulation of intercellular protein trafficking through plasmodesmata in tobacco leaf epidermis. Plant Physiology. **118**, 373-385. The data are presented here with modifications with permission from the American Society of Plant Biologists. Data regarding cellular regulation of protein trafficking were part of a manuscript submitted for publication as: Liang, G., Itaya, A., Ding, B. (2001) Functional polarity of plasmodesmata probed by a viral movement protein.

# INTRODUCTION

Macromolecular trafficking through PD potentially functions to coordinate plant growth, development, and physiology. Therefore, such trafficking is likely regulated between specific cells, tissues and/or organs. Recent work suggests that protein trafficking occurs by two basic mechanisms: specific trafficking and nonspecific trafficking (Imlau et al., 1999; Oparka et al., 1999; Itaya et al., 2000; Crawford and Zambryski, 2000, 2001). Specific trafficking requires interaction between a trafficking molecule and cellular factors. Therefore, the trafficking molecule contains specific motifs (e.g., structural domains or sequences) that are recognized by the cellular machinery (Itaya et al., 2000; Crawford and Zambryski, 2000, 2001). Mutations in such motifs or in the cognitive cellular factors will abolish trafficking. Intercellular trafficking of some viral proteins and plant endogenous proteins discussed in chapter I represent specific trafficking because the mutant forms of these proteins fail to traffic. In contrast, nonspecific trafficking occurs by passive diffusion, which will be discussed in chapter IV.

A significant issue regarding the mechanisms of macromolecular trafficking is how different types of PD function to support the trafficking. In addition to primary formation of PD, modification of existing primary PD, de novo formation of secondary PD, and degeneration of existing PD at certain cellular boundaries during plant development have been well documented (Chapter I; also reviewed in Robards and Lucas, 1990; Lucas et al., 1993; Ding and Lucas, 1996; Ding et al., 1999a). Formation of secondary PD during plant development has been observed in many occasions. For example, secondary PD are formed during pollen mother cell development (Cheng et al., 1987), cell elongation (Schnepf and Sych, 1983; Seagull, 1983), and carpel fusion (Boeke, 1971). They are also observed at graft unions (Kollmann and Glockmann, 1985, 1991; Kollmann et al., 1985) and in protoplast fusion (Monzer, 1991; Ehlers and

Kollmann, 1996). Complex secondary PD (see the definition in Chapter I) are formed between mesophyll cells during tobacco leaf development (Ding et al., 1992). Interestingly, TMV MP is localized only to complex secondary PD, but not to primary PD in transgenic tobacco (Ding et al., 1992), and this function is correlated with the ability of TMV MP to increase the SEL of PD in tobacco mesophyll cells (Deom et al., 1990; Ding et al., 1992). A protein kinase activity that phosphorylates TMV MP is correlated with the development of complex secondary plasmodesmata in tobacco (Citovsky et al., 1993). The arrest in secondary PD formation correlates with accelerated leaf senescence in transgenic tobacco expressing an yeast acid invertase, suggesting that development of complex secondary PD is essential for proper growth of some plants (Ding et al., 1993). The above information strongly implies distinct functions of primary and complex secondary PD. Ding et al., (1993) suggest that they facilitate trafficking of different macromolecules for specific plant functions. However, this hypothesis has not been tested.

PD at specific cellular boundaries may perform distinct functions. Secondary PD between L1 and L2 in the meristem appear to regulate symplasmic domain interactions in the meristem. For example, these PD control polar trafficking of the transcription factor DEF from L2 to L1, but not from L1 to L2 in *A. majus* (Perbal et al., 1996). PD at the bundle sheath and phloem interface have a distinct properties. Their SEL does not change in the presence of TMV MP, whereas SEL of PD between mesophyll cells can be increased by TMV MP (Ding et al., 1992). The formation of complex secondary PD at this interface is not affected by the presence of yeast acid invertase, whereas that in mesophyll cells was arrested (Ding et al., 1993). It is well known that the boundary between bundle-sheath and phloem cells constitutes a barrier for virus infection. Restriction of movement into the phloem was observed with CMV in the transgenic tobacco expression an altered form of 2a protein (Wintermantel et al., 1997), *cowpea*

*chlorotic mottle virus* in a soya-bean resistant line PI 346304 (Goodrick et al., 1991), various tobamoviruses and potyviruses in several host plants (Ding et al., 1998), and RCNMV carrying specific MP mutations in *N. tabacum* (Wang et al., 1998). On the other hand, viruses such as beet western yellows virus (Sanger et al., 1994) and potato leafroll virus (van den Heuvel et al. 1995) are confined to the phloem. Interestingly, unloading of some viruses from the phloem is largely affected by host physiology and the environment (Ding et al., 1999b) and developmental stages of cells or tissues (Sudarshana et al., 1998). These results suggest that the bundle sheath-phloem interface regulates trafficking of various molecules between phloem and nonvascular tissues. Regulation of loading into and unloading out of phloem as well as long distance transport through the phloem is important to orchestrate entire plant physiology.

In this chapter, I present studies on the developmental and cellular regulation of specific protein trafficking using a 3a MP:GFP fusion. Multiple approaches such as biolistic bombardment, transgenic tobacco plants, fluorescence and electron microscopy, and immunolabeling were used. We obtained the first evidence that primary and complex secondary PD have different functions in protein trafficking. Furthermore, we demonstrated that the interface between the phloem and nonvascular tissues controls 3a MP:GFP trafficking.

## MATERIALS AND METHODS

### *Plant material and growth conditions*

Tobacco (*N. 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 gene expression vectors***

Construction of *35S:3a MP:GFP, 35S:M5 MP:GFP,* and *35S:GFP* has been described in Chapter II.

***Biolistic bombardment***

Procedures for biolistic bombardment by a vacuum gun (Biolistic PDS 1000/He system, BioRad, Hercules, California, USA) has been described in Chapter II.

***Construction of binary vectors***

*Constructs for constitutive expression*

*35S:3a:GFP* and *35S:GFP* fusion genes cloned in pRTL2 (Chapter II) were amplified by PCR using a forward primer named "*Sac*I-35*S*"containing the *Sac*I restriction site at the 5' end and the 35*S* promoter sequence at the 3' end (5'C$\underline{\text{GAGCTC}}_{SacI}$GCATGCCTGCAGGTCA3'; see p.33 where you could also annotate the primer sequences similarly), and a reverse primer named "*term-Hind*III" containing the *Hind*III restriction site at the 5' end and the terminal sequence of pRTL2 at the 3' end (5'CCC$\underline{\text{AAGCTT}}_{HindIII}$GCATGCCTGCAGGTCA3'). As mentioned in Chapter II, additional nucleotides are required for primers containing restriction endonucleases at the end of DNA fragments. PCR products were cleaned and digested by restriction endonucleases *Sac*I and *Hind*III and then cloned into a pBIN19 binary vector (kindly provided by Dr. Yinghua Huang, Oklahoma State University) at *Sac*I and *Hind*III sites as described in Chapter II. It should be noted that when a vector is larger than 10 kbp such as a pBIN19, the molar ratio of an insert to a vector should be adjusted to 1:3 for successful ligation. The ligation products were used to transform E. *coli* and transformants were selected by kanamycin resistance (50 $\mu$g/ml). The sequences of the

cloned genes were verified by DNA sequencing. The DNA constructs were biolistically bombarded into tobacco leaves to confirm expression of functional proteins.

*Constructs for phloem specific expression*

The fusion DNA fragment containing the tobacco etch virus (TEV) leader sequence:3a *MP:GFP* in plasmid pRTL2-3a:GFP (Itaya et al., 1997) was cut out by simultaneous double digestion using *Bam*HI and *Eco*RI. The band corresponding to the fusion DNA fragment was separated by electrophoresis, isolated from the agarose gel, and purified with the QIAEX II Gel Extraction System (Qiagen, Cat. # 20021). The purified DNA fragment was polished to produce blunt ends using a PCR thermocycler. The reaction mixture contained the cleaned DNA (1 $\mu$g) in 20.5 $\mu$l of $H_2O$, 1 $\mu$l of dNTPs (2.5 mM each), 2.5 $\mu$l of 10X *pfu* reaction buffer, and 1 $\mu$l of *pfu* polymerase. The reaction conditions were as follows: 65°C for 30 min, 72°C for 10 min, and 4°C storage. The polished DNAs were cleaned for ligation by phenol/chloroform extraction as described in Chapter II.

The pGPTV-Kan binary vector containing the phloem specific commelina yellow mottle virus (CoYMV) promoter at *Sal*I and *Sst*I sites (kindly provided by Dr. Neil Olszewski at the University of Minnesota) was digested with *Sma*I at 25°C overnight. To inhibit self-ligation and hence increase the efficiency of cloning, the *Sma*I digested DNA was dephosphorylated at the 5' end with alkaline phosphatase (Boehringer Mannheim, Cat. # 713 023) per the manufacturer's instructions. The dephosphorylated vector was cleaned by phenol/chloroform extraction. The TEV leader:*3a MP:GFP* fragment was ligated into the *Sma*I site of the binary vector using a DNA Ligation Kit (Panvera, Cat. # TAK6021). The ligation products were used to transform *E. coli* DH5α cells. Positive transformants were selected by kanamycin resistance (50$\mu$g/ml). The correct orientation and the sequences of the inserted genes were verified by DNA sequencing.

***Preparation of Agrobacterium tumefaciens competent cells***

*Competent cells for electroporation*

The protocol for preparing competent cells was obtained from Dr. Yinghua Huang (Oklahoma State University). *A. tumefaciens* LBA 4404 (Clontech) carrying a helper plasmid pAL 4404 was grown at 29°C in 10 ml of YM medium (0.04% yeast extract, 1% mannitol, 0.1% NaCl, 0.02% $MgSO_4(7H_2O)$, 0.05% $K_2HPO_4(3H_2O)$, pH 7.0) containing streptomycin (500 μg/ml; for the helper plasmid pAL 4404) until $OD_{600}$ reached 0.5-0.7. The culture was placed on ice for 15 min, and then spun down at 5,000 rpm for 15 min at 4°C. The pellet was washed in 7 ml of 1 mM HEPES (pH 7.0) twice. The washed cells were resuspended in 200 μl of sterile 1 mM HEPES containing 10% glycerol. Cells were split into 45 μl aliquots, frozen in liquid nitrogen, and stored at –80°C.

*Competent cells for chemical transformation*

A single *A. tumefaciens* colony was grown in 2 ml of YEP (1% bacto-peptone, 1% yeast extract, 0.5% NaCl) medium containing streptomycin (500 μg/ml) at 28°C overnight. The preculture was used to inoculate 50 ml of YEP medium containing streptomycin at 28°C until $OD_{600}$ = 0.5. Cells were harvested by centrifugation at 8,000 rpm for 10 min, and then resuspended in 10 ml of 0.15 M NaCl. Cells were centrifuged again at 8,000 rpm for 10 min and resuspended in 1 ml of 20 mM ice-cold $CaCl_2$. Cells were then split into 100 μl aliquots, frozen in liquid nitrogen, and stored at –80°C.

***Transformation of A. tumefaciens***

Binary vector constructs were isolated and purified from *E. coli* cells using the Qiagen Plasmid Midi Kit (Qiagen, Cat. # 12143) following manufacturer's instructions. The

vectors were used to transform *A. tumefaciens* by electroporation or by chemical transfromation.

*Electroporation*

The protocol was obtained from Dr. Yinghua Huang (Oklahoma State University). The competent cells were thawed on ice. Plasmid (0.3 μg) in 1 μl of $H_2O$ was added to 20-45 μl of competent cells, and incubated on ice for 2 min. The DNA and competent cell mixture were transferred to a prechilled electroporation cuvette (0.2 cm gap). The cuvette was placed into the Gene Pulser (BioRad). Electroporation was executed with an electrical pulse at a field strength of 12.5-16.7 kV/cm for 5 ms. Immediately afterwards, the cuvette was removed from the apparatus, and 1 ml of prechilled YM broth was added to the mixture. The mixture was transferred to a microcentrifuge tube and incubated with gentle shaking at 29°C for 1-2 h. The recovered culture (50 μl) was spread onto a selective YM plate containing streptomycin (500 μg/ml: for the helper plasmid pAL 4404) and kanamycin (50 μg/μl: for pBIN19 or pGPTV-Kan) and incubated at 29°C for 2-3 days. Transformants were subjected to colony PCR to verify that the cells contained plasmid with the correct inserts. Briefly, single bacterial colonies were picked with pipette tips or plastic toothpicks and resuspended in 50 μl of dd$iH_2O$. (Wood toothpicks should not be used to pick colonies since they may contain substances which inhibit PCR, based on discussions from the Arabidopsis network group.) Resuspended cells were boiled for 10 min, and 5 μl of boiled cells was used for PCR. Colony PCR mixture contained 5μl of cells, 40 ng of forward and reverse primers for the insert, 2 μl of dNTPs (2.5 mM each), 2.5 units of *Taq* polymerase, 2 μl of 10X *Taq* polymerase buffer, 2 μl of 25 mM $Mg^{2+}$, and $H_2O$ up to 20 μl of total volume. PCR was performed as described in Chapter II.

*Chemical transformation*

The competent cells were thawed on ice. Plasmid (~1 μg) was added to competent cells, and incubated at 37°C for 5 min. YEP medium (1 ml) was added to the cells and the mixture was incubated at 28°C for 3 h with gentle shaking. Cells were harvested by centrifugation at 2,000 rpm for 3 min, and resuspended in 50 μl of YEP medium. Recovered cells were spread onto the selective YEP plates containing streptomycin (500 μg/ml) and kanamycin (50 μg/μl) and incubated at 29°C for 2-3 days. Colony PCR was applied as described above to verify the plasmid constructs in positive transformants.

***Leaf disc transformation of tobacco plants***

The procedure was based on Horsch et al (1985) with some modifications. The *A. tumefaciens* was precultured in 2ml of 2YT or LB medium containing streptomycin (500 μg/ml) and kanamycin (50 μg/μl) and incubated at 29°C with vigorous shaking for overnight to 3 days until the bacterial growth could be seen by eyes. The preculture was used to inoculate 50 ml of 2YT or LB medium containing the antibiotics. The culture was grown at 29°C with vigorous shaking until $OD_{600}$ reached 1.0.

Healthy-looking leaves from 5 to 6 week-old tobacco plants were sterilized by soaking in 10% Clorox for 20 min and rinsed with dd$iH_2O$. Leaf discs of approximately 0.5 $cm^2$ were cut with a sterile razor blade. Discs were immersed in the *A. tumefaciens* culture for 5 min, and placed on sterile filter papers to eliminate excess liquid. Discs (15-20 of them) were placed with lower epidermis facing down on the cocultivation medium [1X MS salt (Gibco BRL Cat. # 11117-066), 3% sucrose, 1 mg/L 6-benzylaminopurine (Sigma Cat. # 3408), 0.8% bactoagar, pH 5.8] in Petri dishes. Petri dishes were sealed with Parafilm, and placed in a growth chamber controlled at 16 hours light (25°C)/8 hours dark (25°C) cycles for 1-3 days until bacterial growth could be seen at the edge of

leaf discs as a thin (0.5-1 mm) white layer. (It is important not to overgrow bacteria as it may cause death of plant cells.)

Leaf discs were transferred to the shoot regeneration medium [1X MS salt, 3% sucrose, 1 mg/L 6-benzylaminopurine, 0.1 mg/L a-naphthalene acetic acid (Sigma Cat. # 0460), 1X B-5 vitamins, 0.8% bactoagar, pH 5.8; after autoclave 200 mg/L cefotaxime (Sigma Cat. # C7039) was added to eliminate *A. tumefaciens*, and 100 mg/L kanamycin was added to select for pBIN19 or pGPTV-Kan transformants] in Petri dishes (preferably 2 cm high), ensuring good contact between discs and the medium. Cefotaxime is not stable at room temperature, thus the shoot regeneration medium was prepared fresh before use. The 1 X B-5 vitamins were prepared from a 100X B-5 vitamin stock that contained 1.0 g myo-inositol (Sigma Cat. # I3011), 100 mg thiamine-HCl (Sigma Cat. # T1270), 10 mg nicotinic acid (Sigma Cat. # N0765), and 10 mg pyridoxine-HCl (Sigma Cat. # P8666) in 1 L of ddi$H_2O$. Petri dishes were sealed with Parafilm and placed in a growth chamber until shoots regenerated. If leaf discs expanded to move away from the medium, they were cut smaller and recultured.

Calli formed in approximately one week of cluturing, and shoots formed after 2-3 weeks. When shoots grew to approximately 0.5-1 cm long, they were cut at the base, with caution not to take calli, and inserted into the rooting medium (1X MS salt, 3% sucrose, 1X B-5 vitamins, 0.8% bactoagar, pH 5.8; after autoclave 200 mg/L cefotaxime, 100 mg/L kanamycin were added) in Magenta boxes. Magenta boxes were placed in a growth chamber until roots regenerated.

Plantlets with approximately 1 cm roots were removed from the rooting medium, washed to remove agar, and transferred to soil. To harden the plantlets, pots were covered in plastic bags, or placed in a tub covered with plastic wrap for 2-3 days. Plastic covers were removed gradually, and the transgenic plants were kept in the growth chambers. The transgenic plants were examined with a fluorescence microscope for GFP signal.

Seeds were collected from the transgenic plants which showed GFP signal, and progeny plants were used for further analysis.

***Immunoblot analysis of transgenic tobacco plants***

Crude protein extracts were prepared from the transgenic and nontransgenic plants following the protocol described by Vaquero et al. (1994). Fresh leaves (0.1 g) were ground in liquid nitrogen and homogenized in 1 ml of grinding buffer (10 mM KCl, 5 mM $MgCl_2$, 0.4 M sucrose, 10% [v/v] glycerol, and 10 mM 2-mercaptoethanol in 100 mM Tris-HCl buffer, pH 7.5). The homogenate was centrifuged at 1,000 rpm for 10 min at 4°C . The supernatant was collected and used for dot blotting.

For immunoblot analysis, 10 $\mu$l of crude protein extract was blotted on a nitrocellulose membrane. The membrane was incubated in the blocking buffer consisting of 5% (w/v) nonfat dry milk in TBST (25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 2.7 mM KCl, and 0.1% Tween 20) for 1h, then incubated with a rabbit-derived polyclonal antibody against GFP antibody (Clonetech Cat. #8363) at 1:1,500 dilution overnight. After several buffer washes, the membrane was incubated with a goat-derived anti-rabbit IgG conjugated with alkaline phosphatase at 1:10,000 dilution for 1 h. Color reaction was performed using the Nucleic Acid Detection Kit (Boehringer Mannheim, Cat. # 1175 041) following the manufacturer's instructions.

***6(5)-carboxyfluorescein diacetate translocation experiments***

The sink or source status of a tobacco leaf was determined by 6(5)-carboxyfluorescein (CF; Sigma) translocation patterns as described by Roberts et al. (1997). Briefly, CF (50 $\mu$g/ml) was applied to the adaxial surface of a fully mature leaf that was gently abraded with a sandpaper. The leaf was covered with plastic wrap to prevent evaporation. In some experiments, CF was fed through the cut end of a leaf to allow further

translocation throughout the plant. The upper leaves were examined for CF fluorescence at 30 min intervals up to 3 h.

***Fixation of plant tissues for electron microscopy***

Tobacco leaf tissues were fixed for electron microscopy following the procedures described by Ding et al. (1992). For structural studies of plasmodesmata, tobacco leaves were cut into 1 $mm^2$ discs with a razor blade, and then fixed in 4% (v/v) glutaraldehyde/0.1% (v/v) tannic acid buffered with 0.1 M sodium cacodylate, pH 7.2 at room temperature for 3 h. After three buffer washes (20 min each), the samples were postfixed in 2% (w/v) osmium tetroxide in the same buffer at 4 °C overnight. Following three buffer washes and one distilled $H_2O$ wash (20 min each) the samples were soaked in 2% (w/v) aqueous uranyl acetate on ice for 30 min. The samples were subsequently dehydrated in a gradual series of ethanol (25, 50, 70, 90, and 100%, v/v), infiltrated with Spurr's plastic medium (Spurr, 1969), and embedded in Spurr. The embedded samples were placed in an oven at 70°C for 24 h to polymerize the plastic.

For immunolabeling analysis, leaf samples from 3a MP:GFP-transgenic tobacco and non-transgenic tobacco were fixed in 2% (w/v) paraformaldehyde/0.5% (v/v) glutaraldehyde for 1 h. The leaf samples were infiltrated and embedded in LR White resin (Electron Microscopy Sciences, Cat.# 14380) for thin sectioning, and immunolabeled as described below.

Thin sections (70 to 80 nm) were cut with a diamond knife on an ultramicrotome (Dupont Sorbal, MT 6000), and stained with 2% (w/v) uranyl acetate and lead citrate in methanol. All thin sections were examined with an transmission electron microscope (model JEM-100CX II, JEOL) operated at 80 kV.

### *Immunolocalization of 3a MP:GFP*

Thin sections of 3a MP:GFP-transgenic tobacco and non-transgenic tobacco leaf samples were first incubated in TBST buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2% BSA, and 0.1% Tween 20) at room temperature for 1 h and then incubated in a rabbit-derived polyclonal antibody (IgG) against 3a MP (C. Masuta, Hokkaido University, Japan) diluted 1:200 at room temperature for 1 h. The sections were washed with TBST buffer, and incubated in a goat-derived 10-nm gold conjugated anti-rabbit IgG antibody (Sigma) at room temperature for 1 h. The sections were washed with buffer and distilled-water, then stained with 2% (w/v) uranyl acetate and lead citrate in methanol.

### *Colocalization of 3a MP:GFP and callose*

Leaves from 3a MP:GFP transgenic tobacco were cut into 2-3 $mm^2$ segments and fixed in a fixative (3.7% paraformaldehyde, 0.2% picric acid, 50 mM potassium phosphate, and 5 mM EGTA, pH 6.8) at room temperature for 2 h. The segments were washed with buffer (50 mM potassium phosphate and 5 mM EGTA, pH 6.8) and then successively infiltrated with a 2:1 mixture of 20% (w/v) Suc:Tissue Tek optimal cutting temperature compound (Ted Pella, Inc., Redding, CA). The infiltration was completed in three steps: 30% (v/v) of the mixture for 30 min, 60% for 30 min, and 100% for 1 h. Two to three leaf segments were embedded in absolute Tissue Tek and frozen in a CTD cryostat (International Equipment Co., Needham Heights, MA). Cryosections of 10 $\mu$m were obtained and attached to a microscopic slides coated with a mixture of 1% (w/v) gelatin and 0.1% (w/v) chrome alum. The slides with tissue sections were incubated on a warming plate at 40°C for at least 2 h before further processing.

