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**Involvement of the TMV-MP in the Control of
Metabolism and Partitioning in Transgenic Plants**

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CMV, CMV-MP, SEL, TMV, TMV-MP,

Publications & Patents Summary (following page)

Summary of Cooperation (following page)

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ABSTRACT

The function of the 30-kilodalton movement protein (MP) of tobacco mosaic virus (TMV) is to facilitate cell-to-cell movement of viral progeny in infected plants. Our earlier findings have indicated that this protein has a direct effect on plasmodesmal function. In addition, these studies demonstrated that constitutive expression of the TMV MP gene (under the control of the CaMV 35S promoter) in transgenic tobacco plants significantly affects carbon metabolism in source leaves and alters the biomass distribution between the various plant organs.

The long-term goal of the proposed research was to better understand the factors controlling carbon translocation in plants. The specific objectives were:

- A) To introduce into tobacco and potato plants a virally-encoded (TMV-MP) gene that affects plasmodesmal functioning and photosynthate partitioning under tissue-specific promoters.
- B) To introduce into tobacco and potato plants the TMV-MP gene under the control of promoters which are tightly repressed by the Tn10-encoded Tet repressor, to enable the expression of the protein by external application of tetracycline.
- C) To explore the mechanism by which the TMV-MP interacts with the endogenous control of carbon allocation.

Data obtained in our previous project together with the results of this current study established that the TMV-MP has pleiotropic effects when expressed in transgenic tobacco plants. In addition to its ability to increase the plasmodesmal size exclusion limit, it alters carbohydrate metabolism in source leaves and dry matter partitioning between the various plant organs. Expression of the TMV-MP in various tissues of transgenic potato plants indicated that sugars and starch levels in source leaves are reduced below those of control plants when the TMV-MP is expressed in green tissue only. However, when the TMV-MP was expressed predominantly in PP and CC, sugar and starch levels were raised above those of control plants. Perhaps the most significant result obtained from experiments performed on transgenic potato plants was the discovery that the influence of the TMV-MP on carbohydrate allocation within source leaves was under developmental control and was exerted only during tuber development.

The complexity of the mode by which the TMV-MP exerts its effect on the process of carbohydrate allocation was further demonstrated when transgenic tobacco plants were subjected to environmental stresses such as drought stress and nutrients deficiencies. Collectively, these studies indicated that the influence of the TMV-MP on carbon allocation is the result of protein-protein interaction within the source tissue.

Based on these results, together with the findings that plasmodesmata potentiate the cell-to-cell trafficking of viral and endogenous proteins and nucleoproteins complexes, we developed the theme that at the whole plant level, the phloem serves as an information superhighway. Such a long-distance communication system may utilize a new class of signaling molecules (proteins and/or RNA) to co-ordinate photosynthesis and carbon/nitrogen metabolism in source leaves with the complex growth requirements of the plant under the prevailing environmental conditions.

The discovery that expression of viral MP in plants can induce precise changes in carbon metabolism and photoassimilate allocation, now provide a conceptual foundation for future studies aimed at elucidating the communication network responsible for integrating photosynthetic productivity with resource allocation at the whole-plant level. Such information will surely provide an understanding of how plants coordinate the essential physiological functions performed by distantly-separated organs. Identification of the proteins involved in mediating and controlling cell-to-cell transport, especially at the companion cell-sieve element boundary, will provide an important first step towards achieving this goal.

ACHIEVEMENTS

Following our previous study, in which we produced transgenic potato plants expressing the TMV-MP under the control of green tissue- and tuber-specific promoters, during the first phase of this research we produced transgenic plants that express the TMV-MP under a phloem-specific promoter. Using these plants we were able to demonstrate that the TMV-MP can alter both carbohydrate metabolism in source leaves and partitioning of assimilates among the various plant organs, either when expressed only in mesophyll and bundle sheath cells, or when expressed in phloem parenchyma and companion cells (Almon et al., 1997). Interestingly, when restricted to mesophyll and bundle sheath cells, the TMV-MP induced a reduction in the levels of sugar and starch, relative to control potato lines, whereas sugar and starch levels increased above control values when the TMV-MP was restricted to the phloem. These experiments demonstrated that the TMV-MP exerts its influence on carbon allocation, to the various plant organs when expressed in the leaf, and that plasmodesmata interconnecting the bundle sheath/phloem parenchyma (BS/PP) and the companion cells (CC) may represent the site of action. In light of these findings, as well as our earlier results, we proposed that plasmodesmata interconnecting the BS/PP cells and the companion cells sieve element (CC-SE) complex establish a special communication network that plays a central role in regulating carbon metabolism (Wolf and Lucas, 1997; Lucas and Wolf, 1999).

From an agricultural point of view, one of our most important findings was that the TMV-MP can exert long-range influence over resource allocation in transgenic plants expressing this gene in leaves. Transgenic potato plants expressing the TMV-MP under a phloem-specific promoter were found to have a harvest index (percent of dry matter partitioned to the tubers) that was 25% higher than in control plants. An application for a permit to perform field trials with these transgenic potato plants was filed, and we were one of the first research groups to perform field trials with transgenic plants in Israel. Over the past 2 years we have begun exploring the agricultural potential of these potato lines in the experimental fields at Kibbutz SA'AD (a major potato-growing area in Israel).

During the period covered by the current grant we also investigated the influence of environmental factors such as water stress and nutrient deficiency (N, P and K⁺), on the TMV-MP-imposed influence over resource allocation. Our studies demonstrated that although nutrient deficiency resulted in alteration in biomass partitioning, this form of stress did not override the influence of the TMV-MP on resource allocation. However, when transgenic plants

expressing the TMV-MP were subjected to water stress, the root-to-shoot ratio increased to a value comparable to that of control plants subjected to the same water-stress treatment (Balachandran et al., 1997). In addition to the information regarding the mode by which the TMV-MP exerts its influence on plant function, these studies were important in terms of defining the optimal agrotechnical conditions for growing plants expressing viral MPs.

Since the beginning of our studies on the TMV-MP, we have been able to provide important information on the role played by plasmodesmata in terms of cell-to-cell trafficking of information molecules. Collectively, our research has indicated that such trafficking occurs at the level of the plant organ (e.g., within plant leaves), as well as at the whole-plant, or supracellular level. The latter appears to be facilitated by the trafficking of informational molecules via the phloem and these molecules (proteins and RNA) appear to be involved in coordinating the processes of carbon partitioning, export from source leaves and allocation of photosynthates among the various plant organs (Lucas and Wolf, 1999).

One goal that was not achieved in our research is the use of transgenic plants expressing the TMV-MP under an inducible system. We tried to adopt the tetracycline (Tc)-inducible system. Expression of the Tc repressor in plant cells should regulate the expression of the TMV-MP gene which was cloned downstream of the CaMV 35S promoter containing Tet operators adjacent to the TATA box. Transgenic tobacco plants expressing constitutively high amounts of the Tc repressor were transformed with a DNA cassette containing a modified promoter (CaMV and a Tc operator motif that is recognized by the Tc repressor). Several individual plantlets were regenerated on selective media and contained the MP gene. We also transformed tobacco plants expressing high levels of repressor with a reporter gene (GUS) for calibration of the Tc application method. Much effort was invested in calibrating a routine protocol to regulate the gene expression in an on/off manner. However, the system was found to be somewhat leaky and unreliable.

It is important to stress that this model of supracellular control over plant function was developed on the basis of data obtained from two different species, tobacco and potato. As phloem loading in both plants occurs via the apoplasm, our studies could not address the question of the relative importance of the plasmodesmal pathway in terms of it being a rate-determining step in the overall process of phloem loading in these species. To verify the generality of our concept we expanded the research beyond its original scope and began investigating the effect of other viral proteins on carbon metabolism and transport in melon plants.

Source leaves infected with CMV were characterized by high concentrations of reducing sugars and relatively low starch levels. While stachyose was the predominant sugar in phloem sap collected from petioles of control leaves, sucrose was the major sugar in the phloem sap of infected leaves. Moreover, elevations in sucrose concentration were also evident in source leaves that did not exhibit symptoms or contain detectable amounts of virus particles (Shalitin and Wolf, 1999, submitted). Pulse-labeling experiments provided support for the assumption that a high sucrose level in the phloem of CMV-infected plants is associated with an alteration in the process of phloem loading. The fact that this phenomenon was also evident in leaves in which virus particles were still absent indicated that the alteration in sugar transport was controlled by information perceived by these leaves.

The role of the phloem in the delivery of nutrients and hormones has long been appreciated. The data originating from our BARD-funded project has provided a foundation for the concept that the phloem also serves as an information superhighway that plays an important role in the control over carbon metabolism and resource allocation at the whole-plant level.

DESCRIPTION OF COOPERATION

Plant transformation and whole-plant analyses were conducted in Israel, while the cellular studies were performed in Davis. For example, transgenic plants (produced in Israel) were sent to Davis for immuno-histochemical studies and dye-coupling experiments. Dr. Wolf spent a sabbatical leave in Davis and got acquainted with the immuno-histochemical techniques. Both Prof. Lucas and Dr. Wolf were co-organizers of the third international workshop on Basic and Applied Research in Plasmodesmal Biology. Prof. Lucas was invited to be a Lady Davis Visiting Professor at the Faculty of Agriculture in the spring semester of the academic year 1998 and taught a course on basic and applied aspects of plasmodesmata.

RESEARCH ACTIVITIES AND RESULTS

Phloem-specific expression of TMV-MP alters carbon metabolism and partitioning in transgenic potato plants

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Functional viral movement proteins (MPs) are essential for the cell-to-cell movement of plant viruses (Lucas and Gilbertson, 1994; Maule, 1991). Evidence accumulated over recent years has established that these proteins have the capacity to alter plasmodesmal function (Ding et al., 1995; Fujiwara et al., 1993; Noueiry et al., 1994; Wolf et al., 1989). Dye-coupling studies performed on transgenic tobacco plants, expressing the tobacco mosaic virus (TMV) MP, indicated that this protein can induce a significant increase in the size exclusion limit (SEL) of plasmodesmata interconnecting both mesophyll and bundle sheath cells. In control tobacco plants, plasmodesmal SEL is approx. 0.8 kDa, whereas in the presence of the TMV-MP, fluorescent probes larger by a factor of more than 10 could move from cell to cell (Ding et al., 1992; Waigmann et al., 1994; Wolf et al., 1989).

Pursuant to the hypothesis that cell-to-cell movement of sucrose from the site of synthesis to the site of phloem loading, within the vascular bundle, follows a symplasmic pathway, we have used the TMV-MP transgenic tobacco plants to study the role of plasmodesmal function in controlling sugar transport and photoassimilate partitioning between the various plant organs (Balachandran et al., 1995; Lucas et al., 1993; Olesinski et al., 1995). Pulse-chase experiments following $^{14}\text{CO}_2$ labeling, as well as analyses of the diurnal changes in carbohydrate levels, indicated that constitutive expression of the TMV-MP (under control of the CaMV 35S promoter) results in inhibition of sucrose export and accumulation of carbohydrates in source leaves during the day. Moreover, in addition to its effects on the physiology of tobacco source leaves, the TMV-MP causes a significant reduction in dry matter partitioning to roots, yielding a lower root-to-shoot ratio in these transgenic tobacco plants (Balachandran et al., 1995). Here, it is important to note that analyses of transgenic plants expressing temperature-sensitive and C-terminal deletion mutant forms of the TMV-MP demonstrated that this protein exerts its effects on carbon allocation and dry matter partitioning via a mechanism that is independent of its ability to increase plasmodesmal SEL (Balachandran et al., 1995; Olesinski et al., 1995). Collectively, these studies established the pleiotropic nature of the TMV-MP in transgenic tobacco plants.

Further studies, aimed at determining the site where the TMV-MP exerts its effects on the physiology of the plant, included the generation of transgenic potato plants expressing the TMV-MP under tissue specific promoters. In this work it was demonstrated that starch and sugars accumulate in potato leaves to significantly lower levels, compared with control leaves, when the TMV-MP was expressed in green tissue only (under the control of the nuclear photosynthetic ST-LS1 gene promoter). Interestingly, no effect on carbohydrate metabolism, or partitioning of photoassimilates between the various plant organs, was found when TMV-MP expression was restricted to tubers (Olesinski et al., 1996). These results, together with tobacco grafting experiments (Balachandran et al., 1995), identified the shoot as the site where the TMV-MP exerts its influence over the physiology of the plant.

A prerequisite for understanding the mechanism by which the TMV-MP interacts with endogenous factors to cause the observed changes in carbon allocation and transport would be an explicit identification of its site(s) of action. The boundary between the mesophyll and the vascular bundle represent a potential control site for sucrose transport. In potato plants, sucrose is thought to be released from the symplasm of the phloem parenchyma cells into the apoplasmic space, from where it is then actively loaded into the companion cells-sieve element (CC-SE) complex (van Bel, 1993). As the TMV-MP alters carbohydrate metabolism in source leaves, it could be argued that it exerts its effect within the mesophyll, where sucrose synthesis takes place. Alternatively, the TMV-MP may well interfere, directly or indirectly, with the loading of sucrose into the CC-SE complex; in this situation its site of action would be located within the phloem.