The sections were incubated in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 1.8 mM $KH_2PO_4$, pH 7.4) containing 3% (w/v) BSA and 1% (v/v) Nonidet P-40 (Sigma) at room temperature for 30 min. The sections were then incubated with a

monoclonal antibody against callose (Biosupplies Australia, Parkville, Australia) at 1:50 dilution in PBS containing 1% BSA and 0.05% (v/v) Triton X-100 at 37°C for 1.5-2 h. Following PBS washes for 10 to 15 min, the sections were incubated in a goat-derived and Texas Red-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:50 dilution in PBS containing 1% BSA and 0.05% (v/v) Triton X-100 at 37°C for 1.5-2 h. The sections were then washed with PBS, embedded in a mounting medium, and covered with a coverslip. The slides were placed in the dark for at least 1 h prior to microscopic examination.

***Fluorescence microscopy***

Samples were examined under the Nikon fluorescence microscope as described in Chapter II. A filter set consisting of a green excitation filter (546/10nm), a 580 nm dichroic mirror and an orange-red barrier filter (590 nm) was used to visualize Texas Red fluorescence.

***Confocal microscopy***

Some sections were examined with a laser confocal microscope (BioRad MRC 1024) at the imaging center in the Department of Genetics and Cell Biology, University of Minnesota, St. Paul. The confocal microscope was attached to an inverted microscope (Diaphot, Nikon) equipped with a 15 mW krypton/argon laser. For 3a MP:GFP and Texas Red visualization, the 488/522 nm and 568/605 nm filter sets were used respectively. Digital images were collected on a personal computer (model 300 ProSignia, Compaq Computer Corporation, Houston, TX) using LaserSharp software (version 2.1, BioRad) and were further analyzed using an imaging software (version 1.62 NIH Image, National Institute of Health, Bethesda, MD).

# RESULTS

## *Formation of complex secondary plasmodesmata in tobacco leaf epidermis during leaf development*

It was demonstrated that PD undergo structural modification from primary PD to complex secondary PD in tobacco mesophyll cells during leaf development (Ding et al., 1992). To test whether this PD modification also occurs in tobacco epidermis, we conducted a structural analysis of PD in epidermis during plant development. For a systematic analysis, we used leaves at different stages as shown in Fig. 1. Specifically, sink and source status of leaves were used to determine developmental stages of leaves. Tobacco plants studied were approximately 9 weeks old. We employed carboxyfluorescein (CF) to determine sink/source status of a leaf because CF follows the movement of photoassimilates and has been widely used to define the sink-source status of leaves (Roberts et al., 1997; Oparka et al., 1999). Following the established criteria, we defined a young leaf that imported and unloaded CF as a sink leaf, and mature leaf that did not import CF as a source leaf (Fig. 1). Within the same leaf, the maturation occurs from tip to base (Turgeon, 1989; Ding et al., 1992). Therefore, we examined both tip and base regions of each leaf.

In the base of a sink leaf, all PD were simple primary (Fig. 2A and 3). In the tip of the same leaf, approximately 14% of PD had become either H- or Y-shaped branches (Fig. 2C, and 3). The H-shaped PD may have been derived from fusion of two neighboring primary PD, and were called "modified primary PD" (Ding et al., 1993). The Y-shaped PD were probably formed by *de novo* addition of a new cytoplasmic strand to primary PD (Ding et al., 1999a).

In the base of a sink-source transition leaf, approximately 84% of the PD were primary and 16% were either H- or Y-shaped (Fig. 2D, E, and 3). This pattern was

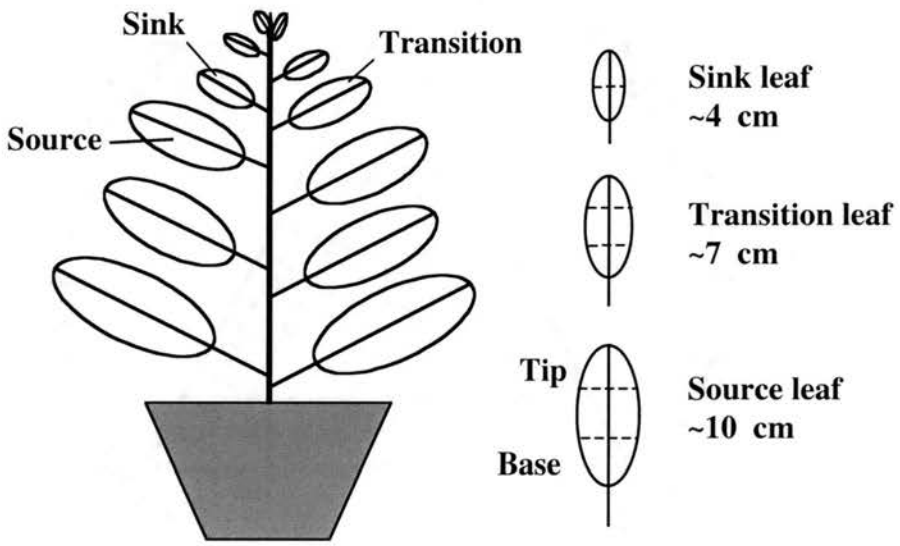


**Figure 1.** Schematic view of a 9-week-old tobacco showing the leaves used in the present study. Physiological status of leaves were determined by CF translocation. For each leaf, both the tip and the base regions were investigated.

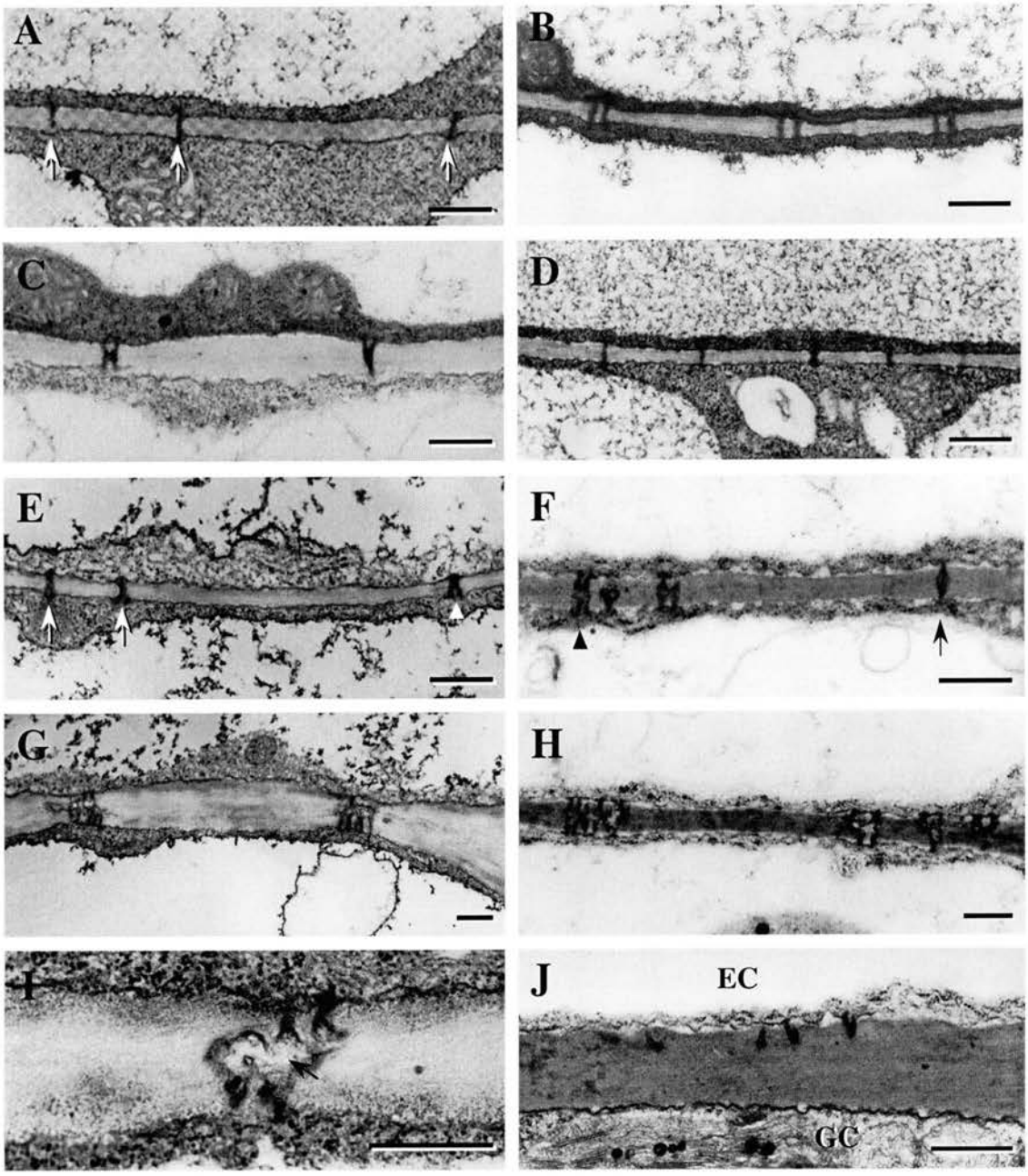
A
B
C
D
E
F
G
H
I
J
EC
GC

**Figure 2.** Structure and development of plasmodesmata between epidermal cells during tobacco leaf sink-to-source transition.

**(A)** Primary PD (arrows) in the base of the sink leaf.
**(B)** Primary PD in the tip of the sink leaf.
**(C)** H- and Y- branched PD in the tip of the sink leaf.
**(D)** Primary PD in the base of the transition leaf.
**(E)** Primary (arrows) and branched (arrowhead) PD in the base of the transition leaf.
**(F)** Primary (arrows) and branched (arrowhead) PD in the tip of the transition leaf.
**(G)** Branched PD in the tip of the transition leaf.
**(H)** Branched PD in the base of the source leaf.
**(I)** Branched PD in the tip of the source leaf. Note the presence of a central cavity in the PD (arrow).
**(J)** Truncated PD between an epidermal cell (EC) and a mature guard cell (GC). Scale bars = 0.5 $\mu$m.

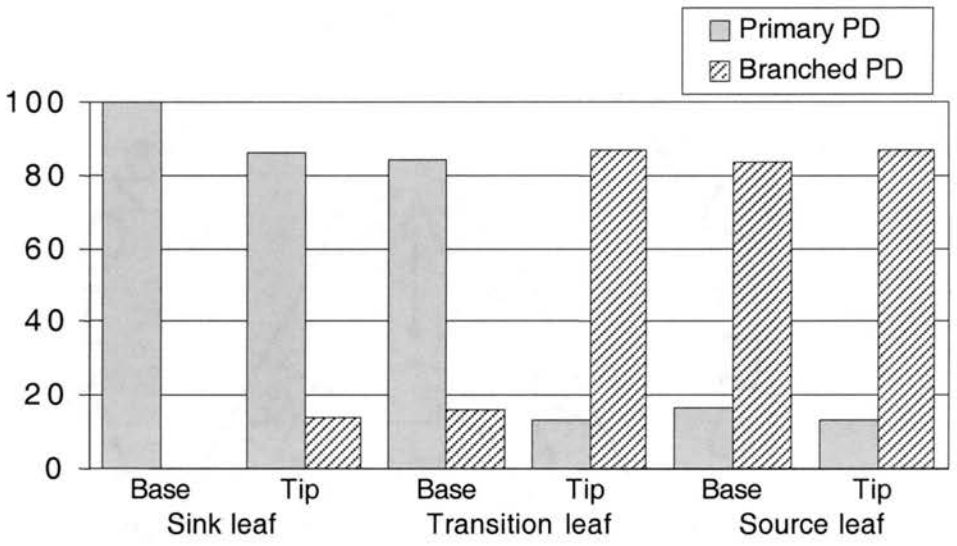


**Figure 3.** Distribution of primary versus branched PD between epidermal cells during tobacco leaf development.

Total number of PD examined for the different leaf regions were 315 for the base of sink leaf, 474 for the tip of sink leaf, 547 for the base of transition leaf, 559 for the tip of transition leaf, 380 for the base of source leaf, and 480 for the tip of source leaf. The PD were counted from 200 to 300 cellular interfaces derived from two different tobacco plants.

similar to that found in the tip of a sink leaf. In the tip of the sink-source transition leaf, 87% of total PD became highly branched (more than three in many cases) and contained a central cavity in the middle lamellar region of the cell walls (Fig. 2F and G). This structural feature and distribution pattern was consistent in both the base and the tip regions of a source leaf (Fig. 2H, I and 3). Therefore, the transition of PD from primary to highly branched occurs most dramatically in the transition leaf. The highly branched PD were termed "complex secondary PD" (Ding et al., 1999).

Truncated PD were observed in the cell walls of an epidermal cell connecting to a mature guard cell (Fig. 2J). These truncated PD might be formed *de novo* after the maturation of a guard cell, or more likely they were remnants of previously existing PD that were sealed during guard cell maturation (Willmer and Sexton, 1979; Willie and Lucas, 1984).

***3a MP:GFP was localized to complex secondary PD, but not to primary PD***

To investigate the cellular localization patterns of 3a MP:GFP, we generated transgenic tobacco plants expressing 3a MP:GFP under the control of CaMV 35S promoter. We obtained three independent transgenic lines expressing 3a MP:GFP. Green fluorescent dots were observed in the cell walls of epidermal and mesophyll cells in source leaves of transgenic plants as they were seen in bombardment experiments (Fig. 4A). Interestingly, the fluorescent dots were also found in the cell walls connecting mature guard cells to surrounding epidermal cells (Fig. 4A). Fluorescent dots were also observed in cell walls of mesophyll cells (Fig. 4B). However, they were absent in sink leaves (Fig.4C) with some exceptions (guard cells, veins, and trichome cells). In transgenic tobacco expressing free GFP, the fluorescence could be observed in both young and mature leaves but there were no fluorescent dots in cell walls. GFP was localized in the nuclei or peripheral regions of cells as it was the case in bombardment experiments (Fig. 4D). To test whether

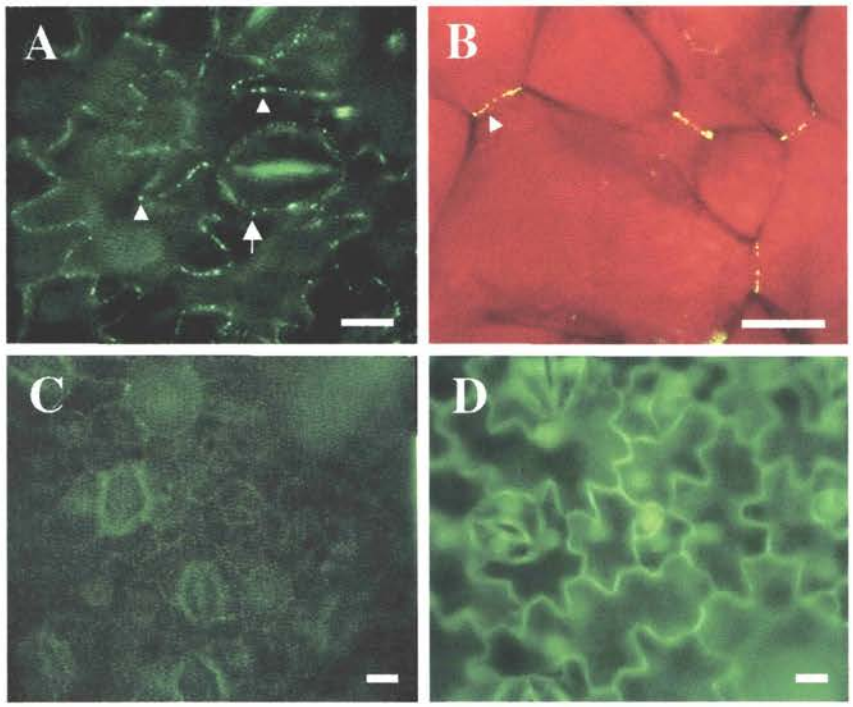


**Figure 4.** Localization of 3a MP:GFP to plasmodesmata during leaf development in transgenic tobacco plants.

**(A)** Tip of a source leaf. Green fluorescent dots (arrowheads) are prominent between all epidermal cells. In addition, such dots are also visible in the part of epidermal cell wall abutting a mature guard cell (arrow).
**(B)** Tip of a source leaf. Fluorescent dots (arrowheads) are present between mesophyll cells.
**(C)** Base of a transition leaf. Green fluorescent dots are absent from most of epidermal cells (except trichomes, guard cells, and veins).
**(D)** Tip of a source leaf from a transgenic tobacco expressing free GFP. Fluorescence is prominent in all epidermal cells and accumulates in the nucleus of every cell.
Scale bars = 40 $\mu$m

3a MP:GFP was not produced in sink leaves, we performed immunoblot analysis using antibodies against GFP. 3a MP:GFP was detected in both young and mature leaves of transgenic tobacco. The same result was obtained in transgenic tobacco expressing free GFP (Fig. 5). Therefore, we concluded that 3a MP:GFP was produced in sink leaves, although it was not localized to the cell walls.

The green fluorescent dots in cell walls likely represented 3a MP:GFP in complex secondary PD since their appearance was correlated with sink to source transition and with development of complex secondary PD. We performed immunolabeling to test this. Given that callose is deposited in PD, we first investigated whether the green fluorescent dots would be co-localized with callose using a monoclonal antibody against callose. As shown in Fig. 6, callose was indeed co-localized with green fluorescent dots of 3a MP:GFP in the cell walls. The result indicated that 3a MP:GFP was localized to PD.

To further confirm the above results and to determine whether 3a MP:GFP was specifically localized to complex secondary PD, we performed immunolabeling followed by electron microscopy on leaf sections from 3a MP:GFP-transgenic tobacco plants using an anti-3a MP antibody and a 10 nm gold-conjugated secondary antibody. By this method, we found that 3a MP:GFP was localized to complex secondary PD in both mesophyll and epidermal cells of transgenic leaves (Fig. 7A and B). In addition, we found that 3a MP:GFP was localized to the truncated PD between guard cells and epidermal cells (Fig. 7C). This explains the fluorescent dots observed in cell walls of guard cells under a fluorescence microscope (Fig. 4A). In contrast, 3a MP:GFP was absent from primary PD in leaves at any developmental stages (Fig. 7D). Therefore, we concluded that 3a MP:GFP was specifically targeted to complex secondary PD.

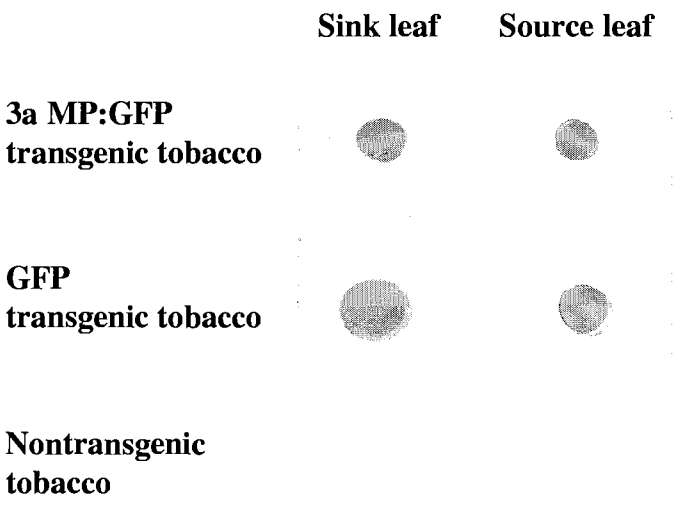


**Figure 5.** Immunoblot analysis showing the presence of 3a MP:GFP and GFP in transgenic tobacco plants.

The transgene products were detected with a rabbit-derived polyclonal antibody against GFP and then with a goat-derived anti-rabbit IgG antibody conjugated to alkaline phosphatase.

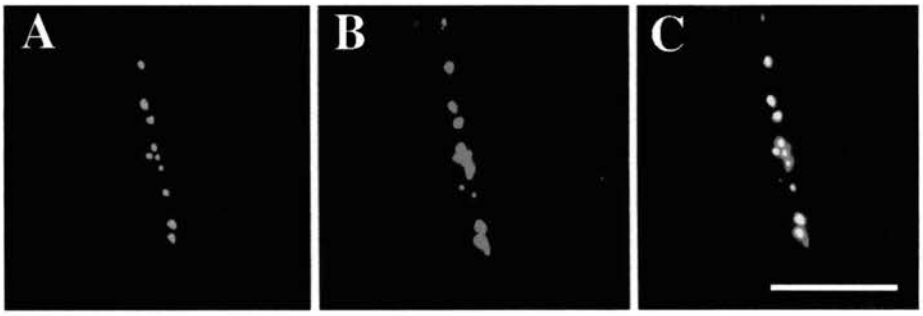


**Figure 6.** Localization of 3a MP:GFP to plasmodesmata in a transgenic tobacco.

**(A)** Confocal image of 3a MP:GFP fluorescent dots in walls between mesophyll cells in a source leaf.
**(B)** Confocal image of callose in the same section, detected with a monoclonal anti-callose antibody and a Texas Red-conjugated secondary antibody.
**(C)** Superimposed confocal images of (A) and (C) demonstrating colocalization of 3a MP:GFP and callose.
Scale bar = 40 $\mu$m.

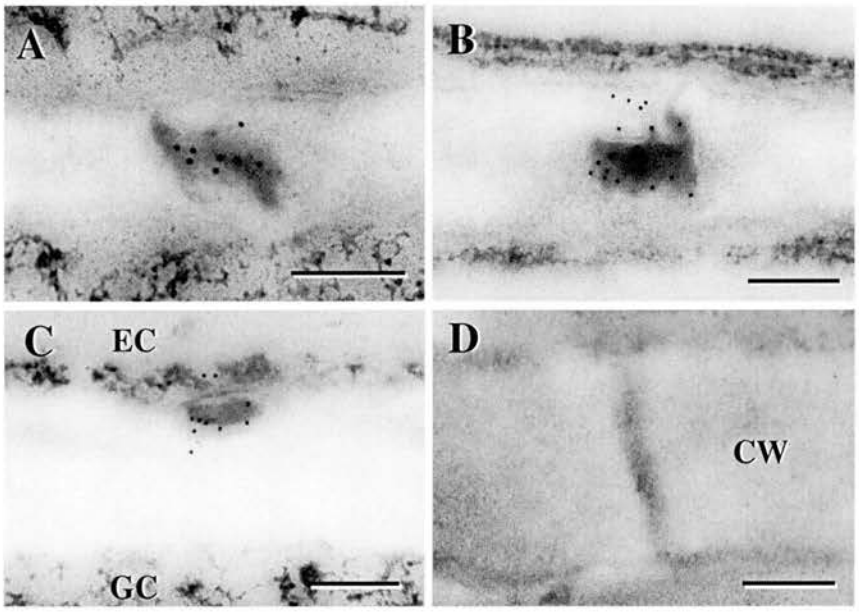


**Figure 7.** Immunolabeling of 3a MP:GFP fusion protein to complex secondary PD in transgenic tobacco plants.

The fusion protein was detected with a polyclonal anti-3a MP antibody and a 10 nm gold-conjugated secondary antibody.
**(A)** Complex secondary PD between two mesophyll cells showing gold labeling.
**(B)** Complex secondary PD between two epidermal cells showing gold labeling.
**(C)** Truncated PD between an epidermal cell (EC) and a guard cell (GC) showing gold labeling.
**(D)** Primary PD from a source leaf showing no gold labeling. CW, cell wall. Scale bars = 0.25 $\mu$m.