To verify the role of phloem cells in TMV-MP-mediated alteration in carbohydrate metabolism and sucrose transport, transgenic plants were generated in which the TMV-MP was expressed under a phloem specific (rolC) promoter (Schmulling et al., 1989; Sugaya et al., 1989). We demonstrate here that the TMV-MP can alter both carbohydrate metabolism in source leaves and partitioning of assimilates between the various plant organs, either when expressed only in mesophyll and bundle sheath cells, or when expressed predominantly in phloem parenchyma and companion cells of transgenic potato plants. These findings are discussed in terms of the mode(s) by which the TMV-MP may exert its influence over carbon metabolism and photoassimilate translocation.

MATERIALS AND METHODS

Reagents, enzymes and plasmids

DNA restriction enzymes were obtained from Boehringer Mannheim unless specified otherwise. Cefotaxime (Claforan) was purchased from Laboratoires Roussel (Paris, France). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and Bio-Rad (Hercules, CA).

Potato transformation, regeneration, and growth conditions

Two cDNA clones encoding the TMV-MP, designated TMV-MP 7 and 11 (Olesinski et al., 1996), were inserted into a Bin19-derived binary vector (Bevan, 1984) containing the phloem-specific promoter rolC (Schmulling et al., 1989; Sugaya et al., 1989) and the octopine synthase polyadenylation signal (OCS). This vector (Fig. 1) was transformed into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983). Tuber discs (3 mm diam) of *Solanum tuberosum* cv. Desiree were co-cultivated with the recombinant *A. tumefaciens* cells. Culture conditions were similar to those previously described by Perl et al. (1991). Regenerated shoots were produced on MS medium (Murashige and Skoog, 1962) supplemented with kanamycin (100 mg/l) and claforan (500 mg/l), and the transformants were selected by rooting on kanamycin (100 mg/l) and claforan (250 mg/l) MS medium. Rooted transformants were grown under sterile conditions at 25°C with a photon flux density (PFD) of 60-160 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a 12-h photoperiod. Plants for regeneration, transformation analysis and physiological experiments were transferred to soil mixture in plastic pots (15 cm diam) and grown in an insect-free, temperature-controlled greenhouse (25 $\pm$ 3°C day/16 $\pm$ 2°C night). Plants were grown under natural sunlight with an average midday PFD of 1200-1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Regenerated plantlets were analyzed for the presence of the transgene using detailed PCR analysis in which the correct orientation of the transgene and its promoter were verified (data not shown). Over 30 individual plantlets were identified to be transgenic for TMV-MP, from which 12 were chosen for further analysis. RNA expression in veins of mature leaves was detected by northern blot analysis. Each samples (50 mg of veins) was denatured at 65°C for 5 min in 70% formamide, 6% formaldehyde, and 200 mg Mops buffer, separated in 1.2% agarose gel containing 6.3% formaldehyde in 20 mM Mops buffer, pH 7.0. RNA was then transferred to a NITRAN (stratagene) membrane and hybridized to TMV-MP-specific ^{32}P -radiolabeled probe (Fig. 2).

Tissue fixation and immunolabeling

Polyclonal antibodies raised against a synthetic TMV-MP peptide were prepared and found to specifically recognize the TMV-MP protein. Immunolabeling studies were performed on phloem expressing TMV-MP plant lines C-7-303 and C-7-312. As controls, we used the vector control

line C-301 and line L-7-203 (expressing the TMV-MP under the ST-LS1 promoter). In addition, tobacco plant line 277 (expressing the TMV-MP under the CaMV 35S promoter) was employed as a well characterized positive control.

Plant tissues (1-2 mm width) from leaf number 6 (leaf #1 was defined as the last expanding leaf to achieve a laminar length of 5 cm) were excised and then fixed for 2 h in 0.5% (w/v) glutaraldehyde and 1.5% (w/v) paraformaldehyde in 50 mM phosphate buffer (pH 7.0). Tissue was then transferred to 1% (w/v) glutaraldehyde and 3% (w/v) paraformaldehyde in 50 mM phosphate buffer (pH 7.0) at 4°C. Plant material was dehydrated through a 20% step-graded ethanol series at 4°C, infiltrated over 2 days through a five-step graded series from 100% (v/v) ethanol to 100% (v/v) LR White (Medium grade, Electron Microscopy Science), and then embedded in LR white resin. Blocks were polymerized by exposure to a temperature sequence of 37°C for 12h, 48°C for 12 h and 60°C for 24 h.

Ultrathin section (60 nm) were cut with a diamond knife and collected on uncoated mesh grids or formvar film-coated slot grids. Sections were pre-incubated for 1 h in blocking solution (50mM Tris-HCL, 150 mM NaCl, 0.1% [v/v] Tween 20, 2% [w/v] bovine serum albumin) and then transferred directly to 90 ml of the antisera solution. After 4 X 15-min washes with blocking solution, the sections were incubated for 1 h in secondary antibody (15-nm or 20-nm diameter gold-conjugated goat anti-rabbit IgG) solution diluted 1:40 in antibody diluent. After several washing steps (2 X 15-min with blocking solution, followed by 2 X 15-min washes with 50 mM Tris-HCL and 150 mM NaCl buffer), sections were stained with uranyl acetate (2% w/v) in distilled water and lead citrate, for 10 min each, and then observed using a Philips (Eindhoven, The Netherlands) electron microscope (model EM410LS) operated at 80 kV.

To quantify the expression of the TMV-MP, the presence of gold-label was analyzed in the various cell types of the different plant lines. Almost 1000 cells were examined and the results represent the mean of at least 40 cells for each cell type.

Microinjection procedures

Lucifer yellow CH (LYCH; molecular mass of 457 Da) and fluorescein isothiocyanate-labeled dextrans (FITC-dextran; molecular masses of 3 or 9.4 kDa) were used in microinjection studies. Attached leaves were microinjected by the method described for tobacco leaves (Ding et al., 1992). In brief, spongy mesophyll cells were impaled with a micropipette and pressure-injected with dye. Movement of dye into neighboring cells was monitored with a Leitz Orthoplan epifluorescence microscope (equipped with a blue BP 390-490 excitation filter) connected to a Hamamatsu (Bridgewater, NJ) image enhancement system (model C1966-20).