***Different roles of primary and complex secondary PD in intercelluar trafficking of 3a MP:GFP during leaf development***

With our finding that 3a MP:GFP was specifically localized to complex secondary PD formed during leaf development, we asked the question of whether this localization was related to intercellular trafficking of 3a MP:GFP. To answer this question, we used biolistic bombardment to produce 3a MP:GFP in nontransgenic tobacco leaf epidermis at successive developmental stages, and monitored intercellular trafficking of 3a MP:GFP.

When 3a MP:GFP was produced in either the base or the tip of a sink leaf where most PD were primary, 3a MP:GFP stayed in single cells and did not traffic from cell to cell (Table 1). Furthermore, no fluorescent dots were observed in the cell walls. This observation is consistent with the immunolabeling result showing that 3a MP:GFP was not localized to primary PD in sink leaves.

In the base of a sink-to-source transition leaf where most of PD were primary, 3a MP:GFP did not traffic from cell to cell (Table 1, Fig. 8A). However, in the tip of the transition leaf where complex secondary PD become dominant, 3a MP:GFP trafficked from cell to cell (Table 1, Fig. 8B). In a source leaf where most PD are complex secondary, 3a MP:GFP was capable of targeting to PD and intercellular trafficking in both the base and tip regions (Table 1, Fig. 8C and D). Our data indicate that intercellular trafficking of 3a MP:GFP only occurred in source leaves in which complex secondary PD were dominant.

***Intracellular localization of 3a MP:GFP and its relationship with trafficking***

There was not a single occasion where 3a MP:GFP trafficked without showing the fluorescent dots in cells walls. When 3a MP:GFP was produced in guard cells, it did not traffic into neighboring cells or show any fluorescent dots in the cell walls (Fig. 8C). Free GFP or M5 MP:GFP failed to localize to PD and did not traffic into neighboring cells

**Table 1.** Intercellular trafficking of 3a MP:GFP in leaf epidermis of *N. tabacum* cv. Samsum NN.

| Leaf | | | | |
|---|---|---|---|---|
| Stage | Region | 3a MP:GFP | M5 MP:GFP | GFP |
| Sink [a] | | 2/174 | 0/40 | 0/200 [c] |
| Transition | Base | 0/34 | --[b] | -- |
| | Tip | 9/20 | -- | -- |
| Source | Base | 14/25 | 0/53 | 0/75 |
| | Tip | 7/13 | -- | -- |

The proteins were produced *in planta* after plasmids containing the respective genes were delivered into the leaf epidermis by biolistic bombardment. Three to four leaves from different plants were used in each experiment, except for the sink leaf, for which 10 samples were used. The data are the number of cells permitting cell-to-cell trafficking of a protein over the total cells producing the protein.

[a] For the first leaf, identical results were obtained when the genes were bombarded into either the tip or the base regions, so the data were pooled.

[b] --, Not available.

[c] See Chapter IV for data obtained with a hand gun.

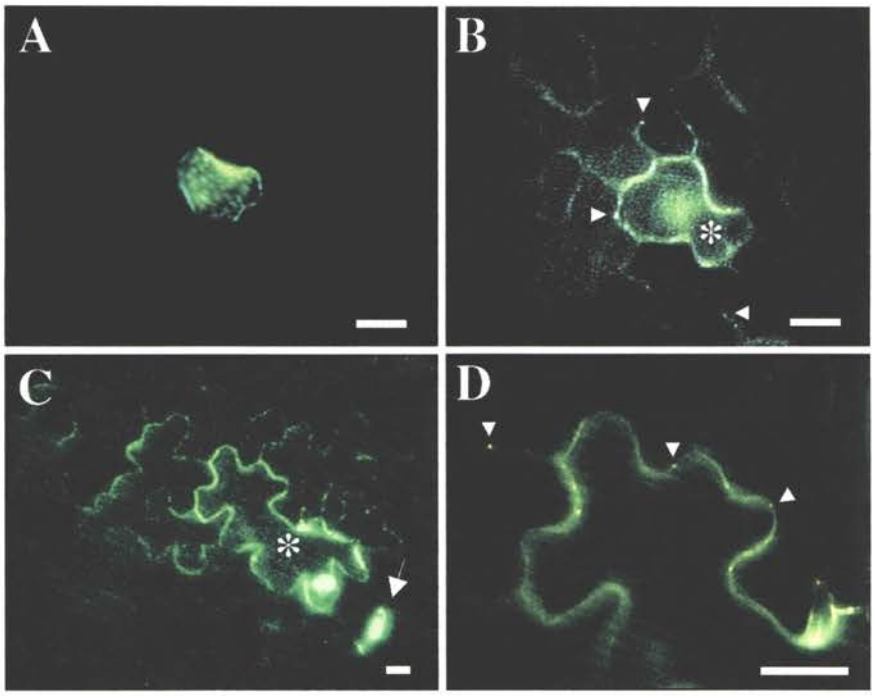


**Figure 8.** Cell-to-cell trafficking of 3a MP:GFP in tobacco leaf epidermis as a function of leaf development.

The fusion protein was produced by biolistic bombardment of the *3a :GFP* fusion gene.
**(A)** 3a MP:GFP produced in the base of a transition leaf, which remains in a single cell.
**(B)** 3a MP:GFP produced in an epidermal cell (asterisk) in the tip of the transition leaf, which traffics into neighboring cells. Note the presence of green dots in the cell walls (arrowheads).
**(C)** 3a MP:GFP produced in the base of a source leaf, which traffics from cell to cell. The asterisk denotes the cell producing the fusion protein. The arrow points to a guard cell that produces 3a MP:GFP but does not permit trafficking of the protein.
**(D)** High-magnification view of **(C)** showing the presence of fluorescent dots in the cell walls (arrowheads).
Scale bars = 20 $\mu$m.

(Table 1, also see Fig. 2 in Chapter II). These observations further support our conclusion that 3a MP:GFP traffics through complex secondary PD. Interestingly, in some cells, 3a MP:GFP was localized to complex secondary PD of the bombarded cell, but failed to move into neighboring cells. Thus, localization of 3a MP to complex secondary PD only is not sufficient for trafficking. Other regulatory factors are likely involved in PD trafficking.

In some cells expressing 3a MP:GFP, we observed filamentous structures (Fig. 9A). 3a MP:GFP was generally confined within such cells and did not move into neighboring cells during the period of observation. Occasionally 3a MP:GFP formed small aggregates (Fig. 9B), followed by large aggregates (Fig. 9C), finally localized to complex secondary PD (Fig. 9D) and trafficked into neighboring cells (Fig 9E). The nature and function of filaments are not known at this point. This structure was not observed in cells expressing free GFP or M5 MP:GFP.

***Cellular regulation of 3a MP:GFP trafficking at the vascular-nonvascular interface in tobacco***

We were interested in finding out how 3a MP:GFP would traffic between vascular and nonvascular tissues as this interface is the control point for many plant viruses to establish systemic infection (Nelson and van Bel, 1998). To investigate 3a MP:GFP trafficking at the vascular-nonvascular interface, we generated transgenic tobacco plants expressing 3a MP:GFP under the control of the phloem-specific commelina yellow mottle virus (CoYMV) promoter (Medberry et al., 1992). We obtained 8 lines of transgenic tobacco plants in which 3a MP:GFP was expressed specifically in the phloem (Fig. 10). Surprisingly, 3a MP:GFP did not traffic out of the phloem into nonvascular tissues at all leaf developmental stages. Detailed analyses revealed that 3a MP:GFP was present in companion cells, sieve elements, and phloem parenchyma cells, but not in

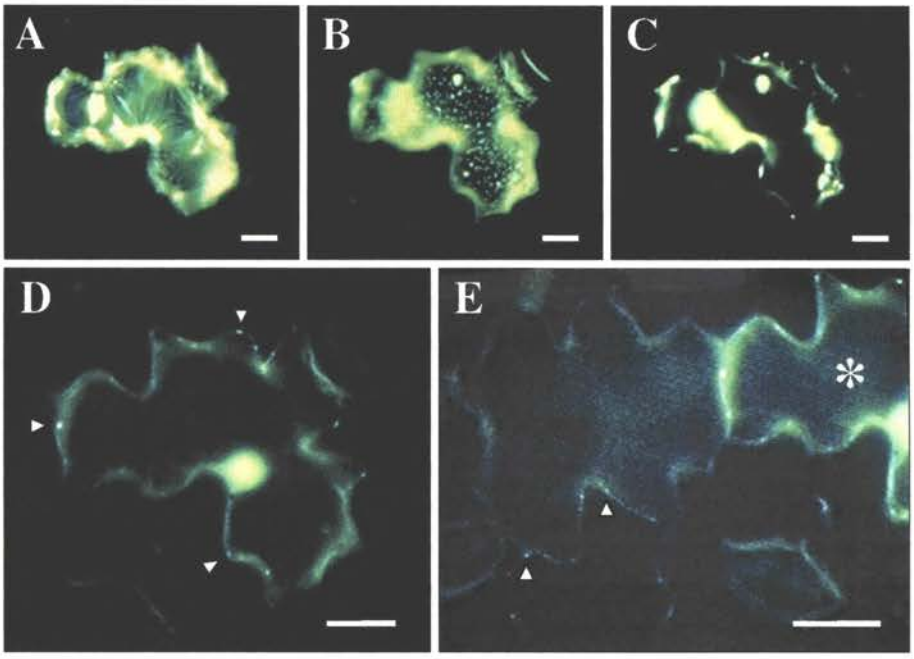


**Figure 9.** Intracellular localization and intercellular trafficking of 3a MP:GFP in a tobacco source leaf.

The fusion protein was produced by biolistic bombardment of the 3a:GFP fusion gene.
**(A)** Image taken 24 h after bombardment. 3a MP:GFP forms filamentous structures in the cytoplasm.
**(B)** Image taken 32 h after bombardment. 3a MP:GFP forms small aggregations in the cytoplasm.
**(C)** Image taken 40 h after bombardment. 3a MP:GFP forms large aggregations. Intercellular trafficking has not been observed.
**(D)** Image taken 54 h after bombardment. Localization of 3a MP:GFP to complex secondary PD are seen as fluorescent dots in the walls (arrowheads).
**(E)** Image taken 54 h after bombardment showing cell-to-cell trafficking of 3a MP:GFP into neighboring cells. The fusion protein is also localized to complex secondary PD in the walls of neighboring cells (arrowheads). The asterisk denotes the cell producing the protein. Scale bars = 20 $\mu$m

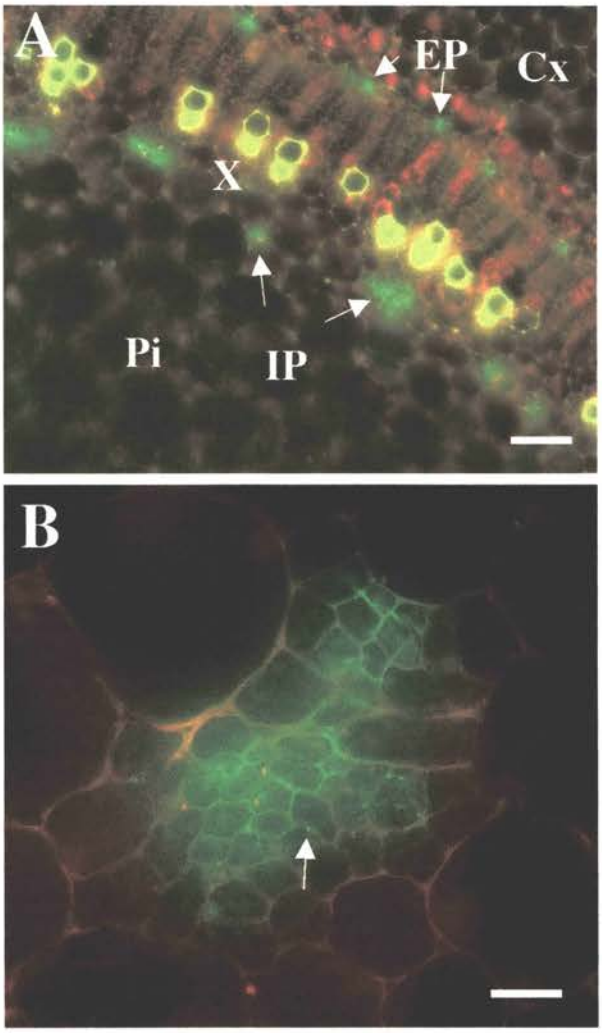


**Figure 10.** Transgenic tobacco expressing 3a MP:GFP under the control of the phloem-specific promoter.

(**A**) Transverse view of a stem of the transgenic plant. 3a MP:GFP is produced in the internal phloem (IP) and the external phloem (EP), but is absent form the xylem (X), pith (Pi) or cortex (Cx). Scale bar = 50 $\mu$m.
(**B**) High-magnification view of phloem cells. 3a MP:GFP is localized to complex secondary PD between phloem cells (arrow). The protein is confined in phloem cells.
Scale bar = 10 $\mu$m.

bundle-sheath cells. Therefore, the interface between bundle-sheath and phloem cells formed a barrier for 3a MP:GFP trafficking out of the phloem. This finding suggests that there exists cellular regulation of 3a MP:GFP trafficking between different tissues.

## DISCUSSION

We have demonstrated that, during tobacco leaf development, PD structures are modified with fundamental changes in protein trafficking functions. Specifically, formation of complex secondary PD is directly related to sink-to-source transition of a leaf. Furthermore, the complex secondary PD are the site of 3a MP:GFP localization, and this specific localization is required for 3a MP:GFP trafficking. This is the first evidence showing the different functions of primary and complex secondary PD in specific protein trafficking. TMV MP was also shown to localize to complex secondary PD in transgenic tobacco plants (Ding et al., 1992). Furthermore, TMV MP could increase SEL of PD in mature leaves, but not in young leaves (Deom et al., 1990; Ding et al., 1992). However, Crawford and Zambryski (2001) recently showed that biolistically expressed TMV MP:GFP fusion protein was localized to PD and was able to traffic intercellularly in sink leaves of *N. tobacum* and *N. clevlandii.* The extent and percentage of TMV MP:GFP movement was even higher in sink leaves than in older leaves. Unfortunately, the types of PD for TMV MP:GFP localization were not examined in this study. This result contradicts the results obtained by us, Deom et al. (1990) and Ding et al. (1992). One noticeable difference was the morphology of cells. In our study, the cells in sink leaves had small, simple and almost symmetric shapes (*e.g.,* circle or square-like; Fig. 8A) except some specified cells such as trichomes, guard cells and veins. As leaves underwent sink-to-source transition, cells became larger and complex in their shape

similar to the shape of jigsaw passels (Fig 4D, 8C). The cells in sink leaves shown by Crawford and Zambryski (2001) appear highly differentiated and have the similar morphology as cells in source leaves in our study (see Fig 4 in Crawford and Zambryski, 2001). It is possible that the leaves studied as sink leaves were actually source leaves in their study. To test this possibility, this study needs to be repeated carefully.

In contrast to developmental regulation of 3a MP:GFP trafficking in leaf epidermis, 3a MP:GFP trafficking from the phloem to the bundle sheath is restricted regardless of plant developmental stages. Specifically, the fusion protein is confined to the phloem cells, and does not traffic into bundle-sheath and other nonvascular cells in the transgenic plants. This result strongly indicates that 3a MP:GFP trafficking is regulated by cell-specific factors in addition to developmental factors. We assume that not only PD structure and components, but also cytosolic factors are involved in regulation of specific trafficking. Such cellular factors may function positively to promote protein trafficking, or negatively to inhibit protein trafficking. Ghoshroy et al. (1998) reported that non-toxic concentration of cadmium inhibits systemic infection of turnip vein clearing virus (TVCV), otherwise an infectious virus, in tobacco. Cadmium has no effect on viral replication, assembly and local movement, but it inhibits virus exit from the vascular tissue into the nonvascular tissue (Ghoshroy et al., 1998). Cadmium may induce the negative cellular factors that inhibit phloem unloading of virus. Alternatively, it may suppress the positive cellular factors that support virus movement. Presence or absence of such cellular factors at different developmental stages, in different cell types, and in response to environmental stimuli may contribute to the regulation of specific protein trafficking.

Although we are not investigating the mechanisms of CMV movement, our findings that 3a MP:GFP does not move in sink leaves or from the phloem into the nonvascular tissues raise the question of how CMV can infect those tissues. It has been

suggested that replicase (Gal-on et al., 1994) and coat protein (Suzuki et al., 1991; Boccard and Baulcombe, 1993; Canto et al., 1997) are required for CMV cell-to-cell movement. Blackman et al., (1998) proposed that CMV traffics into minor vein sieve elements as a ribonucleoprotein complex that contains the viral RNA, coat protein, and 3a MP, with subsequent viral assembly occurring in the sieve element parietal layer. Therefore, other viral proteins than 3a MP appear to be required for viral movement in certain types of tissues.

An interesting phenomenon is that approximately 50% of the epidermal cells producing 3a MP:GFP permit trafficking of 3a MP:GFP into surrounding cells. This indicates that epidermal cells in the same leaf are not as uniform in protein trafficking function as they may appear. Interestingly, even in some cells that do not support 3a MP:GFP trafficking, 3a MP:GFP target to complex secondary PD. Therefore, targeting of the protein to PD is the required step, but not sufficient for 3a MP:GFP trafficking. Presumably, subsequent steps such as translocation of the protein through PD, and release of the protein from PD into the importing cell are other regulatory points for protein trafficking (Ding, 1998). This hypothesis is supported by the observation that 3a MP:GFP is targeted to truncated PD at the guard cell-ordinary epidermal cell junction, even though such truncated PD cannot function to transport any molecules. Whether trafficking of plant endogenous molecules is also regulated at levels of individual cell or cell clusters (mosaic patterns) remains to be determined. If so, the patterns and biological functions of such regulation are of great interest. We have observed filamentous structures formed by 3a MP:GFP in some transfected cells. The nature and functions of these filamentous structures remain to be determined.

The specific targeting of 3a MP:GFP to complex secondary PD can be useful in the study of cell development. In the transgenic plants expressing 3a MP:GFP, the fluorescent dots in cell walls can be interpreted as the presence of complex secondary

PD. In a tobacco sink leaf, targeting of 3a MP:GFP to complex secondary PD can be found in cell walls of guard cells, veins, and in trichome cells, while 3a MP:GFP is not targeted to complex secondary PD in other epidermal cells (data not shown). This implies the early maturation and formation of secondary PD in guard cells, veins, and trichomes. Targeting of 3a MP:GFP to complex secondary PD as fluorescent dots may be used conveniently to investigate PD modification in different parts of a plant such as vascular tissues, roots, stems, flowers when the promoter is active in those tissues. However, fluorescent dots are not definite indicator of complex secondary PD formation because there is a possibility that 3a MP:GFP cannot target to them in the specific tissues. If such phenomenon is observed, it is still interesting to find out the cellular factors responsible for the inability of 3a MP:GFP targeting to complex secondary PD. Relationship between formation of complex secondary PD and macromolecular trafficking other than 3a MP:GFP would further reveal the function of complex secondary PD.

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# CHAPTER IV

# DEVELOPMENTAL AND CELLULAR REGULATION OF NONSPECIFIC PROTEIN TRAFFICKING[1]

[1] Data presented here were published as: Itaya, A., Liang, G., Woo, Y.-M., Nelson, R. S., Ding, B. (2000) Nonspecific intercellular protein trafficking probed by green-fluorescent protein in plants. Protoplasma **213**, 165-175. The data are presented here with modifications with permission from Springer-Verlag (Wien New York).

# INTRODUCTION

Based on a number of microinjection studies, the size exclusion limit (SEL) of PD for passive diffusion was established to be 1 kDa in most cases (Tucker, 1982; Barclay et al., 1982; Goodwin, 1983; Terry and Robards, 1987; Burnell 1988; Weiner et al., 1988; Wolf et al., 1991), 7 kDa in tobacco trichome cells (Waigmann and Zambryski, 1995), and 3 and 10 kDa between companion cells and sieve elements in *Cucubita maxima* (Kempers et al., 1993) and in *Vicia faba* (Kempers and van Bel, 1997). Therefore, macromolecules were generally thought to traffic only in specific manners by interacting with dedicated cellular factors. However, a recent study showed that a non-plant protein, the 27 kDa GFP, that was expressed specifically in companion cells could diffuse into other cells in sink leaves, roots, and flowers in transgenic *A. thaliana* (Imlau et al., 1999). Further analyses of the transgenic plants or grafting experiments indicated that GFP was translocated from companion cells, the site of protein synthesis, into sieve elements and trafficked long distances through the phloem (Imlau et al., 1999). GFP trafficking was also observed in sink leaves of tobacco when it was expressed by biolistic bombardment, by a viral vector, or when it was microinjected (Oparka et al., 1999). GFP trafficking was a function of plant development. Specifically, simple primary PD in sink leaves allowed GFP diffusion, whereas complex secondary PD in source leaves restricted GFP diffusion. Surprisingly, the upper limit of nonspecific trafficking was shown to be 50 kDa (Oparka et al., 1999). Therefore, SEL of PD for diffusion appeared to be much larger than was shown by microinjection studies in some tissues and in some plants.

It is not known whether nonspecific trafficking of GFP observed in *A. thaliana* and tobacco plants is a general phenomenon in plants. If so, is nonspecific GFP trafficking regulated by developmental stages in all plants? Are there cellular regulations for GFP trafficking? Answers to these questions should enhance our understanding of

protein trafficking in plants. To this end, we have conducted a comprehensive analysis of GFP trafficking in several plant species at various developmental stages.

## MATERIALS AND METHODS

### *Plant material and growth conditions*

Tobacco (*N. tabacum* cv. Samsun nn, and *N. tabacum* cv. Xanthi nn) were grown in a growth chamber controlled at a 14 hours light (28°C)/10 hour dark (22°C) cycle. *A. thaliana* (Columbia, Wassilewskija, and Landsberg) were grown at 23°C under 40 W cool white fluorescent light kept on a 16 hours light/8 hour dark cycle. Tomato (*Lycopersicon esculentum* var Rutgers) and cucumber (*Cucumis sativus*) were grown in a growth room controlled at 14 hour light (30°C)/10 hour dark (25°C) cycle.

### *Construction of gene expression vectors*

Construction of *35S:3a MP:GFP, 35S:M5 MP:GFP,* and *35S:GFP* is described in Chapter II.

### *6(5)-carboxyfluorescein diacetate translocation experiments*

The sink and source status of leaves were determined by CF translocation patterns as described in Chapter III.

### *Biolistic bombardment with a vacuum gun*

Procedures for biolistic bombardment by a vacuum gun (Biolistic PDS 1000/He system, BioRad, Hercules, California, USA) are described in Chapter II.

***Biolistic bombardment with a hand gun (Helios Gene Gun, BioRad)***

*Preparation of tubing*

Two mg of 1 μm gold particles (for 10 shots) and the following amount of DNA were used for preparation of tubing: 10 μg of DNA/10 shots for *35S:3a MP:GFP* and *35S:M5 MP:GFP*, and 5 μg of DNA/10 shots for *35S:GFP*. Gold particles were washed with absolute ethanol and ddi$H_2O$ as described in the vacuum gun procedures. One hundred μl of 0.05 M spermidine was added to the gold solution. The mixture was sonicated to disrupt aggregation of gold particles. One hundred μl of 1 M $CaCl_2$ was added to the mixture, and incubated at room temperature for 10 min. Gold particles coated with DNA were then collected by brief spin, washed with 1 ml of absolute ethanol three times, and resuspended in 600 μl (for 10 shots) of absolute ethanol containing 60 μg of polyvinylpyrrolidone (PVP; 0.1 mg PVP/ml ethanol). The DNA solution was used to coat tubing following manufacture's instructions. Coated tubing was stored dry at 4°C.

*Bombardment*

The upper epidermis of leaves or epidermis of stems undetached from a plant were bombarded at a pressure of 150-200 psi per manufacturer's instructions. A diffuser (BioRad) was used to minimize the aggregation of gold particles. Spongy was used to support plants to prevent breakage of tissue by pressure. Bombarded tissues remained attached to a plant in a growth chamber until examined under a fluorescence microscope.

***Fluorescence microscopy***

Trafficking of GFP was examined under a fluorescence microscope as described in Chapter II.

## RESULTS

### *Differences between a vacuum gun and a hand gun*

We have employed a hand gun for biolistic bombardment that does not require detachment of leaves from a plant to confirm the results obtained with a vacuum gun. When tobacco source leaves were used, the results obtained by a hand gun and by a vacuum gun were essentially the same: GFP did not move from cell to cell (Table 1). However, when tobacco sink leaves were bombarded, we obtained different results between the vacuum gun and the hand gun with regard to intercellular trafficking of free GFP. When free GFP was produced by the vacuum gun in a tobacco sink leaf, it stayed in a single cell and did not show intercellular trafficking for up to 5-7 days (Table 1). On the contrary, when GFP was produced by the hand gun in a sink leaf, it showed intensive intercellular trafficking within 12 h (Table 1, Fig. 1A). For 3a MP:GFP, the results obtained by a vacuum gun and a hand gun were the same in both source and sink leaves (data not shown). Therefore, nonspecific trafficking of GFP in sink leaves was affected by the types of guns used while specific trafficking of 3a MP:GFP was not.

We assumed that different treatments used for two different guns caused different physical conditions of a plant that were responsible for the differences in intercellular trafficking of GFP. The first possibility we considered was the detachment of a leaf, which was necessary for a vacuum gun. To test this possibility, we tried different treatments of leaves for the hand gun: 1) detachment of a leaf prior to the bombardment and 2) detachment of a leaf after the bombardment. Furthermore, to avoid variations between leaves, we also tried 3) bombardment of a leaf on a plant, followed by removal of half of the bombarded leaf, and allow the other half to remain attached to a plant. In all cases, GFP produced by a hand gun trafficked from cell to cell (data not shown), therefore, detachment of leaves did not prevent intercellular trafficking of GFP.

**Table 1.** Intercellular trafficking of GFP, produced by two types of gene guns, in leaf epidermis of tobacco.

| Leaf Samples | No. of clusters and of single cells expressing GFP [a] | | No. of cells/cluster (average) | % Cells permitting GFP trafficking [b] |
|---|---|---|---|---|
| | Clusters | Singles | | |
| *N. tabacum* cv. Samsum nn (Vacuum gun)[c] | | | | |
| Sink | 3 | 106 | 4-10 (5.6) | 2.8 |
| Source | 0 | 39 | | 0 |
| *N. tabacum* cv. Samsum nn (Hand gun) | | | | |
| Sink | | | | |
| 16 hpb[d] | 13 | 104 | 3-20 (7.7) | 11.1 |
| 72 hpb | 68 | 42 | 3-40 (7.2) | 61.8 |
| Source | | | | |
| 72 hpb | 2 | 69 | 2 | 2.8 |
| *N. tabacum* cv. Xanthi nn (Hand gun) | | | | |
| Sink | | | | |
| 16 hpb | 62 | 450 | 3-30 (12.6) | 13.8 |
| 72 hpb | 80 | 100 | 3-70 (15.0) | 44 |
| Source | | | | |
| 72 hpb | 2 | 177 | 10 | 1.1 |

[a] A cluster is defined as two or more adjacent cells showing GFP. A single cell is defined as a solitary cell showing GFP fluorescence.
[b] Each percentage is equivalent to the number of cell clusters divided by the sum of the number of cell clusters and the number of single cells.
[c] Data collected three days (72 h) after gene bombardment.
[d] Hours post bombardment.

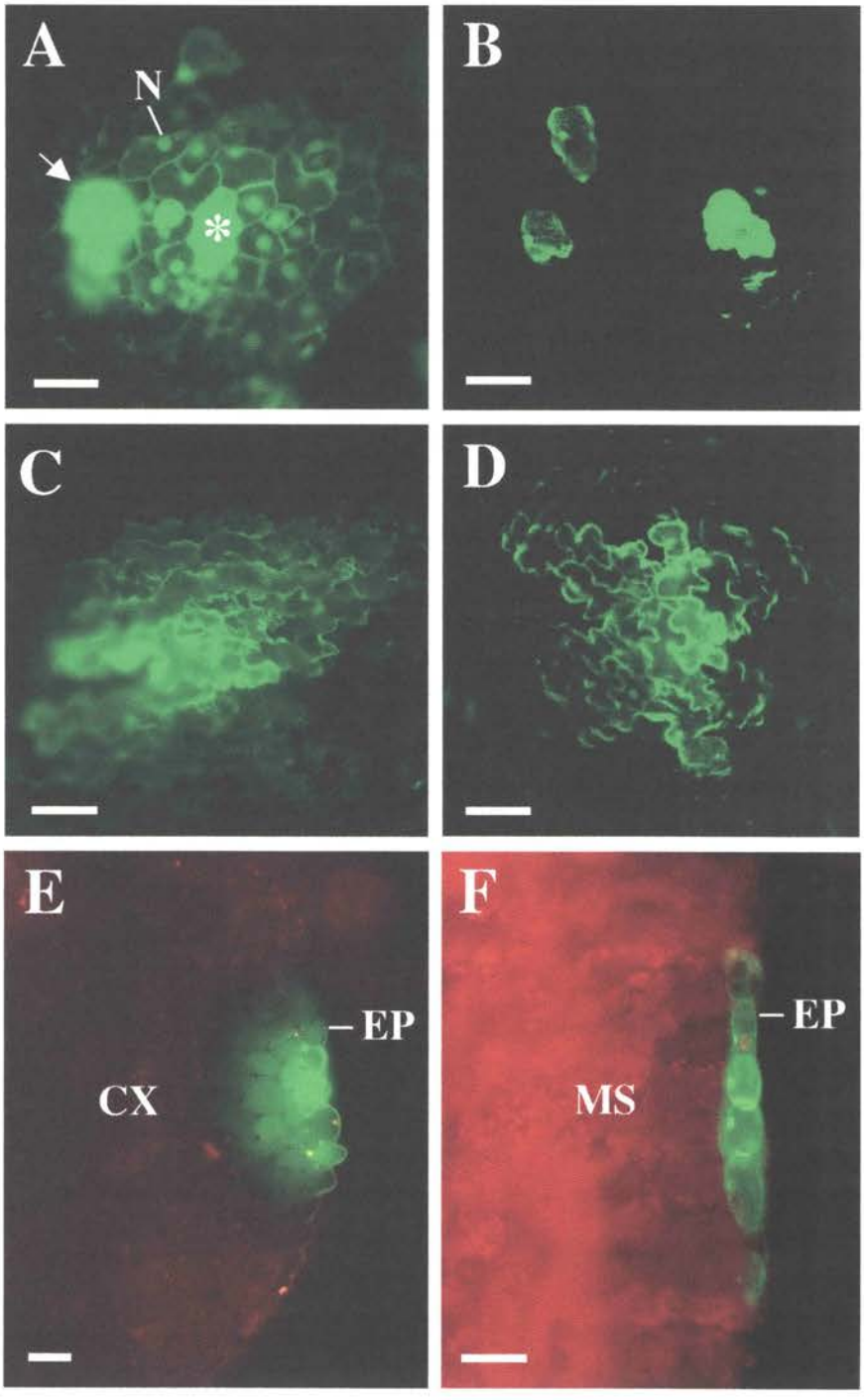
A
N
B
C
D
E
EP
CX
F
EP
MS

**Figure 1.** Nonspecific intercellular trafficking of GFP

**(A)** GFP trafficking in a sink leaf of *N. tabacum* cv. Samsun nn. Asterisk indicates the cell producing GFP. Arrow indicates a trichome cell. (N) Nucleus. Scale bar = 10 μm.
**(B)** GFP in single cells in a source leaf of *N. tabacum* cv. Samsun nn. Scale bar = 40 μm.
**(C)** GFP trafficking in rosette leaf epidermis of *A. thaliana.*
Scale bar = 40 μm.
**(D)** GFP trafficking in cucumber cotyledon epidermis.
Scale bar = 40 μm.
**(E)** Transverse section of cucumber hypocotyl showing trafficking of GFP between epidermal cells (EP) and from epidermis into two layers of cortical cells (CX).
Scale bar = 20 μm.
**(F)** Transverse section of cucumber cotyledon showing lateral trafficking of GFP among epidermal cells and no trafficking from epidermis to mesophyll (MS). Red fluorescence is from chlorophyll. Scale bar = 40 μm.

The second possibility was the vacuum condition applied by a vacuum gun. To test this possibility, we treated leaves in different ways prior to a hand gun bombardment: 1) the vacuum treatment of a leaf for a few seconds and a gradual release of vacuum prior to the bombardment, and 2) the vacuum treatment followed by a rapid release of vacuum (*i.e.* bombardment without gold particles) prior to the bombardment by a hand gun. In either case, GFP produced by a hand gun showed intercellular trafficking (data not shown). Therefore, we could not determine the cause of the different intercellular trafficking patterns of GFP produced by the two different guns in a sink leaf.

We originally utilized a vacuum gun for the investigation of GFP trafficking in plants. Because different effects on protein trafficking by the two guns were found, we repeated experiments with the hand gun. In the following sections, data obtained with the vacuum gun are presented when the results were the same with the hand gun. Otherwise, data obtained with the hand gun are presented.

***Nonspecific intercellular trafficking of GFP during tobacco leaf development***

When GFP was produced in a source leaf, it stayed in single cells and did not traffic into neighboring cells for up to 5 days (Table 1, Fig. 1B). On the contrary, when GFP was produced in a sink leaf, GFP exhibited extensive intercellular trafficking (Fig 1A, Table 1). We examined two tobacco cultivars (*N. tabacum* cv. Samsun nn and *N. tabacum* cv. Xanthi nn) and obtained essentially the same results (Table 1). The trafficking of GFP continued for three or more days, resulting in an increase in the percentage of expressing cells that permitted GFP to traffic as well as in the number of fluorescent cells (up to 70 cells) in a given cluster.

We interpreted a cluster of fluorescent cells as the result of GFP trafficking, but not a result of simultaneous GFP expression in adjacent cells. First, the chance of simultaneous transformation of many continuous cells is very low (1-3%) based on the

expression pattern of GFP in source leaves (Table 1). Second, each cluster often contains a bright fluorescent cell in the center, which is most likely the transformed cell, and fluorescence gradually fades out towards the edge of the cluster (Fig. 1A). Third, both the number of clusters and the number of cells within a cluster increase over time, and they do not appear simultaneously (Table 1). Fourth, guard cells within a cluster are not fluorescent (Fig. 1A), indicating they are not transformed and GFP does not traffic into them because they are symplasmically isolated. Therefore, we concluded that GFP trafficked intercellularly in a sink leaf, but not in a source leaf.

***GFP trafficking in leaf epidermis and inflorescence stems of A. thaliana***

We investigated GFP trafficking in three ecotypes, Columbia, WS, and Landsberg, of *A. thaliana*. For each plant, rosette leaves, cauline leaves and inflorescence stems were examined. Regardless of ecotype, plant age or organs, GFP trafficked out of 30-50% of the total GFP expressing cells (Table 2). The sizes of clusters ranged from 3 to 15 cells with an average of 5 cells (Fig. 1C). Data shown in Table 2 were obtained by the vacuum gun. Essentially the same result was obtained by the hand gun (data not shown). Therefore, the developmental status of a tissue did not affect GFP trafficking in *A. thaliana*. This was sharp contrast with results from tobacco leaves. Furthermore, GFP showed extensive intercellular trafficking in all organs examined in *A. thaliana*.

***GFP trafficking in leaf epidermis, cotyledons and hypocotyls of cucumber***

GFP trafficked intercellularly in cucumber hypocotyls and young cotyledons (7-10 days old) regardless of the types of gene guns used to produce the protein (Fig. 1D, Table 3). The trafficking became limited in old cotyledons (27-30 days), whereas it remained extensive in hypocotyls or in stems throughout the time of observation period (Table 3). GFP trafficked in 17-20% of the cases when GFP was produced in a sink leaf with the

**Table 2.** Intercellular trafficking of GFP, produced by a vacuum gun, in *A. thaliana.*

| Ecotype and Organ [a] | No. of clusters and of single cells expressing GFP [b] | | No. of cells/cluster (average) | % Cells permitting GFP trafficking [c] |
|---|---|---|---|---|
| | Clusters | Singles | | |
| Columbia | | | | |
| Rosette | 71 | 75 | | 47 |
| Cauline | 11 | 15 | 3-16 (5.5) | 42 |
| Stem | 6 | 7 | | 46 |
| WS | | | | |
| Rosette | 17 | 29 | | 37 |
| Cauline | 37 | 40 | 3-10 (5.0) | 48 |
| Stem | 3 | 5 | | 38 |
| Landsberg | | | | |
| Rosette | 20 | 40 | | 33 |
| Cauline | 5 | 7 | 3-15 (5.6) | 42 |
| Stem | 13 | 12 | | 52 |

[a] The ages of plants used were 21-28 days.
[b] A cluster is defined as two or more adjacent cells showing GFP. A single cell is defined as a solitary cell showing GFP fluorescence.
[c] Each percentage is equivalent to the number of cell clusters divided by the sum of the number of cell clusters and the number of single cells.

**Table 3.** Intercellular trafficking of GFP, produced by biolistic bombardment, in cucumber.[ab]

| Plant age (days) | Organ | No. of clusters and of single cells expressing GFP [c] | | No. of cells/cluster (average) | % Cells permitting GFP trafficking [d] |
|---|---|---|---|---|---|
| | | Clusters | Singles | | |
| 7-10 | cotyledon [a] | 9 | 25 | 8-22 (14.7) | 26.4 |
| | hypocotyl [a] | 28 | 42 | 3-15 (8.8) | 40.0 |
| 18-23 | cotyledon [a] | 40 | 86 | 5-14 (10.4) | 31.7 |
| | hypocotyl [a] | 30 | 59 | 4-7 (5.3) | 33.7 |
| | sink leaf [b] | 32 | 127 | 3-30 (10.2) | 20.1 |
| | source leaf [b] | 0 | 27 | | 0 |
| 27-30 | cotyledon [a] | 2 | 74 | 3-4 (3.5) | 2.6 |
| | hypocotyl [a] | 13 | 18 | 4-20 (9.8) | 41.9 |
| | sink leaf [b] | 4 | 20 | 4-30 (13.5) | 16.6 |
| | source leaf [b] | 0 | 5 | | 0 |
| 43 | stem [a] | 3 | 4 | 4-8 (6.0) | 42.8 |

[a] GFP was produced by a vacuum gun in these organs.
[b] GFP was produced by a hand gun in these organs.
[c] A cluster is defined as two or more adjacent cells showing GFP. A single cell is defined as a solitary cell showing GFP fluorescence.
[d] Each percentage is equivalent to the number of cell clusters divided by the sum of the number of cell clusters and the number of single cells.

hand gun (Table 3). GFP produced in source leaves rarely trafficked out of the expressing cells (Table 3). Therefore, GFP trafficking is also regulated by leaf development in cucumber, although the degree of trafficking in sink leaves was less than that in tobacco (Table1 and 3).

***GFP trafficking in tomato***

When GFP was produced in young (8-11 days) tomato cotyledons and hypocotyls, it trafficked from cell to cell. Interestingly, as a plant grew older, GFP trafficking in these organs gradually diminished (Table 4). This was different from the situation in cucumber where GFP trafficking remained intensive in hypocotyls or stems regardless of plant age (Table 4). To our surprise, GFP did not traffic in either sink or source leaves of tomato, in contrast to its trafficking in tobacco or cucumber sink leaves.

***Cellular regulation of GFP trafficking in cucumber***

We were interested in finding out whether GFP would traffic nonspecifically between different tissue layers. We chose to analyze cotyledons and hypocotyls from 10-day old cucumber because GFP was expressed particularly well in these tissues. Additionally, these organs had thick, solid, yet soft tissues that allowed us to obtain good hand sections. Among 56 clusters of fluorescent epidermal cells in the hypocotyl we examined, 39 of these clusters showed GFP fluorescence in 2-3 layers of underlying cortical cells (Fig. 1E). In contrast, in 49 fluorescent clusters of cotyledon epidermal cells, we never detected GFP fluorescence in the underlying mesophyll (Fig. 1F). Therefore, GFP could traffic from epidermal cells into the underlying layers in hypocotyls, but not in cotyledons. This observation indicated that there is a tissue-specific cellular regulation of nonspecific GFP trafficking in some organs.

**Table 4.** Intercellular trafficking of GFP, produced by a vacuum gun, in tomato.

| Plant age (days) | Organ | No. of clusters and of single cells expressing GFP [a] | | No. of cells/cluster (average) | % Cells permitting GFP trafficking [b] |
|---|---|---|---|---|---|
| | | Clusters | Singles | | |
| 8-11 | cotyledon | 22 | 26 | 3-11 (5.1) | 45.8 |
| | hypocotyl | 17 | 22 | 3-10 (5.4) | 43.6 |
| 13-15 | cotyledon | 6 | 35 | 2-4 (2.8) | 14.6 |
| | hypocotyl | 1 | 10 | 6 (6) | 9.0 |
| 18-23 | cotyledon | 0 | 4 | | 0 |
| | hypocotyl | 3 | 17 | 4 (4) | 15.0 |
| | sink leaf | 3 | 66 | nd [c] | 4.3 |
| | source leaf | 30 | 471 | nd | 5.9 |
| 27-30 | cotyledon | 0 | 0 | | 0 |
| | source leaf | 0 | 22 | | 0 |
| | source leaf | 0 | 104 | | 0 |
| | stem | 9 | 501 | nd | 1.7 |

[a] A cluster is defined as two or more adjacent cells showing GFP. A single cell is defined as a solitary cell showing GFP fluorescence.
[b] Each percentage is equivalent to the number of cell clusters divided by the sum of the number of cell clusters and the number of single cells.
[c] Not determined

# DISCUSSION

Plant development exerts a significant control over both nonspecific and specific protein trafficking. In tobacco plants, specific 3a MP:GFP trafficking is restricted in sink leaves, but not in source leaves as discussed in Chapter III. From this result only, it would appear as if primary PD do not facilitate macromolecular trafficking, and complex secondary PD are specified for macromolecular trafficking. However, this is clearly not the case because nonspecific GFP trafficking occurs in sink leaves, but not in source leaves. Oparka et al. (1999) proposed that primary PD are actually free gate for macromolecular trafficking, and secondary PD restrict nonspecific trafficking. If nonspecific protein trafficking occurs in sink leaves, how do cells in such leaves establish or retain their specific identities? There may be retention mechanisms to prevent undesirable diffusion of endogenous low molecular weight proteins as discussed by Imlau et al. (1999). It has been shown that ubiquitin from *C. maxima* with a molecular mass of only ~8 kDa does not traffic through PD in cotyledon mesophyll cells even though coinjection with phloem protein PP2 increased SEL of PD to 20 kDa (Balachandran et al., 1997). GFP trafficking is inhibited when the ER targeting signal is fused to GFP (Oparka et al., 1999; Crawford and Zambryski, 2000). Therefore, cells in sink tissues may control nonspecific trafficking by localizing proteins to specific subcellular sites or forming protein complexes that will not diffuse through PD. Most importantly, the question of whether any plant endogenous proteins would traffic nonspecifically remains to be investigated.

Sink or source status is not always the determinant of nonspecific protein traffics as GFP trafficked in cotyledons of cucumber or tomato. Cotyledons supply nutrients to germinating seedlings, thereby functioning as a source. Interestingly, GFP trafficking in cotyledons diminishes as the plant ages. Therefore, there are developmental factors that restrict GFP trafficking in cotyledons. On the other hand, even though protein trafficking

is greatly regulated by plant development in many cases as discussed above, it is not always true in some organs or in some plants. For example, nonspecific trafficking of GFP occurs in epidermis of leaves and stems regardless of developmental stages in *A. thaliana*. Similarly, GFP trafficking occurs in hypocotyls or stems of cucumber regardless of plant age. Conversely, GFP rarely traffics in either sink or source leaves of tomato plants. It will be revealing to investigate the structures of PD and their relationship with GFP trafficking in these plant species and organs.

Cellular regulation of GFP trafficking occurs at the epidermal-mesophyll interface of cucumber cotyledons. While GFP produced in an epidermal cell traffics into neighboring epidermal cells, it does not traffic into the mesophyll underneath. The mechanisms for this restriction are not understood. Imlau et al. (1999) showed that GFP is excluded from epidermal and cortical files of mature root, and from developing gametophytes. These observations are consistent with previous reports that showed symplasmic isolation of those organs and tissues (Oparka et al., 1994; Weber et al., 1997). Therefore, nonspecific trafficking of GFP can be useful to assess symplasmic domains within a plant.