Determination of biomass partitioning

Initial experiments were performed on second-generation potato plants grown in a temperature-controlled greenhouse (25/16°C day/night) under natural sunlight (13 to 14 h photoperiod); average midday PFD was 1200-1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were harvested 57 days after planting and then separated into shoots (leaves plus stems), roots and tubers and dried in a 70°C oven for 72-96 h. A second set of experiments was conducted on third-generation potato plants that were grown under controlled environment conditions (Convion [Asheville, NC] model PGV36); 22/15°C day/night temperature, relative humidity maintained at 70%, light intensity (metal halide lamps) during the first and last h of either an 8 or 16 h photoperiod was set at 100-150 $\mu\text{mol m}^{-2} \text{s}^{-1}$, while over the rest of the photoperiod it was set at 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (at canopy level).

Starch and sugar determinations

Tissue samples were collected from the youngest fully expanded leaf (nos. 5 or 6, with leaf no 1 being the last leaf to achieve a length of 5.0 cm) at 0800 and 1500 h and carbohydrate content determined as described by Lucas et al. (1993). In brief, soluble sugars were extracted from leaf discs in 80% ethanol. After evaporating the supernatant, sugars were redissolved in H₂O, and filtered through a 0.45-mm membrane HPLC filter (Whatman, Clifton, NJ). Sugars were separated in an analytical HPLC system (LDC Anal., Riviera Beach, FL) fitted with a Sugar-Pak I column (6.5 mm x 300 mm; Waters Associates, Milford, MA) using an LDC refractive-index detector. Starch content was determined in the ethanol-water-extracted leaf discs following starch conversion by amyloglucosidase (Sigma). Starch content, as glucose equivalents, was determined using the Sigma (HK) quantitative glucose determination kit.

Sucrose efflux from source leaves

Phloem exudate was collected from excised petioles of fully expanded potato leaves using the method described by van Bel et al. (1994), with minor modifications. Experiments were conducted in a greenhouse under natural sunlight in the late morning h. The youngest, fully expanded, leaves were detached under water by cutting petioles close to the stem. Excised petioles were immediately placed into a test tube containing 5 ml of 5 mM EDTA (Parafilm was used to seal the mouth of the test tube). Leaves were gently shaken in a water bath that was covered with a glass plate to maintain high relative humidity. After a 3 h exudation period, leaf area was measured and the phloem exudate was lyophilized. Sugars were resuspended in 100 ml distilled water and the sucrose content determined by HPLC.

RESULTS

Cellular localization of the TMV-MP within transgenic potato plants

Cellular localization of the TMV-MP was investigated in several transgenic potato lines using high resolution immuno-histochemical techniques. As indicated in Fig 3. (c,d), TMV-MP was localized mainly to the phloem parenchyma and companion cells when the gene was expressed under the rolC promoter (see also Table 1). Interestingly, label was also detected in bundle sheath cells (Fig. 3d) at a level significantly above that of background which was established using vector control plants (line C-301) and preimmune controls (Fig. 3a,b). Label was also detected in mesophyll cells of these plants, however, the level was not statistically different from those detected in the vector control line (Table 1). Most of the TMV-MP detected by immunogold labeling in the rolC plants was localized to the cytoplasm (Fig. 3c,d). When the TMV-MP was expressed under the control of the promoter from the nuclear photosynthesis gene (ST-LS1), the amount of label in mesophyll cells was almost 6 times higher compared with the values obtained for same cells in rolC plants (Table 1, Fig. 3h). Label in bundle sheath cells was also highest in this plant line, whereas the degree of labeling in companion cells was not significantly different from the background level established using vector control plants (Fig. 3f and Table 1). A relatively low level of label was detected in phloem parenchyma cells of the ST-LS1 plant line, with the value being significantly lower than detected in rolC plants, but significantly higher than that present in the vector control line (Table 1).

Expression of the TMV-MP under the control of the ST-LS1 promoter resulted in an increase in mesophyll plasmodesmal SEL in transgenic potato plants (Olesinski et al., 1996). In the present study, dye-coupling experiments established that 9.4 kDa FITC-dextran did not move out of the injected mesophyll cells (Table 2). Furthermore, only 2 out of 7 injections indicated

movement of 3 kDa FITC-dextran compared with 100% movement of this probe in plant line L-7-203.

Phloem-specific expression of TMV-MP alters dry weight partitioning

When grown for 57 days under greenhouse conditions, the average shoot (leaves+stems) dry weight for rolC transgenic potato plants was 25% higher compared with values obtained for vector control plants (Table 3). In contrast, the average root dry weight of rolC plants was 27% lower compared with control plants. As a result of this TMV-MP-induced alteration in biomass partitioning, the root-to-shoot ratio in the rolC lines was 40% lower as compared with that of vector control plants.

TMV-MP-mediated effects on growth pattern, photoassimilate partitioning, and carbohydrate allocation in potato plants expressing the gene under the ST-LS1 promoter, was evident only when plants had undergone tuber initiation (Olesinski et al., 1996). To determine if this were the case when the TMV-MP was expressed in the phloem, potato plants were next grown in controlled environment chambers under an 8 or 16 h photoperiod. When grown under a short photoperiod (8 h), the root-to-(shoot+tubers) ratio was significantly lower in three out of six rolC lines tested, as compared with control potato lines (Fig. 4). The remaining rolC lines had root-to-(shoot+tubers) ratios that were similar to control values (Fig. 4a). The mean ratio for all rolC plants was 0.04 compared with a mean value of 0.06 for control lines. All six rolC lines tested in this experiment exhibited higher dry matter partitioning to the tubers (harvest index) as compared with control lines (Fig 4b). The average value of percent dry matter partitioned to the tubers (out of the total dry weight) was 53% for the rolC plants, which was significantly higher ($P=0.01$) than the average value obtained for the controls (41%).

No differences in dry weight partitioning, either to the roots or tubers, were found between rolC and vector control lines when plants were grown under long photoperiodic conditions (Fig. 5). It is important to note that, for all plant lines grown under long days, tubers were initiated but partitioning to these organs was significantly reduced compared with plants grown under short day conditions.

Phloem-specific expression of TMV-MP alters carbohydrate metabolism and export from source leaves

Our earlier studies with transgenic tobacco and potato plants expressing the TMV-MP indicated an alteration in carbohydrate metabolism concomitant with the detected change in biomass partitioning (Balachandran et al., 1995; Lucas et al., 1993; Olesinski et al., 1995, 1996). Daytime changes in carbohydrate levels of young, fully mature, potato leaves were analyzed 52 days after planting (Fig. 6). No differences in glucose or fructose levels were detected in transgenic versus control leaves over the sampling period; e.g., in the afternoon, average glucose and fructose values were 24 and 75 $\mu\text{g cm}^{-2}$, respectively, for rolC plants, with the respective values for control plants being 26 and 75 $\mu\text{g cm}^{-2}$. Sucrose levels were similar in the morning, but underwent a significant increase in the afternoon compared with values obtained for the control plants. The average sucrose level in the transgenic lines was 191 $\mu\text{g cm}^{-2}$ as compared with 145 $\mu\text{g cm}^{-2}$ in the control plants (significant at $P=0.05$).

The most pronounced effect of TMV-MP expression in the phloem of transgenic potato plants was reflected by changes in starch metabolism. All transgenic lines contained higher levels of starch in source leaves at the end of the dark period, with an average value of 871 $\mu\text{g cm}^{-2}$ as compared with 420 $\mu\text{g cm}^{-2}$ in the control plants. A similar increase in starch accumulation occurred within source leaves during the photoperiod (about 40% for all plant lines), resulting in starch levels being higher, again, by a factor of two, in the transgenic plants as

compared with either controls (wild-type Desiree or vector control line C-301). Statistical analysis of the differences in starch levels, in the afternoon, between the tested lines, indicated significantly higher levels ($P=0.05$) in the rolC plants. The average value for rolC plants was $1195 \mu\text{g cm}^{-2}$ as compared with $595 \mu\text{g cm}^{-2}$ for control plants.

Analysis of total carbohydrates (starch + sugars) in the afternoon, indicated that the values for the different rolC plant lines were 30 to 100% higher as compared with either control lines (Fig. 7).

As no significant differences in either photosynthetic rate or dark respiration were observed between the various lines tested (data not shown), it was logical to assume that the daytime accumulation of carbohydrates in TMV-MP expressing plant was due to an inhibition of sucrose export from source leaves. An *in vitro* experimental system was employed to study sucrose efflux from petioles of transgenic and control plants (Van Bel et al., 1994). The rate of sucrose efflux from detached leaves of both control lines (Desiree and C-301) was significantly higher as compared with the transgenic lines expressing the TMV-MP (Fig. 8). These results provide indirect evidence that the TMV-MP can influence the processes involved in phloem loading.

DISCUSSION

In the present study, we demonstrate that expression of TMV-MP in the phloem of transgenic potato plants can influence the processes involved in sucrose loading into the phloem and carbon partitioning to various plant organs. These findings are consistent with our earlier studies in which we established that the TMV-MP can alter carbohydrate metabolism in source leaves, and resource partitioning to the lower stems and roots (Balachandran et al., 1995; Lucas et al., 1993, 1996; Olesinski et al., 1995, 1996; Wolf and Lucas, 1994). Our present results further support the hypothesis that the leaf is the site where the TMV-MP exerts these pleiotropic effects.

Immuno-histochemical studies, performed on transgenic potato plants in which *TMV-MP* expression was regulated by the ST-LS1 promoter, established that the MP was present mainly in mesophyll and bundle sheath cells of source leaves (Table 1). Interestingly, when restricted to these photosynthetic tissues, the TMV-MP induced a reduction in the levels of sugar and starch compared with control potato lines (Olesinski et al., 1996). A significant alteration in accumulation of sucrose and starch was also observed in source leaves of rolC-TMV-MP transgenic potato plants, however, in these plants sucrose and starch accumulated to higher levels (Fig. 6), whereas the rate of sucrose efflux from excised petioles was lower than observed with control plants (Fig. 8). The significant differences in the localization site of the TMV-MP between the ST-LS1 and rolC plants, together with the contrasting situation in terms of carbon metabolism in source leaves, indicate that the **specific** influence of the TMV-MP on sugar transport and carbohydrate partitioning in the rolC plants is likely due to TMV-MP expression within the phloem parenchyma and companion cells.

It is important to note that expression of the β -glucuronidase (*GUS*) gene in transgenic tobacco plants, under the control of the rolC promoter, resulted in confinement of *GUS* expression to phloem tissue (Schmulling et al., 1989; Sugaya et al., 1989). Unfortunately, in these studies the specific cells were not identified in which *GUS* was present. Immunolocalization of the TMV-MP in the rolC transgenic potato plants indicated that protein was present predominantly in the phloem parenchyma and companion cells (Fig. 3, Table 1). However, immunogold label above the background level was detected in bundle sheath and mesophyll cells. This finding can be explained on the basis of either limited tissue-specificity of

the promoter, or cell-to-cell movement of the TMV-MP from the site of synthesis to neighboring tissues. Here it is important to note that immunocytochemical studies, performed on transgenic tobacco plants expressing a foreign inorganic pyrophosphatase under the control of the *rolC* promoter, indicated that the protein was restricted to the phloem of the inner and outer midrib and the minor veins (Lerchl et al., 1995). Based on these results, together with the finding that the TMV-MP has the ability to move between mesophyll cells (Nguyen et al., 1996; Waigmann et al., 1994), it would appear reasonable to assume that immunodetection of TMV-MP, in bundle sheath cells of *rolC*-TMV-MP potato plants, is a consequence of its capacity to traffic through the plasmodesmata that connect the CC/PP to these cells.

The relationship between the presence of the TMV-MP in specific tissues within source leaves and its established pleiotropic effects on carbon metabolism and resource partitioning remains to be elucidated. In this regard, it is generally accepted that transport of sucrose from the mesophyll to the region of the vascular bundle follows a symplasmic pathway. A recent study on a maize mutant demonstrated that aberrant plasmodesmal structure, at the bundle sheath-vascular parenchyma interface, resulted in an inhibition of sucrose export and carbohydrate accumulation in source leaves (Russin et al., 1996). This study established the prominent role of plasmodesmata in symplasmic transport of photoassimilates (Stitt, 1996).

Expression of sucrose synthase in the phloem of potato (Yang and Russell, 1990), *Arabidopsis* (Martin et al., 1993) and companion cells of maize (Nolte and Koch, 1993), together with the finding that PPi in phloem sap of *Ricinus communis* is catalysed by sucrose synthase (Geigenberger et al., 1993), provided the basis for the hypothesis that PPi is required for phloem metabolism. A direct approach to study the role of PPi in phloem metabolism included production of transgenic tobacco plants in which the *Escherichia coli* inorganic pyrophosphatase was expressed in the phloem only (Lerchl et al., 1995). Deletion of PPi from the phloem of these plants resulted in accumulation of photoassimilates in source leaves, a decrease in ATP/ADP ratio and respiration rate in the midribs and an inhibition of export rate (Geigenberger et al., 1996; Lerchl et al., 1995). These results suggest that energy limitation, due to shortage of PPi, may inhibit the process of sucrose loading into the CC-SE complex and reduce the rate of export from source leaves. Since accumulation of carbohydrates and reduction in export rate from source leaves was also evident in the transgenic potato plants expressing the TMV-MP in the phloem (Figs. 7 and 8), it is possible that energy limitation may be responsible, in part, for these effects in transgenic potato plants expressing the TMV-MP under the control of the *rolC* promoter. This hypothesis cannot explain the effect of the TMV-MP on resource allocation nor can it provide a satisfactory explanation for the developmental control over the effects exerted by the TMV-MP (Lucas et al., 1996). In any event, we are presently examining the metabolic status of phloem cells in lines expressing the TMV-MP under the different tissue specific promoters.