We have observed differences between two biolistic bombardment systems, a vacuum gun and a hand gun with regard to intercellular trafficking of GFP in sink leaves of tobacco and cucumber. We speculate that certain conditions required for a vacuum gun caused the closure of PD that prevented movement of GFP in sink leaves. Crawford and Zambryski (2001) speculated that the high pressure (900-1,300 psi) used for a vacuum gun might affect the protein trafficking. However, the distance between the gun and a leaf was greater with a vacuum gun (10-15 cm) than with a hand gun (~2 cm), thus actual pressure at the leaf surface during bombardment is not known.

Although we do not know the cause of the different results obtained by the two guns for sink leaves of tobacco and cucumber, we believe that results obtained by a hand

gun are more biologically relevant than a vacuum gun because of the evidence of GFP trafficking in sink leaves in transgenic plants (Oparka et al., 1999; Imlau et al., 1999). Therefore, to study protein trafficking in a plant, a hand gun will be the better tool since it appears to affect plant physiology less than a vacuum gun. A vacuum gun could be useful in cases where plant physiology is less significant, for example testing the function and expression level of promoters.

In conclusion, our results indicate that nonspecific trafficking of GFP occurs in a variety of plant species. Nonspecific trafficking is regulated by multiple mechanisms involving developmental, cellular, organ- and species-specific factors. The molecular mechanisms of such regulation remain to be determined. Determination of whether plant endogenous proteins can traffic nonspecifically is an important task for future research.

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# CHAPTER V

# DEVELOPMENT OF GENETIC APPROACHES TO ISOLATE CELLULAR FACTORS FOR MACROMOLECULAR TRAFFICKING

# INTRODUCTION

Macromolecular trafficking in a plant is highly regulated by developmental, cellular, organ-specific, or species-specific factors as discussed in previous chapters. Although a large amount of effort has been devoted to the discovery of endogenous factors that facilitate macromolecular trafficking, little is known to date. Biochemical components of PD are largely still unknown. A few candidates have been reported. These include actin (White et al., 1994), myosin (Radford and White, 1998; Blackman and Overall, 1998), centrin (Blackman et al., 1999), calcium-dependent protein kinase (Yahalom et al., 1998) and others with unknown identity (Epel et al., 1996; Blackman et al., 1998) based on immunolabeling studies. A protein kinase from tobacco is speculated to be a component of complex secondary PD (Citovsky et al., 1993). Whether these proteins are true structual or functional components is still an outstanding issue. TMV MP has been shown to interact or colocalize with actin filaments (McLean et al., 1995) and microtubules (Heinlein et al., 1995; McLean et al., 1995). These data suggest the involvement of the cytoskeleton in viral intracellular and intercellular trafficking. However, the conclusive evidence for the necessity of the cytoskeleton in viral movement is not yet available. Besides interacting with the cytoskeleton, TMV MP also interacts with a pectin methylesterase (PME) as shown in yeast two-hybrid analysis, and this interaction may be required for cell-to-cell movement of TMV (Dorokhov et al., 1999; Chen et al., 2000). The tomato spotted wilt tospovirus MP was shown to interact with a DnaJ homologue by yeast two-hybrid (Soellick et al., 2000). One of the DnaJ functions is the regulation of chaperone HSP70. Therefore, involvement of chaperone activity in cell-to-cell trafficking of MPs has been speculated (Kragler et al., 1998; Soellick et al., 2000). Kragler et al. (1998) showed that a putative PD receptor in the cell wall fraction interacted with KN1 and 3a MP. This putative PD receptor itself had an ability to traffic intercellularly

(Kragler et al., 1998). These biochemical and cell biological studies have provided important insights about potential PD components and cellular factors for viral movement. However, conclusive evidence for the role of these components and factors is still lacking.

Genetic and molecular studies of plant structure and function have advanced dramatically. *A. thaliana* is an ideal plant for genetic and molecular studies because of its small size, short generation time, self-fertilizing nature, and production of large number of seeds (Meinke et al., 1998). Genome sequencing of this organism has been completed (Pennisi, 2000; Walbot, 2000; Willmann, 2001). A large collection of genetic markers and physical makers is available for map-based cloning (Meinke et al., 1998).

A few *A. thaliana* mutants or ecotypes in which the movement of viruses is altered or restricted have been identified. The *RTM1* locus is a dominant allele that restricts long-distance movement of tobacco etch virus (TEV; Mahajan et al., 1998). The *RTM1* gene encodes a protein homologous to *Artocarpus integrifolia* lectin (jacalin) (Chisholm et al., 2000). How RTM1 functions to restrict the long distance movement of TEV remains to be determined. Recessive mutation *cum1* affects accumulation of CMV in a local leaf (Yoshii et al., 1998a), and *cum2* affects accumulation of both CMV and turnip crinkle virus (TCV; Yoshii et al., 1998b). Since *cum1* and *cum2* mutations do not affect replication of these viruses in protoplasts, proteins encoded by *CUM1* and *CUM2* genes are presumably involved in cell-to-cell movement of these viruses (Yoshii et al., 1998b). Lartey et al. (1998) isolated the *A. thaliana* mutant *vsm1* (virus systemic movement) in which systemic movement of turnip vein clearing virus and tobamoviruses is blocked while local movement in the inoculated leaf is not. The viral systemic movement within the *vsm1* plants appears to be blocked at the step of entry into the phloem tissue.

Although *A. thaliana* mutants that affect virus movement will help us clarify the mechanisms of virus movement and plant-pathogen interactions, they may not be the best tools to understand PD functions and mechanisms of macromolecular trafficking because viruses usually encode several multifunctional proteins that are involved in movement. Viral movement can be affected at any stage of the viral life cycle, which may complicate studies of the function of a mutated gene. Nevertheless, these studies demonstrate the great potential of genetic approaches to study intercellular transport. Therefore, we decided to establish a genetic system to focus on the investigation of protein trafficking. We have obtained transgenic *A. thaliana* that express 3a MP:GFP fusion protein under the 35S promoter or the CoYMV phloem specific promoter. Investigation of a single viral protein and its interaction with cellular factors will give us more direct and specific ideas about the mechanisms of intercellular protein trafficking.

## MATERIALS AND METHODS

### *Plant material and growth conditions*

*Arabidopsis thaliana* (Columbia and Wassilewskija) were grown at 23°C under 40 W cool white fluorescent light kept on a 16 hour light/8 hour dark cycle. Detailed description for the preparation of hydroponic growth solution can be obtained from the web at http://arabi4.agr.hokudai.ac.jp/arabie/protocols/culture/culture.html The details are reproduced below with the permission of Dr. Satoshi Naito (Hokkaido University, Sapporo, Japan).

To make 10 litres of hydroponic medium for *A. thaliana*:

1) Dissolve 7.08 g of Hydroponic A mixture in about 1 litre of deionized water. (takes a while to dissolve - we use a sonicator to speed up the process)
2) Add the dissolved A mixture to your 10 litre container, fill the container up to about 4 litres with deionized water.

3) Dissolve 4.72 g of Hydroponic B mixture in about 1 litre of deionized water. (we use the same 2L flask and rinse 3 times with deionized water between solutions)
4) Add the dissolved B mixture to your 10 litre container.
5) Add 10ml of 1000X Hydroponic C solution to your 10 litre container.
6) Fill up the solution to 10 litres.
7) Go and water your Arabidopsis plants!

Preparation of stock solutions:

Hydroponic A mixture

Hydroponic A is a mixture of the following:

$NaH_2PO_4$ (anhydrous) ..... 18.1g
$Na_2HPO_4$ (anhydrous) ..... 3.7g
$MgSO_4$ (anhydrous) ..... 18.1g
$KNO_3$ ..... 30.4g
Fe(III).EDTA ..... 0.5g

To make Hydroponic A mixture:
(1) Weigh $NaH_2PO_4$ (anhydrous) and $NaH_2PO_4$ (anhydrous).
(2) Pass through a sieve about 1mm mesh.
(3) Crush clumps that do not pass the sieve with a mortar and pestle.
(4) Repeat steps (2) to (3) until a fine powder is obtained.
(5) Weigh $MgSO_4$ (anhydrous).
(6) Repeat steps (2) to (4) with the $MgSO_4$.
(7) Weigh $KNO_3$.
(8) Repeat steps (2) to (4) with the $KNO_3$.
(9) Transfer the powder mixture to a bottle/container.
(10) Add Fe(III).EDTA and mix well.
(11) Hydroponic mixture A can be stores at room temperature.

Hydroponic B mixture

This is just Calcium Nitrate crystals. Since Calcium Nitrate degrades upon dehydration, anhydrous crystal cannot be obtained. If Calcium Nitrate crystals are mixed with the Hydroponic A mixture, some of the crystals in the mixture will be dissolved into the water of the Calcium Nitrate crystals. Therefore, we add the Calcium Nitrate separately when preparing hydroponic medium

$Ca(NO_3)_2.4H2O$

Hydroponic C mixture

This is a 1000X stock solution of micro-nutrients. Dissolve the following in 1 litre of deionized water.

$MnSO_4.4H_2O$ ..... 2.3g
$H_3BO_3$ ..... 1.85g
$ZnSO_4.7H_2O$ ..... 0.29g
$CuSO_4.5H_2O$ ..... 0.24g
$(NH_4)6Mo_7O_{24}.4H_2O$ ..... 0.03g
$CoCl_2.6H_2O$ ..... 0.03g

***Construction of binary vectors***

Construction of binary vectors containing *35S:3a MP:GFP* or CoYMV:*3a MP:GFP* is described in Chapter III.

***Transformation of A. thaliana by vacuum infiltration***

The procedure was based on the method described by Bechtold et al. (1993) with some modifications. A 3" pot was filled with soil and the surface was covered with a piece of pored (3-5 $mm^2$) mesh cloth. Five to six seeds were planted in each pot. Approximately 3 weeks later, emerging bolts were clipped off to encourage growth of multiple secondary bolts. Infiltration was performed five to 8 days after the clipping when secondary bolts were well formed.

*A. tumefaciens* harboring the desired plasmids was precultured in 2-3 ml of LB medium containing streptomycin (500 $\mu$g/ml) and kanamycin (50 $\mu$g/$\mu$l) and incubated at 29°C with vigorous shaking for 4-5 days prior to infiltration. The entire preculture (2-3 ml) was used to inoculate 400 ml of LB medium containing the antibiotics. The culture was grown at 29°C with vigorous shaking until $OD_{600}$ reached 1.0~1.2. This culture contained enough cells for infiltration of at least six pots.

Cells were harvested by centrifugation at 5,000 rpm for 10 min at room temperature, and resuspended in 600 ml ($OD_{600}$ approximately 0.8) of infiltration medium [0.5 X Murashige & Skoog salts, 1 X Gamborg B5 vitamins, 1% sucrose, 0.044 $\mu$M benzylamino purine (BAP, 10 $\mu$l per liter of a 1 mg/ml stock in DMSO), 0.02% (v/v) SilwetL-77 (Lehle seeds, Cat. #VIS-01)]. The bacterial solution (200-300 ml) was transferred to a 1 L beaker, and plants were inverted to have bolts and rosettes leaves immersed in the solution. The beaker and plants were placed in a bell jar. Vacuum was drawn at 40 mmHg (=-50 kPa) for 5-10 min and then released gradually. The vacuum condition and time could vary depending on vacuum equipment and growth conditions in

each laboratory. Darkened, water-soaked tissue is indicative of over-infiltration and may result in the death of plants. The same bacterial solution was used for three different pots (i.e., three pots/infiltrate). After infiltration, plants were removed from the beaker. Excessive bacterial solution was removed by blotting plants on paper towels, and plants were laid on their side in a plastic container. The plastic container with plants was covered with plastic wrap to keep humidity for one day. The next day, the plastic cover was removed and plants were set upright. Plants were left without watering for 5-7 days until soil became dry. Plants were watered regularly afterwards, and seeds ($T_0$ seeds; naming system is given below) were collected after 3-4 weeks.

***Seeds grown on kanamycin medium for transformant selection and segregation analysis***

All procedures were conducted under sterile conditions in a laminar hood. Seeds were placed in a 2.5 ml glass vial, sterilized in 70% EtOH for 30 s followed with 50% Clorox/0.001%(v/v) Tween 20 for 6 min and then rinsed with sterile distilled water at least 6 times or until the odor of Clorox vanished. For transformant selection, seeds ($T_0$ seeds) were suspended in a sterile 0.1% agarose solution and plated with a glass pipette on the MS agar medium (1X MS salt, 3% sucrose, 1X B-5 vitamins, 0.8% bactoagar, pH 5.8, 100 mg/L kanamycin, 200 mg/L cefotaxime). After plating, the plates were sealed with Parafilm, and placed at 4°C for 3 days (this is not necessary if seeds have been placed at 4°C before plating), then at 23 °C under 40 W cool white fluorescent light kept on a 16 hour light/8 hour dark cycle for approximately 1-2 weeks. The kanamycin resistant phenotype could be seen at the cotyledon stage. Susceptible plants at this stage turned white and died, wheras resistant plants continued to grow. Resistant plants were transferred to soil at 4 to 6-leaves stage. The transplanted plants were covered with plastic bags or Saranwrap for 2 to 3 days to keep moisture. The cover was gradually

removed. Positive transformants showing 3a MP:GFP expression were identified by fluorescence microscopic examination of leaf samples as described in Chapter II. Seeds from such plants were collected.

For segregation analysis, sterile seeds were placed on the sterile filter paper, and then placed on the MS agar medium (cefotaxime is not necessary) using sterile foecepts. Fifty seeds were placed on one plate.

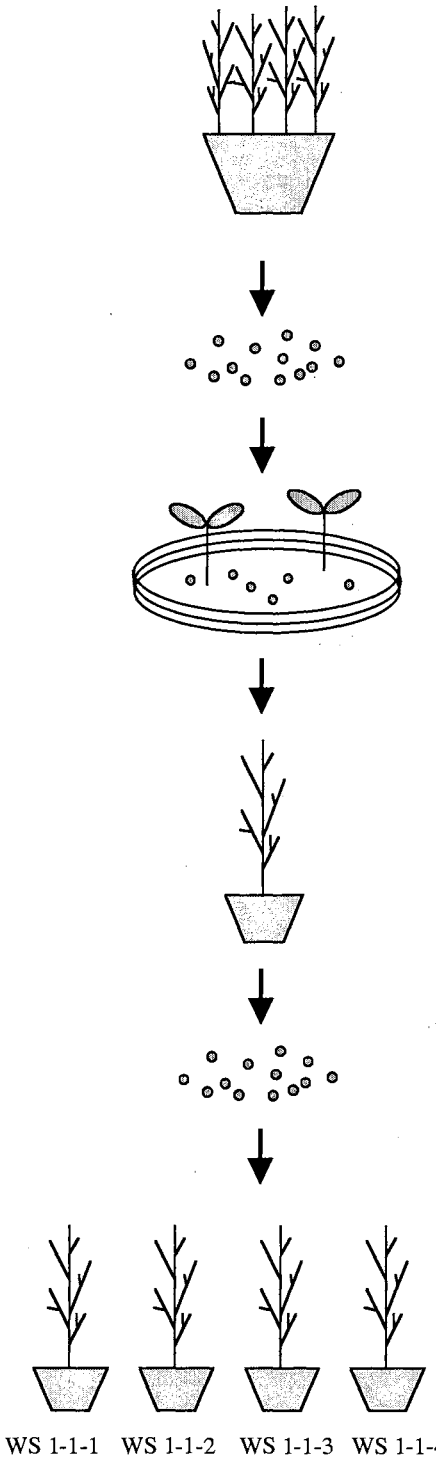
***Naming system for transgenic plants***

For genetic analysis, we utilized the following system to name the transgenic lines and progeny seeds (Fig. 1). The plants infiltrated with *A. tumefaciens* were called $T_0$ plants. The seeds produced by $T_0$ plants were called $T_1$ seeds. Plants germinated from $T_1$ seeds were $T_1$ plants. The seeds produced by $T_1$ plants were $T_2$ seeds. Subsequent generations were named accordingly.

To name an individual plant, the type of transgene was indicated first (e.g., 35S:*3a MP:GFP*, or CoYMV:*3a MP:GFP*), followed by the ecotype (e.g., 35S:*3a MP:GFP,* WS for Wassilewskija), and then by the number of the pot in which the plant was first grown (e.g., 35S:*3a MP:GFP,* WS1 was from pot#1). Identification of the pot number was important because transgenic plants obtained from the same pot might be originated from the same parental plant, and thus might not be independent transgenic lines. When more than one transgenic plants were obtained from the same pot, each plant was named by numbers after a hyphen (e.g., 35S:*3a MP:GFP,* WS1-1).

The progeny plants originated from the same $T_1$ plant ($T_2$ plants) were individually named by numbers (e.g., 35S:*3a MP:GFP,* WS1-1-1). Therefore, $T_2$ plants WS1-1-1 and WS1-1-2 were siblings produced by $T_1$ plant WS1-1.

$T_1$ plants were most probably heterozygous at the transgene loci. Thus, the number of transgenic loci could be determined by the segregation of $T_2$ plants.




*A. tumefaciens* infiltration
**$T_0$ plants**

**$T_1$ seeds**
**WS 1**
Ecotype The number of the pot

Selection on the Km medium
**$T_1$ plants**

Transplantation to soil
**$T_1$ plants** (heterozygous)
**WS 1-1**
Individual $T_1$ plants

**$T_2$ seeds**

| Segregation $Km^r$ (+/ ) : $Km^s$ (-/-) | T-DNA loci |
|---|---|
| 3 : 1 | 1 |
| 15 : 1 | 2 |
| 63 : 1 | 3 |

$T_2$ seeds were placed on Km medium for segregation analysis. $Km^r$ (+/ ) $T_2$ plants were transplanted to soil to collect $T_3$ seeds.

**$T_2$ plants, WS 1-1-1**
Individual $T_2$ plants

**Figure 1.** Naming system for transgenic plants.

For example, if the ratio of transgenic phenotype to nontransgenic phenotype was 3:1, the number of the transgene locus would be one. In this case, one fourth of $T_2$ plants would be homozygous for the transgene locus. Homozygous plants could be identified by the segregation of $T_3$ plants. All $T_3$ plants should show a transgenic phenotype if the parental $T_2$ plant was homozygous, whereas $T_3$ plants would segregate with 3:1 ratio if the parental $T_2$ plant was heterozygous. $T_2$ plants identified as homozygous were labeled +/+ (e.g., 35S:*3a MP:GFP,* WS1-1-1, +/+). The progeny of the homozygous plants were labeled based on their generations (e.g., 35S:*3a MP:GFP,* WS1-1-1, +/+, $T_3$).

***Determination of 3a MP:GFP transgene copy number in a transgenic plant***

Our mutagenesis analysis requires that a transgenic *A. thaliana* plants contains one insert of *3a MP:GFP* gene. To determine the transgene copy number, we used Southern hybridization based on the fact that there is a unique restriction site *Acc*I in the *3a MP* gene sequences. For CoYMV construct, if the transgenic plant contains only one copy of T-DNA, digestion with *Acc*I followed by hybridization with a *3a MP* probe will give one defined band of 1.64 kbp and one band of unknown size (larger than 3.8 kbp; Fig. 2A). If the plant contains more than one copy of T-DNA, it will give an additional band of 4.2 kbp or larger (Fig 2A). For 35S construct, if the plant contains one copy of T-DNA, it will give two bands of unknown size (larger than 1840 bp and 3698 bp respectively; Fig. 2B). If the plant contains more than one copy of T-DNA, it will give an additional band of 5.5 kbp or larger (Fig. 2B).

*Genomic DNA isolation*

Genomic DNA from transgenic *A. thaliana* was extracted with CTAB (Cetyl-trimethyl-ammonium bromide) as described in Murray and Thompson (1980) with some modifications. Plant tissues were ground in liquid nitrogen using a mortar and a pestle.

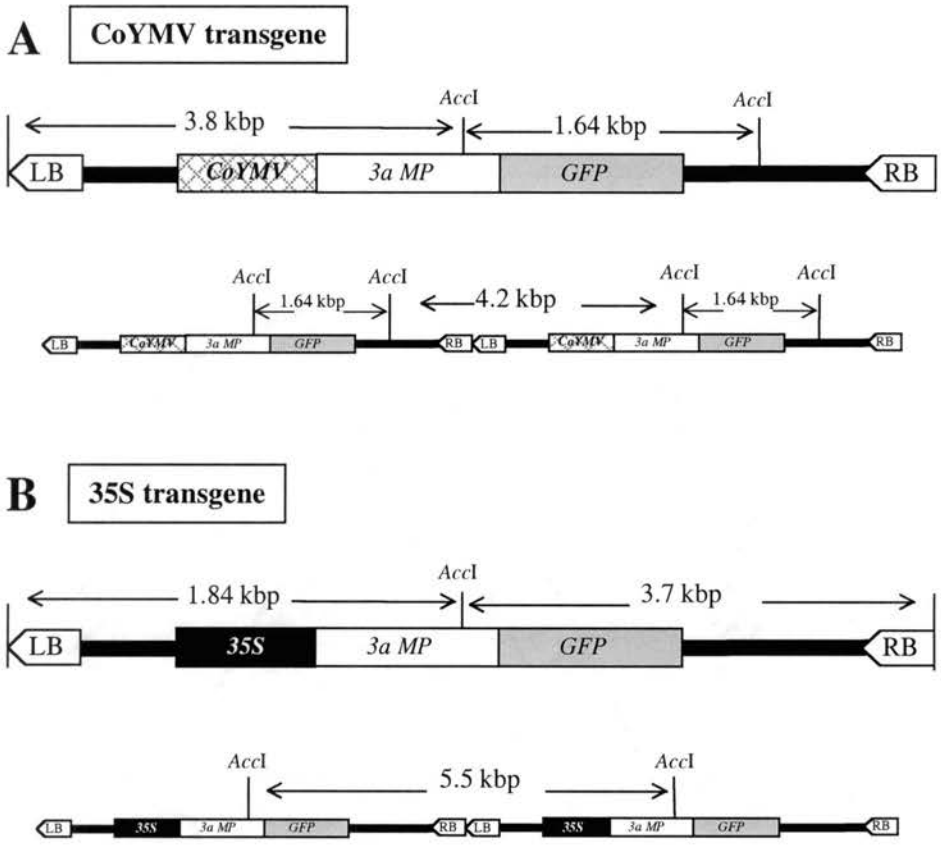



**Figure 2.** Restriction maps of the transgenes.

**(A)** The CoYMV transgene. If the transgenic plant contains only one copy of T-DNA, digestion with *Acc*I followed by hybridization with a *3a MP* probe will give one defined band of 1.64 bp and one band of unknown size (larger than 3.8 kbp). If the plant contains more than one copy of T-DNA, it will give an additional band of 4.2 kbp or larger.