We recently advanced an hypothesis that trafficking of regulatory (information) molecules, through plasmodesmata, may establish a special supracellular communication network between the companion and mesophyll cells which operates to regulate carbon partitioning (Balachandran et al., 1995; Lucas et al., 1996; Wolf and Lucas, 1996). According to this model, the supracellular communication system facilitates efficient orchestration of the network of biochemical and physiological processes involved in photosynthate metabolism and transport. The TMV-MP may interfere at several loci within this putative communication network. The presence of a high level of TMV-MP within companion cells (or phloem parenchyma cells) may interfere with the trafficking of endogenous signaling molecules (into or out of the phloem) and thus induce an alteration in carbon metabolism within the source leaf.

Photoassimilate partitioning and carbohydrate metabolism in ST-LS1-TMV-MP source leaves was altered only after the potato plants had undergone tuber initiation (Olesinski et al., 1996).

Similar developmental control over TMV-MP-mediated effects on resource partitioning was observed in rolC potato plants (Figs. 4, 5). Clearly, tuber development imposes a higher demand for photoassimilates. If this demand is regulated by long-distance transport of information molecules, the TMV-MP that is localized to either the phloem or mesophyll cells appears to be capable of interfering with this trafficking to alter the endogenous control mechanism for photosynthate partitioning only at this specific stage of plant development. Recent findings on transgenic tomato plants overexpressing sucrose phosphate synthase (Micallef et al., 1995) and nitrate reductase deficient tobacco mutants (Scheible et al., 1997), provided further support for the concept that long-distance signals are involved in the regulation of resource partitioning.

Elucidation of the mechanism(s) by which the TMV-MP affects this putative endogenous control system, involved in regulating photosynthate transport in the phloem, may well open many biotechnological avenues for the manipulation of plant function. Future studies will focus on identifying the components of this endogenous control system.

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Table 1. Statistical analysis of immunogold labeling of TMV-MP in different cell types of transgenic potato plants expressing the TMV-MP under the rolC (C-7-303, C-11-312) or the ST-LS1 (L-7-203) promoter. Tobacco plants (line 277) expressing the TMV-MP under the CaMV 35S promoter and empty cassette transformed potato plants (vector control line C-301) served as controls for these experiments. Data are presented as the number of gold particles detected per 20 μm^2 .

Plant line (promoter)	Cell type				
	Mesophyll	Bundle Sheath	Phloem Parenchyma	Companion Cells	Sieve Element
C-301 (vector control)	4 c	4 c	0.4 c	4 c	3 a
C-7-303 (rolC)	11 c	16 b	59 a	83 a	4 a
C-11-312 (rolC)	16 c	18 b	71 a	93 a	6 a
L-7-203 (ST-LS1)	76 a	26 a	15 b	22 c	4 a
277 (CaMV 35S)	58 b	19 b	17 b	47 b	5 a

Values followed by the same letter within a column are not statistically different at the 5% level using Duncan's multiple comparison test.

Table 2. Mobility of fluorescent probes through the symplasmic pathway of leaf mesophyll of transformed potato plants expressing the TMV-MP under the rolC (C-7-303, C-11-312) or the ST-LS1 (L-7-203) promoters, or a vector control line (C-301). Data are presented as the number of injections showing movement of the specified probe (values in parentheses represent total number of injections).

Plant line	LYCH	FITC-dextran	
		3 kDa	9.4 kDa
C-301	9 (9)	0 (9)	0 (8)
C-7-303 and C-11-312	7 (7)	2 (7)	1 (8)
L-7-203	3 (3)	6 (6)	11 (14)

Table 3. Growth analysis of 57-day-old transgenic potato plants expressing the TMV-MP under the rolC promoter (lines C-7- and C-11-) compared with vector control plants (line C-301). Plants were grown in a greenhouse under natural sunlight (13 to 14 h photoperiod) with an average midday photon flux density of 1200-1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. (Values represent the mean $\pm$ SE with 5 plants of each line being used in each experiment.)

Plant line	Dry weight (g plant organ ⁻¹)			Root:shoot Ratio
	Shoots	Roots	Tubers	
C-301	4.8 $\pm$ 0.6	0.75 $\pm$ 0.20	8.4 $\pm$ 1.1	0.15 $\pm$ 0.03
C-7-301	5.5 $\pm$ 0.4	0.48 $\pm$ 0.12	10.4 $\pm$ 0.7	0.09 $\pm$ 0.02
C-7-303	6.3 $\pm$ 1.2	0.37 $\pm$ 0.06	9.5 $\pm$ 1.3	0.06 $\pm$ 0.01
C-7-304	4.2 $\pm$ 0.5	0.59 $\pm$ 0.15	6.5 $\pm$ 0.9	0.14 $\pm$ 0.03
C-7-308	6.4 $\pm$ 0.5	0.58 $\pm$ 0.06	8.9 $\pm$ 0.9	0.09 $\pm$ 0.01
C-7-315	7.2 $\pm$ 0.7	0.52 $\pm$ 0.10	9.7 $\pm$ 0.9	0.07 $\pm$ 0.01
C-11-307	6.1 $\pm$ 0.6	0.54 $\pm$ 0.26	11.0 $\pm$ 0.9	0.09 $\pm$ 0.04
C-11-308	5.7 $\pm$ 0.5	0.62 $\pm$ 0.24	8.9 $\pm$ 1.0	0.11 $\pm$ 0.04
C-11-311	6.5 $\pm$ 0.5	0.66 $\pm$ 0.13	8.8 $\pm$ 1.0	0.09 $\pm$ 0.02
C-11-312	5.5 $\pm$ 0.7	0.58 $\pm$ 0.29	14.1 $\pm$ 3.9	0.12 $\pm$ 0.08
C-11-315	5.5 $\pm$ 0.3	0.74 $\pm$ 0.16	10.4 $\pm$ 0.9	0.14 $\pm$ 0.03
C-11-316	6.6 $\pm$ 0.8	0.33 $\pm$ 0.07	10.4 $\pm$ 1.1	0.05 $\pm$ 0.01
rolC (average) ^a	6.0 $\pm$ 0.8	0.55 $\pm$ 0.12	9.9 $\pm$ 1.8	0.09 $\pm$ 0.03

^aData average for all 12 plant lines used in the study.

Figure Legends

Figure 1. Structure of the chimeric binary plant expression cassettes (pBin-rolC) used for potato transformation.

Figure 2. Northern analysis of TMV-MP RNA (20 μ g per lane) in veins of transgenic potato plant lines C-7-303, C-7-308, C-7-304, C-11-311, C-11-312 (lanes 2-6, respectively) and the wild type Desiree (lane 1). Tobacco plant line 277 expressing the TMV-MP under the CaMV 35S promoter served as a positive control (lane 7).

Figure 3. Immunogold labeling of TMV-MP in transgenic potato plants.

(A) and (B): Electron micrographs of sections cut from leaves of rolC plants immunogold labeled with pre-immune sera. (C), and (D): Sections cut from leaves of rolC plants immunogold labeled with TMV-MP antisera. High level of labeling was localized in the phloem parenchyma and companion cells as compared with bundle sheath cells. (E) and (F): Sections cut from leaves of potato plant line L-7-203 (expressing the TMV-MP under the ST-LS1 promoter) and immunogold labeled with TMV-MP antisera. Label was rarely detected in phloem cells whereas numerous gold particles were observed in mesophyll cells. Bs, bundle sheath; Cc, companion cells; Ch, chloroplast; Ics, intercellular space; Mc, mesophyll cell; Pp, phloem parenchyma; and Se, sieve element. Bar = 500 nm.

Figure 4. Root-to (shoot+tubers) ratio (A) and harvest index (percent of dry matter partitioned to the tubers) (B) of 56-day-old TMV-MP (C-7-, C-11-) and control (C-301, C-302) potato plants. For these experiments, plants were grown under controlled environment conditions; 22/15°C day/night temperatures and 8 h photoperiod. (Values represent the mean $\pm$ SE; 3-5 plants were used for each line.)

Figure 5. Root-to (shoot+tubers) ratio (A) and harvest index (percent of dry matter partitioned to the tubers) (B) of 56-day-old TMV-MP (C-7-, C-11-) and control (C-301, C-302) potato plants. Plants were grown under controlled environment conditions; 22/15°C day/night temperatures and 16 h photoperiod. (Values represent the mean $\pm$ SE; 3-5 plants were used for each line.)

Figure 6. Morning (A,C,E,G) and afternoon (B,D,F,H) carbohydrate levels measured in leaves of transgenic potato plants expressing the TMV-MP under the rolC promoter, as well as in wild-type (untransformed) and vector control potato plants. Plants were grown for 52 days under greenhouse conditions of 25°C/16°C day/night temperatures and natural sunlight (13-14 h photoperiod and average midday PFD of 1200-1500 μ mol m⁻² s⁻¹). Leaf disks were analyzed for glucose (A,B), fructose (C,D), sucrose (E,F) and starch (G,H) levels. (Values represent the mean $\pm$ SE of 6 plants.)

Figure 7. Afternoon total carbohydrate levels measured in leaves of transgenic potato plants expressing the TMV-MP under the rolC promoter, as well as in wild-type (untransformed) and vector control potato plants. Plants were grown for 52 days under greenhouse conditions of 25°C/16°C day/night temperatures and natural sunlight (13-14 h photoperiod and average midday PFD of 1200-1500 μ mol m⁻² s⁻¹). Values represent the mean $\pm$ SE of 6 plants.

Figure 8. Sucrose content in the phloem exudate obtained from excised petioles of potato plants. Leaves were cut from 6-week-old plant lines C-7-, C-11-, C-301 and wild-type Desiree that had been grown under greenhouse conditions of 25°C/16°C day/night temperatures and natural sunlight (13-14 h photoperiod and average midday PFD of 1200-1500 μ mol m⁻² s⁻¹). Values represent the mean $\pm$ SE of 5 plants.

Figure 1.

Figure 2

Figure 3

Figure 4. **Figure 5**

Figure 6

Figure 7

Figure 8

Influence of Environmental Stress on Biomass Partitioning in Transgenic Tobacco Plants Expressing the Movement Protein of Tobacco Mosaic Virus¹

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The influence of various environmental factors on biomass partitioning between shoots and roots in transgenic tobacco (*Nicotiana tabacum*) plants expressing the movement protein (MP) of tobacco mosaic virus (TMV) was investigated. TMV-MP-expressing transgenic plants exhibited a root-to-shoot ratio that was approximately 40% below that of transgenic vector control plants. When transgenic plants expressing the TMV-MP were subjected to water-stress conditions, the root-to-shoot ratio was increased to a value comparable to that of control plants subjected to the same water-stress treatment. Although the root-to-shoot ratio was increased by N or P deficiencies, the TMV-MP-induced alteration in biomass partitioning was not overcome. Surprisingly, under K⁺-deficient growth conditions, both TMV-MP-expressing and control plants exhibited reduced root-to-shoot ratios when compared with plants grown in the presence of sufficient K⁺. Furthermore, plant growth under K⁺-deficient conditions did not alleviate the influence of the TMV-MP over resource allocation to the roots. These results are discussed in terms of possible mechanisms by which stress signals could cause an alteration in biomass partitioning between shoots and roots in control and transgenic tobacco plants expressing the TMV-MP.

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Abbreviations: MP, movement protein; SEL, size exclusion limit; TMV, tobacco mosaic virus.

Transgenic tobacco (*Nicotiana tabacum*) plants expressing the MP of TMV exhibit an increase in sugar and starch levels within mature leaves, as well as a reduction in partitioning of biomass to roots, when compared with vector control plants (Lucas et al., 1993; Balachandran et al., 1995; Olesinski et al., 1995). The mechanism(s) through which the TMV-MP affects carbon allocation and biomass partitioning in transgenic tobacco plants has yet to be elucidated. It is well established that in TMV-MP-expressing transgenic tobacco plants plasmodesmal SEL is increased from control values of 800 D to greater than 9.4 kD (Wolf et al., 1989; Ding et al., 1992). Although it was hypothesized that this TMV-MP-induced increase in SEL would result in the enhanced export of fixed carbon out of source leaves, translocation during the photoperiod was actually decreased, with the concomitant increase in the levels of sugar and starch (Olesinski et al., 1995). Transgenic tobacco plants expressing TMV-MP mutants, in which the domain responsible for the increase in plasmodesmal SEL was deleted, further demonstrated that the effect of the TMV-MP on carbon allocation and biomass partitioning was independent of its influence over plasmodesmal SEL (Balachandran et al., 1995). Studies in which a temperature-sensitive mutant of TMV was employed confirmed that the effect

of the TMV-MP on SEL was independent of its effects on carbon allocation (Olesinski et al., 1995).