**(B)** The 35S transgene. The transgenic plant contain a single copy of T-DNA will give two bands of unknown size (larger than 1.84 kbp and 3.7 kbp respectively) . If the plant contains more than one copy of T-DNA, it will give an additional band of 5.5 kbp or larger.

LB, left border. RB, right border.

The ground tissues (200-400 mg) were homogenized in an equal volume of fresh 2% CTAB extraction buffer (100 mM Tris-HCl pH 8.0, 2% CTAB, 1.4 M NaCl, and 20 mM EDTA pH 8.0) pre-warmed at 60°C. The homogenate was incubated at 60°C for 30 min. An equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added to the homogenate, and mixed gently by inverting the tube for 5 min. The mixture was centrifuged at 12,000 rpm for 15 min. The upper phase was transferred to a new tube, and chloroform extraction was repeated one more time. The aqueous phase was transferred to a new tube, and an equal volume of 1% CTAB solution (50 mM Tris-HCl pH 8.0, 1% CTAB, 10 mM EDTA pH 8.0) was added to the tube. The mixture was incubated at room temperature for 1 h, and centrifuged at 8,000 rpm for 10 min. The pellet was resuspended in 450 μl of 1 M NaCl (the tube was heated to 56°C to dissolve the pellet if necessary). Absolute ethanol (900 μl) was added to the DNA solution, and incubated at -20°C for at least 20 min. The mixture was centrifuged at 12,000 rpm for 5 min. The pellet was washed twice with 1 ml of 70% EtOH and dissolved in 100-400 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA).

Genomic DNA (10-20 μg) was digested with restriction enzyme *Acc*I. The *Acc*I digested DNA was separated by electrophoresis through a 0.7% agarose gel in TBE buffer. After electrophoresis, the DNA was transferred to a nylon membrane by standard capillary transfer (Maniatis et al, 1983) and immobilized by a UV-crosslinker.

*Probe preparation and hybridization*

The fusion gene *3a MP:GFP* cloned in pRTL2 (Chapter II) was cut out by simultaneous double digestion with *Nco*I and *Bam*HI. The corresponding DNA fragment was isolated from the agarose gel and purified as described in Chapter II. This DNA fragment (1 μg) was randomly labeled with DIG-11-dUTP by using the DIG Highprime DNA kit (Roche, Cat. # 1585 614) following manufacturer's instructions. Hybridization was conducted at

55°C overnight, and signal detection was performed by using DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Cat. # 1175 041) following manufacturer's instructions.

***Mutagenesis***

The procedure was based on the method described by Lightner and Caspar (1998). Methanesulfonic acid ethyl ester (EMS) is a strong carcinogen and volatile, therefore, it must be handled in a functional fumehood with extreme caution.

Absorbent diapers were placed in the hood to contain spills. A magnetic stirring plate and a sealable chemical waste container (larger than 1 gallon) were placed inside the hood. Two-to-three thousands seeds (approximately a volume of 100 $\mu$l in an Eppendorf tube) and 100 ml of $H_2O$ were placed in a 250 ml plastic disposable beaker (a glass beaker was not used because seeds tend to stick to the wall). As many as half of the seeds could be lost during washing steps, thus an excessive amount of seeds should be used for this procedure. A metal paper clip was placed in the beaker for stirring. A piece of Styrofoam (1 cm thick) was placed between the beaker and the stir plate to insulate the beaker. Stirring speed was adjusted as slow as possible because rapid stirring would damage the seeds. EMS (300 $\mu$l) was added to the beaker, using a filter-plugged pipet tip, to give a final EMS concentration of 0.3% (v/v). The beaker was covered with aluminum foil.

After 15 h, the EMS solution was decanted to the waste container. Distilled, deionized water (120 ml) was added to the beaker and stirred for approximately 1 min. Seeds were allowed to settle and the water was decanted into the waste container. The washing procedure was repeated seven more times. Seeds were washed one more time with 120 ml of water and transferred to a 1 L flask. A new metal clip (stir bar) and

500 ml of water were added to the beaker, and seeds were stirred for 30 min. The water was transferred to the waste container, and seeds were resuspended in water for plating.

Seeds were dispersed onto the soil with a disposable plastic pipette. To determine a germination rate and a mutation rate, six hundred seeds were sown in 30 pots (20 seeds/pot). The rest of the seeds were distributed evenly into 30 pots without counting. After germination, the number of seedlings was thinned to 10 for each pot.

All materials used for EMS treatment and the EMS solution (including the washing solution) were treated with 3 M NaOH for decontamination. EMS should be left in the minimum concentration of 1 M NaOH for 30 min before disposal.

***Naming system for EMS mutagenized plants***

EMS-treated and subsequent progeny plants were named using a parallel system as for the transgenic plants. Seeds treated with EMS were called $M_1$ seeds (equivalent to $T_1$ for transgenic plants). Plants grown from $M_1$ seeds were called $M_1$ plants. $M_1$ plants produced $M_2$ seeds. Since $M_2$ seeds population would contain seeds homozygous at the mutagenized loci, $M_2$ plants would be used to screen for mutants defective in 3a MP:GFP intercellular trafficking.

## RESULTS

***Generation of transgenic A. thaliana expressing 3a MP:GFP under the control of the CaMV 35S promoter or the CoYMV phloem-specific promoter***

*A. thaliana* ecotypes, Columbia and Wassilewskija, were used for vacuum infiltration transformation. Approximately 30 plants of each ecotype were infiltrated with *A. tumefaciens* carrying either 35S:*3a MP:GFP* or CoYMV:*3a MP:GFP* constructs. Columbia was more sensitive to *A. tumefaciens* infiltration under our experimental

conditions, and tended to die or set few seeds at later stages of growth. As a result, we obtained only Wassilewskija transgenic plants.

For the CoYMV construct, 5 lines of transgenic plants (WS2-1, 9-1, 9-2, 9-3, 12-1) survived to produce $T_2$ seeds. Segregation analysis of $T_2$ plants by kanamycin resistance showed that lines WS2-1, 9-1, 9-3, and 12-1 contained the transgene at one locus (ratio of +/ :-/- = 84:19, 147:40, 88:22, 55:16 respectively). Subsequent kanamycin segregation analysis of $T_3$ plants led to isolation of homozygous plants WS2-1-3, 2-1-4, 9-3-5, 12-1-1 and 12-1-3. Southern hybridization analysis showed that WS12-1 contained one copy of the transgene. Therefore, homozygous seeds of WS 12-1-1, +/+ or WS12-1-3, +/+ will be used for EMS mutagenesis.

For the 35S construct, 4 lines of transgenic plants (WS1-1, 2-1, 3-1, 4-1) were obtained. For unknown reasons, the growth of 35S-transgenic plants on kanamycin media was arrested at 2-4 rosette leaves stage and plants would die eventually if they were left on Kanamycin media. Therefore, segregation analysis was done by using the GFP fluorescent signal as the transgenic trait. By segregation of fluorescent signal in $T_2$ plants, WS1-1, 3-1, 4-1 were shown to contain the transgene at one locus (ratio of +/ :-/- = 31:11, 23:13, 34:15). Southern hybridization analysis showed the line WS1-1 contained one copy of transgene. Homozygous seeds from this line were obtained (WS1-1-4, +/+), and will be used for EMS mutagenesis.

***Phenotype analysis of transgenic A. thaliana***

The $T_2$ transgenic plants of the line CoMYV:*3a MP:GFP*, WS12-1 were examined under the fluorescence microscope. In these plants, 3a MP:GFP was detected in the phloem, but not in other tissues, as was the case in tobacco (Fig 3A). The fluorescent signal was strong in mature leaves, but weak or none in young leaves. This phenotype was consistent in 100 homozygous $T_3$ plants of WS 12-1-1, +/+ or WS12-1-3, +/+.

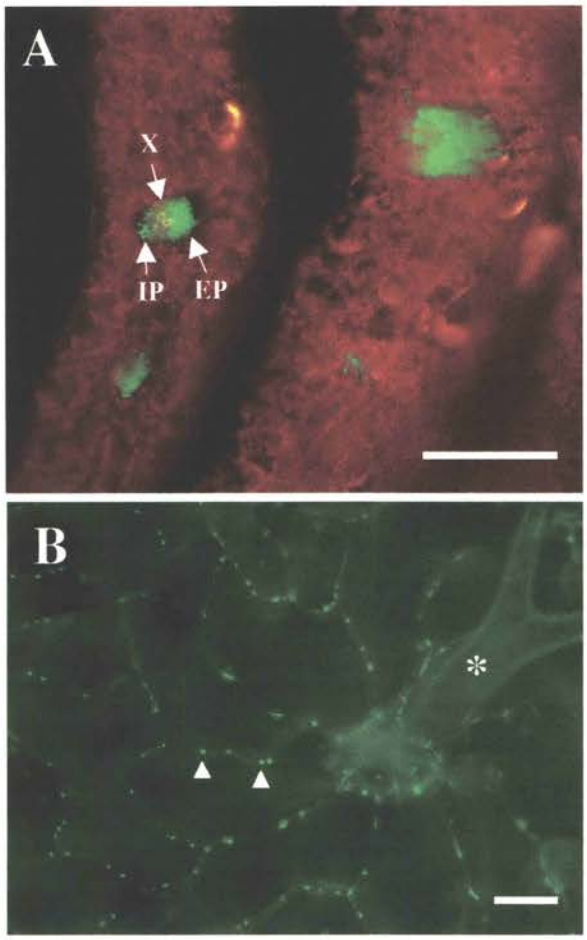



**Figure 3.** 3a MP:GFP expression in the transgenic *A. thaliana* (WS) leaves.

**(A)** Transverse views of leaves expressing the fusion protein under the CoYMV phloem specific promoter. The protein does not traffic into neighboring nonvascular cells. The xylem (X), external phloem (EP) and internal phloem (IP) of a class I vein are indicated. Scale bar = 200 $\mu$m
**(B)** The leaf epidermal cells expressing the fusion protein under the CaMV 35S promoter. The protein is localized to the cell walls (arrowheads). The asterisk denotes a trichome cell. Scale bar = 10 $\mu$m

For the 35S:*3a MP:GFP* transgenic plants, $T_2$ plants of the line WS1-1 were examined. 3a MP:GFP was found in the cell walls as fluorescent dots (Fig 3B). These dots probably represent localization of the fusion protein to PD, as in tobacco. Fluorescent dots were distinct in mature leaves or cotyledons, but not in younger leaves. 3a MP:GFP was also observed as aggregations in the cytoplasm. In general, fluorescent signal was weak in younger leaves and became stronger as leaves matured. This phenotype was consistent in 100 homozygous $T_3$ plants of WS1-1-4, +/+.

***Mutation rate of EMS mutagenesis***

A pilot EMS mutagenesis was conducted for CoYMV-transgenic plants under the condition described in Materials and Methods. Six hundred EMS-treated seeds ($M_1$ seeds) were examined for their germination and mutation rates. Germination rate of $M_1$ seeds was approximately 87% (524/600). Therefore, survival of mutagenized seeds was high. Albino sectors on a plant or abnormal morphologies are indication of successful mutagenesis (Lightner and Caspar, 1998). We found approximately 6.8% of $M_1$ plants to contain albino sectors (41/600), and 7.6% of $M_1$ plants showed abnormal morphologies such as reduced number of leaves, or abnormal shapes (46/600). These results were higher than the expected rate (0.1-1.0%; Lightner and Caspar, 1998).

Because of the extensive amount of work involved, my thesis contribution to this project was to obtain the plant materials ready for mutagenesis experiments. I succeeded in this goal. Extensive phenotypic analysis and mutant screening of the transgenic plants are being carried out by other students and postdocs in Dr. Biao Ding's lab.

# DISCUSSION

Macromolecular trafficking is highly regulated during plant development and at various cellular boundaries. This control must be exerted by cellular factors that act positively or negatively on trafficking. These cellular factors can be present in the cytoplasm or in PD. We have generated transgenic *A. thaliana* expressing 3a MP:GFP under the control of the CaMV 35S promoter and the phloem specific CoYMV promoter, respectively. Screening of mutant plants derived from these transgenic plants is expected to lead to identification of plant factors that are involved in intercellular trafficking of 3a MP:GFP. EMS mutagenesis was chosen as it has a high mutation rate and generally results in various degrees of mutations from null to weak or temperature-sensitive alleles (Haughn and Somerville, 1987; Feldmann et al., 1994; Lightner and Caspar, 1998).

In the 35S:3a MP:GFP-transgenic plants, 3a MP:GFP is localized in the cell walls, presumably to PD. These transgenic plants will be useful in mutant screening to identify the cellular factors that are important for localization of 3a MP:GFP to PD. Absence of green fluorescent dots in the cell walls will provide the first visible mutant phenotype for screening. In the CoYMV:3a MP:GFP-transgenic plants, 3a MP:GFP is confined in the phloem and does not traffic out of the phloem. These transgenic plants will be useful to genetically identify the cellular factors that are important for trafficking between the phloem cells and out through the nonvascular tissue boundary. We will screen for mutants in which 3a MP:GFP traffics out of the phloem.

Detailed analyses are needed to determine if an observed mutant phenotype is due to mutation in the *3a MP* gene, the promoter sequences, the *GFP* gene, or a plant gene. This can be achieved through sequence analysis of the transgene. Although mutations in plant genes are of paramount interest, mutations in the *3a MP* gene can also be interesting in revealing the protein structures important for trafficking.

Besides a forward genetic approach described here, a reverse genetic approach can also be useful to identify cellular factors and their roles in intercellular trafficking. Some of candidates for the reverse genetics include a protein kinase (Citovsky et al., 1993), a DnaJ homologue that interacts with tomato spotted wilt tospovirus MP (Soellick et al., 2000), chaperone (Kragler et al., 1998; Soellick et al., 2000), putative PD receptor (Kragler et al., 1998), actin (White et al., 1994), myosin (Radford and White, 1998; Blackman and Overall, 1998), centrin (Blackman et al., 1999), calcium-dependent protein kinase (Yahalom et al., 1998), and putative PD components with unknown functions (Epel et al., 1996; Blackman et al., 1998) as discussed in the introduction. A collection of *A. thaliana* T-DNA knock-out lines is available at the Biotechnology Center, University of Wisconsin, Madison. It will be revealing to investigate intercellular trafficking or localization of 3a MP:GFP in knock-out mutants of the gene of interest. The *3a MP:GFP* fusion gene can be expressed in those mutants transiently by biolistic bombardment or stably by crossing with the 3a MP:GFP-transgenic plants we generated.

The obtained mutants could also be analyzed for trafficking of macromolecules other than 3a MP:GFP in order to determine if the mutation affects macromolecular trafficking generally, or if it affects 3a MP:GFP trafficking specifically. The effect of a mutant gene on viral systemic movement could also be investigated. Finally, any changes in the morphology, development, or physiology of a mutant plant could be addressed in order to understand the functions of the mutated genes on plant growth and development. Complementation studies with mutants having similar phenotypes for macromolecular trafficking may reveal whether they are different alleles of the same gene, or different genes involved in different steps of a macromolecular trafficking pathway.

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# CHAPTER VI

# RNA TRAFFICKING AND GENE REGULATION IN PLANTS

# INTRODUCTION

RNA trafficking is potentially an important method to regulated plant of gene expression regulation (Jorgensen et al., 1998; Ding et al., 1999; Citovsky and Zambryski, 2000) and regulation of the defense mechanism represented by gene silencing (Matzke and Matzke, 1995; Covey et al., 1997; English et al., 1997; Ratcliff et al., 1997; Voinnet and Baulcombe, 1997; Palauqui et al., 1997; Jorgensen, et al., 1998; Waterhouse et al., 1998; Fagard and Vaucheret, 2000). Intracellular trafficking of RNA, such as nuclear import and export of RNA, has been extensively investigated, and the importance of such trafficking for gene expression has been discussed (Izaurralde and Mattaj, 1992; Zapp, 1992; Wilhelm and Vale, 1993; Izaurralde and Mattaj, 1995; Gerace, 1995; Pennisi, 1998; Nakielny and Dreyfuss, 1999). However, little is known about functions and mechanisms of intercellular and systemic trafficking of RNA.

As discussed in Chapter I, microinjection studies on RCNMV provided the first direct evidence for the existence of cellular machinery for RNA trafficking (Fujiwara et al., 1993). Subsequent studies demonstrated that the intercellular trafficking of viral RNA is a general phenomenon (Noueiry et al., 1994; Ding et al., 1995; Rojas et al., 1997). Some plant endogenous mRNAs also traffic intercellularly or systemically. These include *Knotted1* mRNA from maize (Lucas et al., 1995), *SUT1* mRNA from tobacco, potato, and tomato (Kühn et al., 1997), *CmPP16* mRNA from pumpkin (Xoconostle-Cazares et al., 1999), and *CmNACP* mRNA from pumpkin (Ruiz-Medrano et al., 1999). These findings strongly support the idea that informational molecules such as RNA can traffic via the phloem thoughout the entire plant. However, the consequences of such trafficking, i.e., the function(s) of such trafficking, is not understood.

Viroids provide an excellent tool to study RNA trafficking in a plant at various levels. Viroids are small (246-499 nt), single-stranded and covalently closed circular

RNA molecules that infect plants (Mathews, 1991). An extraordinary feature of viroids is that they are unlikely to encode any proteins. Nevertheless, they are able to replicate autonomously and infect plants systemically. To date, viroids are the only RNA molecules known that are capable of nuclear import and export (Woo et al., 1999), cell-to-cell trafficking (Ding et al., 1997), and systemic trafficking (Palukaitis, 1987; Zhu et al., 2001). Since viroids are unlikely to encode any proteins, all infection steps must be facilitated by direct interactions between the viroid genome and host plant factors. Thus, viroid infection represents a unique system to study plant endogenous mechanisms for RNA trafficking. Furthermore, if RNA trafficking in plants indeed functions to regulate gene expression as proposed (Jorgensen et al., 1998; Ding et al., 1999; Citovsky and Zambryski, 2000), it is possible that viroids take advantage of the plant machinery and invade the plant regulatory systems which eventually results in the abnormal gene expression in plant and disease symptoms. Therefore, studies on plant gene expression patterns during viroid infection may provide insights about RNA trafficking and its relationship to gene regulation in plants.

A number of studies have investigated host responses to viroid infection. Viroid infection causes accumulation of pathogenesis-related proteins (PR proteins; Camacho-Henriquez and Sänger, 1982; Gadea et al., 1996; Rodrigo et al., 1993; Tornero et al., 1997a; Vera et al., 1989). Some PR proteins are hydrolytic enzymes such as chitinase and protease (Vera et al., 1989; García et al., 1990). Viroid infection also increases the expression of an RNA dependent RNA polymerase (RdRp) in tomato (Schiebel et al., 1998). RdRp is required for post-transcriptional gene silencing (PTGS) in *A. thaliana* (Dalmay et al., 2000) and in *Neurosopora* (Cogoni and Macino, 1999). It is possible that the induced RdRp activity in viroid-infected tomato is one of the plant defense responses involving PTGS. Although the above-mentioned studies have been useful to understand defense responses of plants against viroid pathogens, no studies have been undertaken to

analyze comprehensively and systematically the differential gene expression patterns of plants upon viroid infection. Such information would be very helpful to fully understand viroid-plant interactions and possibly also the endogenous systems for RNA trafficking and gene regulations.

To investigate gene expression profiles during viroid infection, we used *Potato spindle tuber viroid* (PSTVd)-infected tomato (*Lycopersicon esculentum* Mill cv."Rutgers") as our experimental system. PSTVd is the best characterized viroid, and tomato is the classic experimental host for PSTVd. We compared tomato gene expression patterns elicited by two strains of PSTVd, a mild and a severe (RG1) strain that cause mild and severe symptoms, respectively (Owens et al., 1996). Sample tissues were collected at different time points post inoculation to obtain gene expression patterns during the infection process. We employed PCR-based representational difference analysis (RDA) to enrich differentially expressed genes (Diatchenko et al., 1996; Akopyants et al., 1998), and macroarray to monitor the expression patterns of thousands of genes simultaneously (Lanfranchi et al., 1996; Bernard et al., 1996; Piétu et al., 1996; Geng et al., 1998; Jordan 1998). By these means, we detected 48 tomato genes whose expression is induced or enhanced by viroid infection, and 8 genes whose expression is decreased or suppressed by viroid infection. The expression patterns of the above genes and their relationship to pathogenicity, differences in genes expression during virus and viroid infections and possible mechanisms of viroid-mediated gene regulation are discussed.

## MATERIALS AND METHODS

### *Plant material and growth conditions*

Tomato (*L. esculentum* var Rutgers) was grown in a growth chamber controlled at a 18 hour light (26°C)/6 hour dark (26°C) cycle. Plants were watered daily with commercial plant nutrients Miracle-Gro for Tomatos as instructed by manufacturer (Scotts Miracle-Gro products Inc.).

### *In vitro transcription of viroid RNA and inoculation of plants*

Plasmids containing viroid cDNA of mild strain (code 13-71) and RG1 strain (code 13-74; kindly provided by Dr. Robert Owens, USDA/ARS Beltsville, Maryland) were linealized by *Hind*III digestion and cleaned by phenol/chloroform extraction. *In vitro* transcription mixture contained 1 $\mu$g of linealized DNA, 10 $\mu$l of 100 mM DTT, 5 $\mu$l of rNTPs (20 mM each; Amersham, Cat. # 27-202-501), 2.5 $\mu$l of RNasin ribonuclease inhibitor (Promega, Cat. # N2111), 3 $\mu$l of T7 polymerase (Promega), 20 $\mu$l of 5X transcription buffer, and DEPC treated $H_2O$ up to 100 $\mu$l. The mixture was assembled at room temperature to prevent precipitation of components. The mixture was incubated at 37°C for 3-4 h. RNA was precipitated by adding 1/10 volume of 10M ammonium acetate ($NH_4Ac$) and 1 volume of isopropanol and incubated at -20 °C for 15 min, and then recovered by centrifugation at 1,400 rpm for 15 min. The pellets were resuspended in DEPC-$H_2O$ and adjusted to a final concentration of 10 ng/$\mu$l. The viroid RNAs could be stored in aliquots at -20°C up to one month without losing infectivity.

Celite powder and 4 $\mu$l of inoculum (10 ng/$\mu$l) were applied to each cotyledon of a 9-10 day-old tomato plant (80 ng/plant). Cotyledons were rubbed very gently 2-3 times with caution not to damage the tissues (damage can be seen as dark regions). DEPC-$H_2O$ was used in mock inoculation. Tobacco mosaic virus (TMV: 1.5 $\mu$g virions/plant) was

used for virus inoculation. After 30 min, celite powder was washed away with a gentle stream of water.

***Plant tissue sampling***

Leaf samples were collected from individual plants at 5 days, 10 days, 17 days, and 25 days post inoculation. Since we did not know which leaves contained viroids, we collected at least one half of a leaf from all branches of a plant except old branches with yellowing leaves. Yellow leaves were avoided as they contain substances which would co-precipitate with RNA during RNA extraction. Leaf samples were wrapped immediately in aluminum foil after the detachment, frozen in liquid nitrogen, and stored at –80°C.

***Dot blot analysis***

Viroid infection was confirmed by dot blot analysis. A piece of leaf tissue (5 $mm^2$) was homogenized in 50 $\mu$l of AMES buffer (0.5 M sodium acetate, 10 mM $MgCl_2$, 20% ethanol, 3% SDS, and 1 M NaCl) in an Eppendorf tube. The homogenate was incubated at room temperature for 10 min. Chloroform:isoamyl alcohol (24:1; 200 $\mu$l) was added to the homogenate, vortexed for 1 min, and then centrifuged at 14,000 rpm for 5 min. The supernatant was transferred to a new Eppendorf tube. The extract (2 $\mu$l) was blotted onto a Hybond $N^+$ nylon membrane (Amersham) and crosslinked using the auto-crosslink setting (120,000 $\mu$J) on the Stratalinker UV crosslinker (Stratagene).

Digoxigenin (DIG) labeled minus-stranded PSTVd probe was made by *in vitro* transcription as described above, except that the *Eco*RI-linearized plasmid pST65-B5 was used as a template and the reaction mixture contained DIG-11-dUTP (Roche, Cat. # 1 573 152) at a final concentration of 0.1 mM.

The blotted nylon membrane was prehybridized in a hybridization solution consisting of 50% deionized formamide, 5% dextran sulfate (mw 500,000), 1% BSA, 150 μg/ml tRNA, 300 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA. Prehybridization was carried out at 65°C for 1 h. The DIG-labeled viroid RNA probe was added to hybridization solution at a final concentration of 400 ng/ml. The hybridization was carried out at 55°C for 12-18 h. The membrane was washed with 2X SSC twice at 55°C for 15 min, and with 0.1% SSC/0.1% SDS at 65°C for 30 min. Hybridization signal was detected using the DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Cat. # 1175 041) following the manufacturer's instructions.

***Total RNA extraction from plants***

Total RNA was extracted from leaves of a pool of 3-5 plants with the same treatment (*i.e.* viroid inoculation, mock inoculation or virus inoculation) in order to minimize the effect of variations among individual plants. Extraction of total RNA was performed by using Trizol (Gibco BRL, Cat. # 15596-018) following the manufacturer's instructions with some modifications. Trizol (1 ml) was placed in a 2 ml tube, and pre-warmed at 55 °C. Plant tissues were ground with a pestle in liquid nitrogen in a mortar. The ground plant tissue of 200-400 mg were added to the 2 ml tube with pre-warmed Trizol, and immediately homogenized by vortexing. The homogenized tissue was incubated for at least 5 min at room temperature, and centrifuged at 1,400 rpm for 10 min at 4 °C. The supernatant was transferred to a new 2 ml tube containing 250 μl of chloroform, mixed vigorously by vortexing for 30 s, and then incubated for 5 min at room temperature. The mixture was centrifuged at the maximal speed for 15 min at 4 °C. The aqueous phase was transferred to a new 2 ml tube containing 600 μl of Trizol, and mixed vigorously by vortexing. Chloroform (200 μl) was added to the tube, further vortexed for 30 s, and then incubated for 5 min at room temperature. The mixture was centrifuged at 1,400 rpm for

15 min at 4 °C. (For the following steps, all glassware were baked at 180°C for at least 4 h, and plasticware and solutions were treated with DEPC at 37°C overnight followed by autoclaving to inactivate RNase.) The aqueous phase from the last Trizol and chloroform treatment was transferred to a new 1.5 ml tube with 250 μl of 0.8 M sodium citrate/1.2 M NaCl, and 250 μl of isopropanol. The mixture was incubated for 10 min at room temperature, and centrifuged at 12,000 g for 10 min at 4°C. The pellet was washed in 1 ml of 75% ethanol, and centrifuged at 7,500 g for 5 min at 4°C. The pellet was dried under a vacuum for 5 min, and resuspended in 50 μl of DEPC-$H_2O$. The solution was incubated for 10 min at 60 °C to dissolve RNA. The RNA solution was split into various aliquots (*e.g.*, 5 μg, 10 μg, 20 μg, 40 μg, 80 μg, 160 μg) and stored at -80°C.

***mRNA isolation and cDNA library subtraction***

mRNA was isolated with the Dynabeads mRNA Purification Kit (Dynal, Cat. # 610.06) following the manufacturer's instructions. The amounts of mRNA isolated from total RNAs were as follows: 0.5-1.0 μg from 70 μg of total RNA of 5 days post inoculation (dpi) plants, 1.2-1.4 μg from 160 μg of total RNA of 10 dpi plants, 1.1-1.9 μg from 160 μg of total RNA of 17 dpi plants, and 1.7-1.9 μg from 200 μg of total RNA of 25 dpi plants.

Subtracted cDNA libraries were constructed by using the PCR-Select cDNA Subtraction Kit (Clontech, Cat. # K1804-1) following the manufacturer's instructions. The method is based on the hybridization kinetics (*i.e.* the fact that abundant DNA species hybridized faster than rare species). Therefore, differentially expressed genes between two biological samples should remain as ssDNA while common DNAs form dsDNA. ssDNA can be selected and enriched by PCR using specific primers.

Briefly, total cDNA was reverse transcribed from mRNA of two different samples. cDNAs containing target sequences (differentially induced or enhanced genes)

were called "test cDNAs" and reference cDNAs were called "driver cDNAs". Both tester and driver cDNAs were digested with *Rsa*I. Therefore the subtracted libraries contained mostly partial cDNAs (100-1000 bp). The *Rsa*I-digested tester cDNAs were divided into two populations, and each population was ligated to a different adapter. Excessive amount of *Rsa*I-digested driver cDNAs without adapters were added to each *Rsa*I-digested tester cDNA populations and allowed to hybridize. The two populations were then mixed together and hybridized further. PCR was performed using tester specific primers to amplify only hybrids of tester-specific genes. Nested primers was used to reduce non-specific PCR products. Forward subtractions (subtraction using cDNAs from viroid-infected plants as tester and mock-inoculated plants as driver) and reverse subtraction (mock-inoculated plants as tester and viroid-infected plants as driver) were performed for all time points (5, 10, 17, and 25 dpi) to detect both induced and suppressed genes during viroid infection. Additional 7 min of 72°C incubation was added at the end of the final PCR cycle to ensure the addition of single 3'-A overhang by *taq* polymerase at each end of the PCR product. Subtracted cDNAs (4 μl of the final PCR products) were shotgun cloned into TOPO TA Cloning vector (Invitrogen, Cat. # K4550-40) that were supplied linearized with single 3'-T overhangs to enable direct ligation of the PCR products. Ligation mixture (2 μl) was used to transform 50 μl of *E. coli* DH5α competent cells as instructed by the vendor (Invitrogen, Cat. # K4550-40). Transformants were selected by ampicillin resistance (100 μg/ml). If the number of colonies obtained was less than 20, the subtracted cDNAs were diluted four times and subjected to shotgun cloning again as described above. Typically, 60-130 colonies were obtained with each transformation experiment.

***Plasmid isolation and macroarray construction***

The work described here was conducted at the Samuel Roberts Noble Foundation, Ardmore, OK. Single *E. coli* colonies harboring the subtracted cDNAs were picked manually and used to inoculat 1.5 ml of LB medium containing ampicillin (100 μg/ml) in 96-well blocks. Bacteria were grown for 20 hours at 37 °C. Ninety-six-well plates of 15% glycerol bacterial stock (100 μl of bacterial culture plus 100 μl of 30% glycerol) were prepared using a BIOMEK 2000 (Beckman) robotic workstation.

The rest of bacterial culture was centrifuged at 2,500 rpm for 10 min at 4 °C to harvest cells. Plasmids were isolated by an alkaline lysis method using a 96-well plate format on the BIOMEK 2000 robotic workstation. Briefly, cells were resuspended in 250 μl of TE containing RNase A (40 μg/ml) and RNase T1 (40 U/ml), and lysed by adding 250 μl of 0.2 N NaOH/1% SDS. The mixture was neutralized by adding 250 μl of 3 M NaOAc (pH 4.8), and incubated at -20°C for at least 8 hours to precipitate proteins and cell debris. The mixture was centrifuged at 3,000 rpm for 45 min at 4 °C, and 400 μl of supernatant was transferred to a new 96-well block. After precipitation by adding 1 ml of 95% ethanol and incubating at -20°C for at least 2 hours, plasmids were collected by centrifugation at 3000 rpm for 30 min at 4 °C and washed twice in 500 μl of 70% ethanol. The pellets were resuspended in 100 μl of ddi$H_2O$ and centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant containing plasmids was transferred to new 96-well U-bottom polypropylene microtiter plates (Corning, Cat. # 3790) with caution not to transfer any debris. Typically, 0.05-0.3 μg/μl of plasmid can be obtained by this method.

Plasmids from 8 microtiter plates were spotted, in duplicate, in a 4 X 4 format on an 8 X 12 cm Hybond $N^+$ nylon filter (Amersham, Cat. # RPN119B). This was accomplished with a 96 pin plate (BIOMEK Part # 609004) on the BIOMEK 2000 robotic workstation (Fig. 1). Filters were submerged in a denaturation solution (1.5 M NaCl, and 0.5 M NaOH) for 2 min, in a neutralization solution (1.5 M NaCl, and 0.5 M

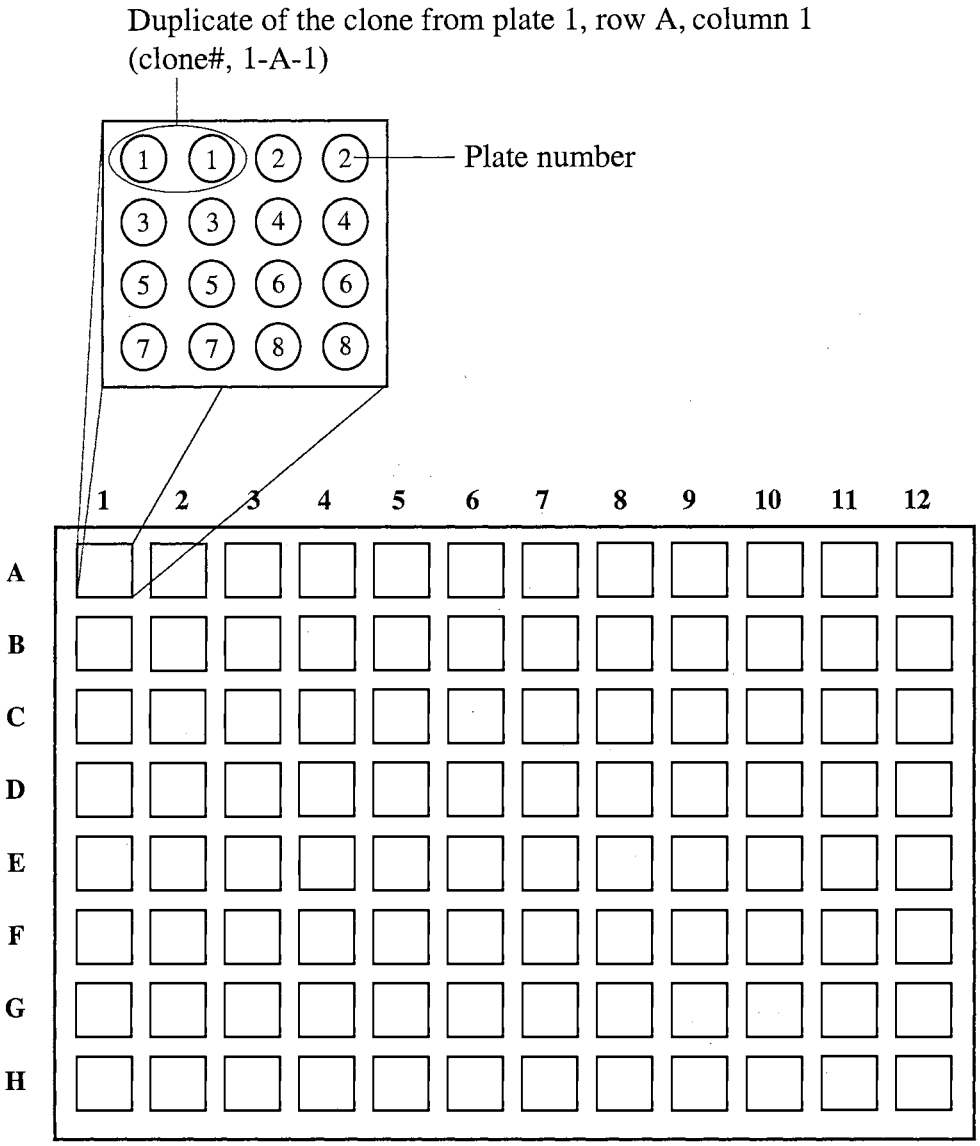


**Figure 1.** Arraying system of clones on a macroarray. Clones from eight 96 well plates were blotted on a macroarray with a 96 pin plate on the BIOMEK robotic station. Each clone was blotted in duplicate.

Tris-HCl, pH 8.0) for 5 min, and rinsed in a 0.2 M Tris-HCl (pH 7.5) and 2X SSC solution for no more than 30 s. The filters were briefly blotted on a piece of Whatman 3MM paper, and crosslinked by 120,000 J UV light (Stratalinker UV crosslinker, Stratagene). The filters were stored at -20°C.

***Preparation and labeling of complex probes***

mRNA from 20 -160 μg of total RNA was isolated by using a Qiagen Oligotex mRNA Kit (Qiagen, Cat. # 70042) essentially following the manufacturer's instructions. The purified mRNAs (0.5-2.0 μg) were concentrated in a Speed Vac centrifuge and adjusted to a volume of 15.6 μl with DEPC-$H_2O$. $Oligo(dT)_{12-18}$ primer (2 μg; GibcoBRL, Cat. # 18418-012) was added to mRNA. The mixture was incubated at 70°C for 10 min, then cooled down on ice to allow annealing. The complex probes were prepared by reverse transcription in the presence of 60 μCi of [α-$^{33}$P] dATP and 60 μCi of [α-$^{33}$P] dCTP. The reverse transcription mixture contained 19.6 μl of mRNA/Oligo(dT) mixture, 9 μl of 5X RT buffer, 0.9 μl of 0.1 M DTT, 0.5 μl of RNasin Ribonuclease Inhibitor (Promega, Cat. # N2111), 1 μl of dNTPs (10 mM dGTP, dTTP, and 1 mM dATP, dCTP), 6 μl of [α-$^{33}$P] dATP (New England Nuclear, 3,000 Ci/nmol) and 6 μl of [α-$^{33}$P] dCTP (New England Nuclear, 3,000 Ci/nmol). The mixture was incubated at 42°C for 2 min, then 2 μl of SuperScriptII (Gibco BRL, Cat. # 18064-014) was added and further incubated at 42°C for 2 h.

The reaction was stopped by adding 1 μl of 0.2 M EDTA (pH 8.0). Unincorporated radioactive nucleotides were removed by passing the reaction mixture through G-50 spin columns (Amersham, Cat. # 27-5335) following the manufacturer's instructions. Typically, 50-80% of radioactive nucleotides were incorporated into the probes giving a total probe activity of 0.4-1.0 X $10^8$ cpm. The probes were stored at -20°C.

***Hybridization conditions***

Hybridization of a complex probe to a macroarray was performed within rotating glass tubes in a hybridization oven (Midwest Scientific). Filters were prehybridized in 7 ml of a hybridization solution (0.5 M $NaHPO_4$, pH 7.2, 1 mM EDTA, 7% SDS) at 55 °C for at least 1 h. Probes (total activity of 1.0-3.0 X $10^7$ cpm) were boiled for 10 min, resuspended in 1 ml of the hybridization solution and then added to the filters (already in 7 ml of hybridization solution) with caution to avoid direct contact between the probes and filters. Hybridization was carried out at 55°C for 18-24 h. Filters were washed for 15 min in 1.5X SSPE/0.2% SDS, 15 min in 0.5X SSPE/0.2% SDS, and 15 min in 0.2X SSPE/0.1% SDS. All washes were carried out at 65°C. After washing, dried filters usually had an activity of 4-8 kcpm as measured by a Geiger counter. The filters were exposed to phosphor screens (Molecular Dynamics, Sunnyvale, CA) for 12-72 h.

***Imaging and analysis of hybridization signals***

The phosphor screens were scanned with the phosphoimager system Storm 820 (Molecular Dynamics). The scanned images were processed or edited with the Image Quant 5.1 or the IQ tools 2.1 softwares (Molecular Dynamics). The Array Vision 5.1 software (IMAGING Research Inc.) was used for quantification of signal intensities. This software performs dot identification, dot quantification, and local background signal subtraction.

Normalization of signal intensities was conducted based on the log space linear regression normalization method obtained from website 'http://afgc.stanford.edu/~finkel/talk.htm'. The Excel file template for calculation was kindly provided by Dr. Sakae Agarie at University of Nevada. Clones that showed more than two-fold reproducible (for duplicates within a macroarray, and between macroarrays

after independent hybridization) enhanced and decreased expressions as compared to the control were chosen for sequencing. Dots with weak hybridization signals that were less than the median of signal intensities of all of the dots on the entire membrane were removed from analysis because the expression ratio of such weak signals might not be reliable. Because of the stringent criteria used for selection of clones, some differentially expressed gene might have been eliminated if they were rare transcripts, or if the amount of DNA spotted on the membrane was low. Among 100 clones sequenced, 4 of them were revealed to be chimeric (more than one gene ligated and inserted into the vector) that required further subcloning to identify the differentially expressed genes. DNA sequence data were subjected to BLAST database search to identify the genes or assign putative functions. DNA sequences will be submitted to Genbank.

## RESULTS

### *Symptoms caused by viroid infection*

Symptoms of PSTVd infected tomato are leaf curling and growth stunt. We did not observe apparent necrotic regions on the infected leaves. Severe strain (RG1)-infected plants started showing symptoms at 13 dpi, while mild strain started showing symptoms at 17 dpi. RG1 infection resulted in much more severe symptoms than mild strain infection at 22 dpi (Fig 2).

We also inoculated tomato plants with TMV in order to compare gene expression patterns between viroid infection and virus infection. TMV-infected plants started showing symptoms as early as 3-5 dpi. Symptoms caused by TMV were mosaic patterns on the leaves and leaf curling. However, the infected plants started recovering from the disease symptoms around 15-17 dpi, and eventually became symptomless. This

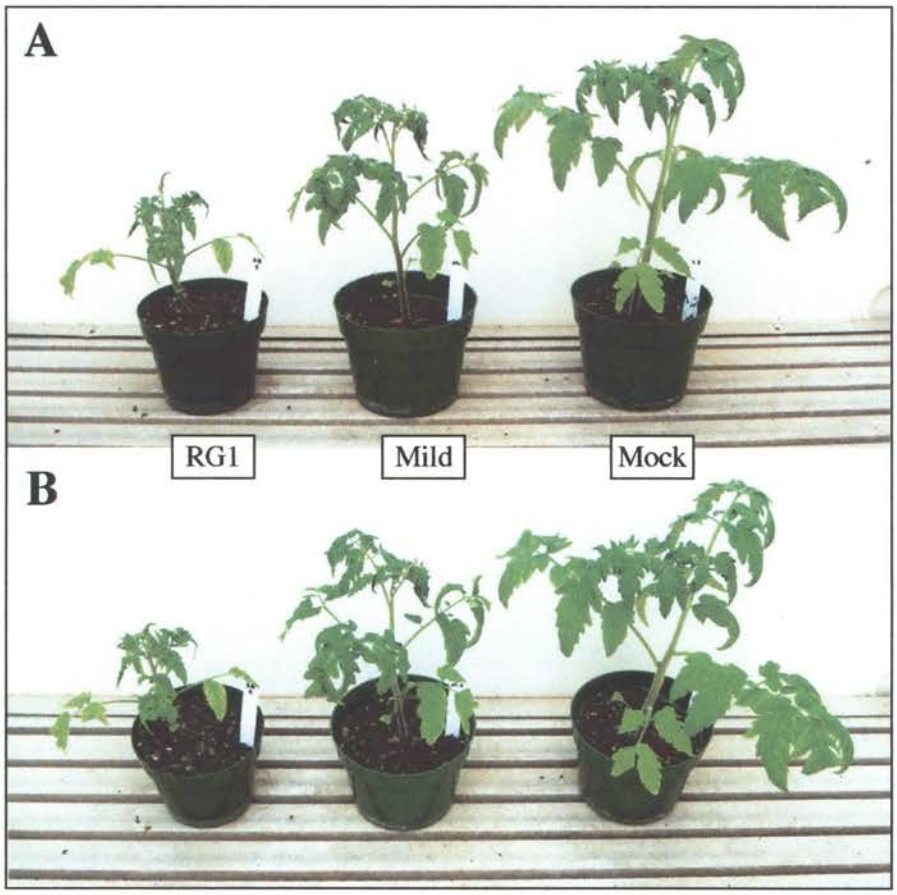


**Figure 2.** Symptoms of PSTVd infection on *L. esculentum* L. cv Rutgers 22 days after inoculation.

**(A)** Ten day-old cotyledons were inoculated with 40 ng of *in vitro* transcribed PSTVd RNA. Left: RG1-infected plant. Middle: mild strain-infected plant. Right: mock-inoculated plant.
**(B)** The same plants in **(A)** viewed from a different angle.

recovering phenotype may be due to gene silencing against TMV as proposed by Covey et al. (1997) and Ratcliff et al. (1997).

***Differentially expressed genes during viroid infection***

The induced genes by PSTVd infection at 25 dpi were summarized in Table 1. A number of genes previously known to be induced by pathogens or stress showed enhanced expression in RG1-infected plants (Fig 3). Mild strain infection also enhanced expression of some of the genes, albeit to a lesser extent. The genes that showed enhanced expression by both RG1 and mild strain infections include those encoding catalase 1, chitinase, cyclophilin, elongation factor, heat shock proteins, a protein homologous to human P23 tumor protein and PR-1b. Induction of *PR-1b* gene by viroid infection was reported previously (Tornero et al., 1994), and the expression level was especially strong in our experiment; ~65 fold by RG1 and ~12 fold by mild strain compared to control. Chlorophyll a-b binding protein genes (*Cab1-3, 9, 10b*), and PSI subunit psaL gene were also induced by both RG1 and mild strain infection.

Additionally, RG1 induced many known genes which were reported from other systems: 1) genes induced by citrus exocortis viroid (CEV), such as *CEV*-1 and *P69e* encoding subtilisin-like protein (Tornero et al., 1997b; Jordá et al., 2000), 2) genes encoding stress/defense-related proteins such as PR16, pre-pro-cystein proteinase, and wound-induced proteins, 3) genes for cell wall-related proteins such as β-glucanase, glycine-rich protein and homologue of cell wall protein from tobacco, 4) genes for proteins that are involved in protein synthesis and turnover such as ribosomal proteins, ubiquitins, ubiquitin extension proteins, and 5) gene encoding other proteins such as ADP/ATP translocator, calreticulin, clock protein, histone, putative endonuclease, small GTP binding protein, and sucrose transporter (SUT1). We also isolated 6 genes of unknown functions and 3 genes with no match in the database from the RG1-infected

**Table 1.** Expression profile of genes induced by PSTVd infection at 25 days post inoculation

| Clone No. (Plate No.-Row-Column) | BLAST hit | Encoded Protein | Fold-Induction[a] | | |
|---|---|---|---|---|---|
| | | | RG1 | Mild | TMV |
| 1-F-6, 8-E-7 | U89839.1 | ADP/ATP translocator | 3.1 | 1.8 | 1.1 |
| 4-G-11 | AB015472.1 | Arabidopsis unkown function | 2.4 | 1.6 | 0.9 |
| 5-B-7 | AC007399.1 | Arabidopsis unkown function | 2.3 | 1.7 | 1.4 |
| 6-A-1 | AL391151.1 | Arabidopsis unkown function | 3.0 | 1.3 | 1.2 |
| 6-G-1 | AC007071.6 | Arabidopsis unkown function | 2.8 | 1.8 | 1.7 |
| 6-G-8 | AC016662.7 | Arabidopsis unkown function | 2.5 | 1.4 | 1.2 |
| 8-G-9 | AC015450.5 | Arabidopsis unkown function | 3.2 | 0.9 | 1.0 |
| 6-H-4, 6-F-11 | X74906.1 | β-glucanase | 5.6 | 1.4 | 1.1 |
| 5-H-12, 11-F-3, | U21112.1 | Cab (Lhcb1-3) | 7.3 | 13.1 | 2.2 |
| 9-A-6 | M32606.1 | Cab 10b | 2.5 | 2.8 | 1.3 |
| 10-C-1 | X61287.1 | Cab 9 | 3.0 | 2.5 | 0.9 |
| 1-D-6 | Z71395.1 | Calreticulin | 2.4 | 1.5 | 1.1 |
| 1-H-12, 8-D-5, 8-H-8,6-H-3 | M93719.1 | Catalase1 | 9.9 | 2.0 | 0.9 |
| 1-H-3 | Y19032.1 | Cell wall protein | 2.8 | 1.1 | 0.9 |
| 6-H-10 | X71593.1 | CEV induced protein (CEV-1) | 5.0 | 1.7 | 0.9 |
| 7-B-6 | Z15141.1 | Chitinase | 4.2 | 1.6 | 1.0 |
| 4-A-7 | U02608.1 | Chitinase B4 | 4.1 | 3.0 | 1.3 |
| 1-B-6 | AF043247 | ClassI chitinase | 7.6 | 3.3 | 1.0 |
| 2-A-9 | UMMS | Clock protein | 2.5 | 1.8 | 1.0 |
| 3-D-5 | M83237.1 | Cloning vector | 2.5 | - | 2.3 |
| 5-E-1, 9-B-1 | M55019.1 | CyP cyclophilin | 3.1 | 2.0 | 0.5 |
| 6-F-2 | X55689.1 | Glycine rich protein | 2.7 | 1.2 | 1.0 |
| 7-H-3 | M96549.1 | Heat shock cognate protein | 8.8 | 3.7 | 1.3 |
| 6-G-11 | X54030.1 | Heat shock protein-2 | 5.2 | 2.6 | 0.9 |
| 3-F-1 | X83422.1 | Histone | 2.4 | 1.2 | 1.5 |

| Clone No. (Plate No.-Row-Column) | BLAST hit | Encoded Protein | Fold-Induction[a] | | |
|---|---|---|---|---|---|
| | | | RG1 | Mild | TMV |
| 1-F-7, 3-F-12, 7-D-10 | Z37160.1 | Homolog to human P23 tumor protein | 4.9 | 2.1 | 1.1 |
| 2-G-8 | Y18312.1 | Intrinsic protein, PR16 ? | 2.3 | 1.7 | - |
| 1-E-4, 4-B-1, 5-G-7, 9-G-7, 10-A-3 | X14449.1 | LeEF-1α | 5.1 | 3.7 | 1.2 |
| 6-C-11 | X80386.1 | Mitochondrial 34kDa | 1.5 | 2.4 | - |
| 3-A-7 | | No hit | 2.7 | 1.9 | 1.1 |
| 6-E-6 | | No hit | 2.3 | 1.5 | 1.2 |
| 7-A-7 | | No hit | 2.2 | 0.9 | 1.6 |
| 2-F-6 | X64344.1 | Polyubiquitin | 3.4 | 1.5 | 1.0 |
| 4-A-1, 7-A-9, 7-B-3, 6-H-11, 6-G-9, 6-G-5 | Y08804.1 | PR-1b | 64.6 | 11.9 | - |
| 1-D-10, 2-C-11, 5-D-2 | Z14028.1 | Pre-pro-cystein proteinase | 2.6 | 1.4 | 0.7 |
| 10-D-7 | D83007.1 | PS I subunit psaL | 4.1 | 5.9 | 0.7 |
| 4-D-3 | AJ293762.1 | Putative endonuclease | 4.5 | 1.6 | 0.7 |
| 1-H-2 | AF026079.1 | Ribosomal protein | 2.3 | 1.5 | 1.2 |
| 6-B-1 | AJ278460.1 | Ribosomal protein | 2.0 | 1.5 | 2.0 |
| 6-D-12 | X64562.1 | Ribosomal protein L2 | 2.7 | 0.7 | 0.5 |
| 7-C-5 | Z23161.1 | Ribosomal protein S15 | 2.7 | 1.0 | 1.1 |
| 8-E-11 | AF210431.1 | Small GTP binding protein | 2.0 | 1.4 | 0.8 |
| 4-C-4 | Y10149.1 | Subtilisin-like protein, p69e | 2.6 | 1.4 | 0.9 |
| 8-A-11 | X82275.1 | Sucrose transporter 1(SUT1) | 2.0 | 1.0 | 1.0 |
| 6-D-1 | X58253.1 | Ubiquitin (ubi3) | 2.2 | 1.1 | 1.1 |
| 8-C-7 | Y10024.1 | Ubiquitin extension protein | 3.1 | 1.8 | 1.3 |
| 3-C-2, 6-G-2 | U89764.1 | Wound-induced protein | 3.7 | 1.5 | 1.4 |

[a] Induction folds were caluculated by the log regression method (see metarials and methods). The average of 4 data points of each gene are presented. Induction folds between 2.0 and 4.9 are indicated as red characters, and greater than 5.0 are highlited in pink. The accession numbers of the registered genes that share the highest homology with identified clones and their putative functions are presented.


**Figure 3.** Macroarrays (768 clones from subtracted cDNA library) hybridized with a complex probe prepared from mRNA of indicated plant samples.

Some of the differentially expressed genes are indicated with circles. RG1-induced genes: PR1b ⬭, catalase ⬭, elongation factor-1α ⬭. RG1-suppressed genes: *Cab5* ⬭, hypothetical protein ⬭, NADH protochlorophyllide ⬭, Pectinmethylesterase ⬭.

sample. These RG1-induced genes were marginally or not induced by the mild strain. On the other hand, two genes, the gene encoding the mithochondrial 34kDa and an unknown gene from *Medicago sativa* (Genbank accession number: gi|3885510|AF084200.1) were induced only by the mild strain. The unknown gene from *Medicago sativa* was special not only because it was specifically induced by the mild strain, but also because it was induced by 6.16 fold at 5 dpi when most of genes were not significantly induced or suppressed in PSTVd-infected plants.

We did not detect as many suppressed genes as induced genes (Table 2). Interestingly, *Cab 4* was suppressed by both RG1 and the mild strain and *Cab 5* was suppressed only by RG1. Pectin methylesterase-like gene was found to be suppressed by both strains. This protein has been shown to interact with TMV MP, and the interaction may be important for intercellular movement of TMV (Dorokhov et al., 1999; Chen et al., 2000).

***Differential gene expressions in viroid- and virus-infected tomato***

Some stress/defense related genes induced by viroid infection such as catalase, cyclophilin, and PR-1b genes were also induced by TMV infection (Table 3). The maximal induction of those genes by TMV was at 5 dpi, the earliest time point in our experiment, whereas induction of those genes by the viroid started at 17 dpi and continued to increase until 25 dpi, the latest time point in our experiment. Genes encoding elongation factor and heat shock proteins were also induced by TMV, with the maximal induction at 10 dpi (Table 3). The genes that were induced by TMV, but not by viroid include those encoding Cab 3a (at 5 dpi), plastidic aldolase, plastidic cystein synthase, Rubisco activase, threonin deaminase, and wound-induced protein inhibitor (at 10 dpi; data not shown). TMV suppressed genes for Rubisco subunit rbc3A (at 5 dpi) and wound-induced proteinase inhibitor (at 25 dpi; data not shown).

**Table 2.** Expression profile of genes suppressed by PSTVd infection at 25 days post inoculation

| Clone No. (Plate No.-Row-Column) | BLAST hit | Encoded Protein | Fold-suppression[a] | | |
|---|---|---|---|---|---|
| | | | RG1 | Mild | TMV |
| 8-E-2, 9-A-9, 10-H-8, 11-F-1 | M17558.1 | Cab-4 | **2.9** | **2.3** | 0.6 |
| 6-C-4, 8-D-11, 2-F-4, 11-H-3 | M17559.1 | Cab-5 | **2.0** | 1.3 | 0.9 |
| 9-H-8 | X67843.1 | Cathepsin D inhibitor | **3.0** | **3.8** | **9.8** |
| 1-B-1 | M94135.1 | Chloroplast carbonic anhydrase | **2.0** | 1.2 | 1.6 |
| 7-E-2 | AJ279852.1 | Hypothetical protein | 1.7 | **2.4** | **2.8** |
| 10-H-3 | X59282.1 | Metallocarboxypeptidase | **2.5** | **3.4** | **7.3** |
| 7-D-4 | AF243523.1 | NADH protochlorophyllide 3 | **2.3** | **2.3** | 1.2 |
| 7-H-12, 8-A-10, 4-H-11, 12-G-1 | S66607.1 | Pectin methylesterase-like sequence | **3.9** | **3.1** | 1.4 |

[a] Suppression folds were caluculated by the log regression method (see metarials and methods). The average of 4 data points of each gene are presented. Suppression folds greater than 2.0 are indicated in blue characters. The accession numbers of the registered genes that share the highest homology with identified clones and their putative functions are presented.

**Table 3.** Expression patterns of the genes induced by RG1 and TMV during time course

| | RG1 | | | | TMV | | | |
|---|---|---|---|---|---|---|---|---|
| Gene | 5 dpi | 10 dpi | 17 dpi | 25 dpi | 5 dpi | 10 dpi | 17 dpi | 25 dpi |
| PR-1b | 0.9 [a] | 0.7 | **15.7** | **64.6** | **4.6** | 0.5 | 1.6 | 1.0 |
| Catalase | 1.1 | 1.1 | **2.3** | **9.9** | **2.0** | 1.4 | 1.2 | 0.9 |
| Cyclophilin | 1.3 | 0.8 | 1.3 | **3.1** | **4.8** | 1.1 | 1.3 | 0.5 |
| Elongation factor | 0.9 | 0.9 | 1.4 | **5.1** | 1.2 | **3.2** | 1.3 | 0.8 |
| Heat shock protein | 1.1 | 1.1 | 1.7 | **8.8** | 1.1 | **2.3** | 1.0 | 1.3 |

[a] Induction folds were calculated by the log regression method (see materials and methods). The average of 4 data points of each gene are presented. Fold-inductions greater than 2.0 are indicated as red characters.

## DISCUSSION

We have demonstrated that two strains of PSTVd could elicit differential gene expression in infected tomato plants, using RDA to enrich the differentially expressed genes, and macroarray to monitor the expression patterns. The presence in our collection of some defense related genes and genes that were previously reported to be induced by viroid infection indicates the general validity of our approach. However, we did not detect some of the genes with our method that were shown to be induced by viroid, such as genes encoding RdRp (Schiebel et al., 1998) and a 55 kDa protein kinase viroid (PKV; Hammond and Zhao, 2000). Such genes could have escaped our detection due to the criteria (see materials and methods) we used to select genes for sequencing. It should be noted that the relative expression levels obtained by macroarray analysis may not be accurate because of the limited sensitivity of this technique and variation between each hybridization. When accurate expression levels are required for specific purposes, northern hybridization should be the preferred method. Nevertheless, macroarray provides high throughput analysis of a large number of genes.

The induced expression by both the mild strain and RG1 of catalase 1, chitinase, cyclophilin, elongation factor, heat shock protein, and PR-1b genes seem to correlate with the appearance of disease symptoms. They were also induced by TMV infection (except for chitinase), but at a much earlier stage (5-10 dpi) when TMV-infected plants showed symptoms. Therefore, expression levels of some of genes individually or in combinations may be partially responsible for the development of disease symptoms. They are general defense response genes as they are induced by both a viroid and a virus. Interestingly, expression of some members of *Cab* genes (*Cab1-3, 3a, 9, 10b*) are induced, whereas expression of the other members of *Cab* genes (*Cab 4* and *5*) are

suppressed by PSTVd infection. These results suggest potential differences in the function and differential regulation of different members in a gene family. Induction of genes such as those encoding elongation factor, ribosomal proteins, ubiquitins, ubiquitin extension proteins and heat shock proteins by PSTVd suggest the active synthesis and degradation of proteins during viroid infection. Similar phenomenon was also observed during viral or fungal infection (Aranda et al., 1999; McCafferty and Talbot, 1998). In the case of viral infection, induced expression of these host genes are speculated to be involved in viral protein synthesis. On the other hand, viroids do not encode proteins. Therefore, induced expression of these genes by PSTVd must be involved in rapid turnover of plant endogenous proteins. The functional significance of such protein turnover in host response and symptom development remains to be understood.

We isolated 6 genes of unknown function and 3 novel genes induced by RG1 and 2 genes of unknown function induced by the mild strain. Elucidating the functions of these genes during normal plant growth and development and in viroid infection could be of great interest. Potential approaches to study the function of these genes include reverse genetics or over-expression of these genes.

The genes that are strongly induced (such as PR-1b or catalase genes) or suppressed by PSTVd have potential applications. For example, the promoter of the gene can be fused to a reporter gene such as *GFP*. A transgenic plant expressing such a fusion gene can be useful in many ways. First, the expression of the reporter gene can be used to monitor the progression of viroid infection. It can be particularly useful when infectivity of many viroid mutants is to be tested. Second, spatial and temporal analyses of reporter gene expression and viroid localization can reveal whether reporter gene expression requires the direct presence of the viroid in a cell or whether it is triggered by a systemic signal generated by localized viroid replication. Third, the expression patterns of the reporter gene can be used to track either viroid movement (if the presence of viroid is

necessary for induction) or the movement of systemic signals. Beyond understanding viroid infection, such studies can be important to understand the regulation of phloem transport and movement of signaling molecules during plant growth and development.

The mechanism(s) of differential expression of host genes during viroid infection is not understood. Many stress-related genes are probably induced during a general defense and/or stress response. On the other hand, some genes may be induced or suppressed by the viroid, but not by the virus, or only by a specific strain of the viroid. Our data suggest that a viroid can alter the expression patterns of some plant genes by specific interactions. How a viroid modifies host gene expression remains an open question. Possibilities include 1) silencing of host gene expression as postulated by Papaefthimiou et al. (2001), 2) interference with mRNA splicing (Diener, 1981; Dickson, 1981), 3) interference with RNA export out of the nucleus, and 4) direct activation of gene expression. Possibilities 2) and 3) may be remote because only a selective group of genes are affected. Direct silencing or activation of genes is possible because these could depend on sequence homology interactions.

In conclusion, we have demonstrated that viroid infection can alter host gene expression patterns. This is significant because viroids do not encode any protein. Viroids must utilize host endogenous systems for their trafficking, and probably alter host gene expression patterns by interacting with host endogenous factors and regulatory systems. Therefore, further investigation on genes whose expression is specifically altered during viroid infection will be important to understand the mechanisms of their regulation. This may lead to the discovery of RNA-based gene regulation systems in addition to the well understood protein transcription factor-based gene regulation.

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# CHAPTER VII

# SUMMARY AND FUTURE PROSPECTS

Plasmodesmata (PD) establish a cytoplasmic network of cell-to-cell communication that is essential for plant biology. My Ph. D. dissertation project was devoted to investigating the regulation of macromolecular trafficking, especially protein trafficking through PD. We have developed a new set of research tools to address this regulation. These include biolistic bombardment, GFP fusion, tissue specific transgenic expression, and transgenic *A. thaliana* for genetic analysis.

With biolistic bombardment and other methods including molecular biology and cell biology, we showed that macromolecular trafficking fell into two categories: specific and nonspecific trafficking. Specific trafficking requires interaction between a trafficking molecule and cellular factors, whereas nonspecific trafficking occurs by passive diffusion. Both specific and nonspecific trafficking are regulated developmentally and cellularly. Specific trafficking was investigated with 3a MP:GFP fusion protein. We have shown that development of complex secondary PD in tobacco leaf epidermis is directly related to sink-to-source transition. 3a MP:GFP is specifically targeted to complex secondary PD, and this is correlated directly with its intercellular trafficking. Our finding provided the first evidence showing different functions of primary and complex secondary PD in protein trafficking. In addition, we showed that phloem-derived 3a MP:GFP cannot traffic across the interface between the phloem and bundle sheath. Important future task will be to test whether bundle-sheath- or mesophyll-produced 3a MP:GFP can traffic into the phloem. Furthermore, experiments are needed to elucidate the molecular mechanisms that regulate protein trafficking. For this purpose, it is important to identify the specific motifs (sequences or structures) in a trafficking molecule that are required for specific trafficking, and the cellular factors that interact with such motifs to facilitate trafficking.

We investigated nonspecific trafficking with GFP in several plant species and organs. In contrast to specific trafficking of 3a MP:GFP, intercellular trafficking of GFP

occurs in tobacco sink leaves, but not in source leaves. This observation is consistent with reports from other research groups (Imlau et al., 1999; Oparka et al., 1999). It is possible that primary PD allow nonspecific trafficking by diffusion, while branched PD become more selective and restrictive. On the other hand, GFP traffics in *A. thaliana* leaf and stem epidermis and in stems or hypocotyls of cucumber regardless of developmental stages. Furthermore, GFP can traffic from epidermis to cortex in cucumber hypocotyls, but not from epidermis to mesophyll in cucumber cotyledons. Taken together, our results demonstrate that PD trafficking is dynamic and is regulated by multiple mechanisms involving types of tissues, organs, species, developmental stages, physiological and environmental conditions. Arising questions are whether there are plant endogenous macromolecules that traffic nonspecifically. And if so, what are the functions of those molecules and how is nonspecific trafficking regulated in addition to specific trafficking during plant growth and development?

Most needed is probably genetics in this field because little is known about cellular factors involved in intercellular trafficking. Although genetic analysis is time consuming, it is perhaps the most productive approach to identify cellular factors in all aspects: PD components, cytosolic factors, developmental factors, and cell- or organ-specific factors. We have developed transgenic *A. thaliana* plants expressing 3a MP:GFP in specific tissues. These plants are expected to play a significant role in genetic studies of the above factors.

There is ample evidence for RNA trafficking in plants. However, the biological functions of RNA trafficking are poorly understood. It is speculated that RNA may function directly or indirectly to regulate gene expression at various levels (Jorgensen et al., 1998; Wassenegger and Pelissier, 1998; Ding et al., 1999; Wassenegger, 2000; Matzke et al., 2000). In our study, we used PSTVd as a biological probe and investigated the gene expression patterns during viroid infection. We have obtained several genes that

are specifically regulated by the viroid. Mechanisms of such regulation remain to be elucidated. If the mechanisms of RNA trafficking or RNA-mediated gene regulation are revealed, it would be possible to block RNA trafficking or RNA-mediated gene regulation to understand their biological significance.

During my Ph. D. program and throughout this dissertation work, I have focused on PD-mediated intercellular trafficking and its importance in cell-to-cell communication. PD-mediated cell-to-cell communication is a unique feature of plants and probably one of the fundamental differences between plants and animals. However, it should not be forgotten that cell wall-mediated cell-to-cell communication (e.g. receptor mediated signal transduction) also plays important roles in plants as in animals (Clark et al., 1997; Hake and Char, 1997). Moreover, intercellular protein trafficking has been reported in the animal (culture cells; Elliot and O'Hare, 1997; Phelan et al., 1998), and gene silencing triggered by dsRNA also happens in animals (Fire et al., 1998; Fire, 1999; Cogoni and Macino, 1999; Bass, 2000; Hammond, 2000). Therefore, plants and animals are different in many ways, but they also share common features. I would be gratified if my research in the past and in the future could contribute not only to plant biology, but also to other disciplines of biology as a whole.

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# APPENDIX

**Table 1.** The list of primers used in this study

| Primer name | DNA sequence |
|---|---|
| *Nco*I-3a | GGCATGCCATGG$_{NcoI}$CTTTCCAAGGTA$_{3a}$ |
| 5'GFP-3'3a | CCCTTGCTCA$_{GFP}$CCATAAGACCGTTAACCAC$_{3a}$ |
| 5'3a-3'GFP | TGGTTAACGGTCTTATGG$_{3a}$TGAGCAAGGGCG$_{GFP}$ |
| GFP-*Bam*HI | GCGCGGATCC$_{BamHI}$TTACTTGTACAGCTCG$_{GFP}$ |
| *Nco*I-GFP | GGCATGCCATGG$_{NcoI}$TGAGCAAGGGCGA$_{GFP}$ |
| *Sac*I-35*S* | CGAGCTC$_{SacI}$GCATGCCTGCAGGTCA |
| *term-Hind*III | CCCAAGCTT$_{HindIII}$GCATGCCTGCAGGTCA |

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