A hypothesis advanced to account for the TMV-MP-induced changes in carbon partitioning and allocation suggests that the TMV-MP interferes with an endogenous signal transduction pathway involved in regulating biomass partitioning between shoots and roots (Balachandran et al., 1995; Lucas et al., 1996). Partitioning of photosynthetically fixed carbon among various organs of a plant depends on the physiological and developmental stages of the organs, and it is well known that environmental growth conditions can influence this partitioning (Wardlaw, 1990; Geiger and Servaites, 1991; Mooney and Winner, 1991). However, the integrated regulatory mechanisms involved in root-to-shoot communication remain to be elucidated (Wardlaw, 1990). In this regard, TMV-MP-expressing tobacco plants appear to represent an ideal experimental system on which to study the control mechanisms involved in biomass partitioning within the plant.

In response to environmental cues, plants readjust partitioning of photosynthates to maintain a functional equilibrium between shoot and root growth, since they are interdependent (Mooney and Winner, 1991). In this study we investigated the effects of environmental factors such as water stress and nutrient deficiency (N, P, and K'), both well known to influence biomass partitioning between roots and shoots, on TMV-MP-expressing tobacco plants. Under the imposed stress, total plant dry weight was reduced and the plant responded by partitioning more biomass to its root system (control and TMV-MP lines). Water stress was found to override completely the influence of the TMV-MP over the root-to-shoot ratio. However, imposed nutrient deficiency conditions did not overcome the MP effect. These results are discussed in terms of possible mechanisms by which stress signals could cause an alteration in biomass partitioning between shoots and roots in control and transgenic tobacco plants expressing the TMV-MP.

MATERIALS AND METHODS

Plant Material and Biomass Measurements

Transgenic tobacco (*Nicotiana tabacum*) line 277 expressing the TMV-MP and vector control line 306 (plasmid vector only) were provided by Dr. Roger Beachy of the Scripps Institute (La Jolla, CA). We chose one line of transgenic tobacco plant for the present experiments, since previous studies with a number of independent transformed lines clearly established that expression of the TMV-MP reduces biomass partitioning to roots and alters carbon allocation in all of these transgenic plants (Lucas et al., 1993; Balachandran et al., 1995; Olesinski et al., 1995). Seeds were germinated on a soil mixture and 4-week-old seedlings were transplanted into plastic pots containing sand. These seedlings were grown for 2 more weeks before water stress was imposed, because the small 4-week-old plants were not expected to survive the stress condition. In nutrient deficiency studies 5-week-old seedlings were transferred to glass jars containing the appropriate hydroponic culture medium (N, P, and K'). Plants were either grown in a greenhouse or growth chamber, as described below for specific experiments. At the end of each treatment, plants were harvested, separated into leaves, stems, and roots, and dried in a 70°C oven for 72 h. Root-to-shoot ratios were calculated for each plant based on dry weight. Significance between treatment means was determined by analysis of variance.

Water-Stress Treatments

Six-week-old transgenic tobacco plant lines 277 and 306 were subjected to water stress by withholding water on alternate days. These water-stressed plants received 2X Hoagland solution (Hoagland and Arnold, 1938) once on alternate days, whereas well-watered controls received 1/2X Hoagland solution twice daily; in each situation, water delivery raised the soil to field capacity. Plants were grown in the greenhouse during mid-summer under a 16-h photoperiod. Light intensity at midday was adjusted to 1200 to 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, whereas in the morning and evening the values ranged between 700 and 950 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The temperature regime in the greenhouse was $30^{\circ}\text{C} \pm 2.0/ 22^{\circ}\text{C} \pm 2.0$, day/night. Water-stressed plants exhibited severe wilting of mature leaves and this stress condition was imposed for 23 to 25 d, after which time the plants were harvested to determine dry matter partitioning.

Water-stress studies were repeated three times, and results of one representative experiment are reported.

Nutrient-Stress Treatments

In N-stress experiments, 5-week-old transgenic tobacco lines 277 and 306 were grown in 1-L glass jars filled with a nutrient solution containing 8 mm (high), 5 mm (moderate), 2 mm (low), or 0.5 mm (very low) nitrate-N. All other macro- and micronutrients were maintained at control levels (Lucas et al., 1993). All nutrient solutions were aerated and replaced every other day. Replacement of nutrients every other day was sufficient for plants supplied with both adequate and deficient nutrients. Under nutrient deficient conditions, the nutrient solution was depleted very slowly by both genotypes, thus MP-transgenic plants did not experience less nutrient stress than the control line. Plants were grown in a greenhouse under early autumn conditions. The natural photoperiod (10 h; intensity, 1200-1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) was extended using high-intensity metal halide lamps (600-700 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for an additional 4 h. Plants were grown for 25 d under the specified N regime and then harvested to determine dry matter partitioning, at which time they were 60 d old. These experiments were repeated three times, and results of one representative experiment are reported. Photosynthetic rates were measured on leaf numbers 4 or 5 (leaf 1 being the last leaf to attain 5 cm in length) of high- and low-N grown plants, as described in Balachandran et al. (1995).

P and K' experiments were carried out essentially as described for N-stress studies, except that plants were grown in a controlled environment chamber (model PGV36, Conviron, Permbina, ND; $28^{\circ}\text{C}/22^{\circ}\text{C}$, day/night temperatures with RH maintained at 70%). Light intensity (metal halide lamps) at the beginning (2 h) and end (2 h) of a 14-h photoperiod was set at 100 to 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (at canopy level), whereas in the middle of the period it was increased to 500 to 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Two nutrient levels were used in these studies: 1 mm (high) and 0.05 mm (low), and 7 mm (high) and 0.5 mm (low). Plants were harvested 25 to 27 d after treatment initiation and analyzed for dry matter partitioning. These experiments were repeated twice and representative results from one experiment are reported. Photosynthetic rates were measured on leaf numbers 4 or 5 of high- and low-P-grown plants, as described in Balachandran et al. (1995).

RESULTS

Effect of Water Stress on Biomass Partitioning

Consistent with our earlier studies (Lucas et al., 1993; Balachandran et al., 1995), under well-watered conditions the biomass of TMV-MP-expressing tobacco plants (line 277) was reduced in comparison with vector control (line 306) plants. From the data presented in Table 1, it is clear that the major effects of the TMV-MP are exerted on the stem and root tissues, with the latter being reduced to 40% of the root mass present in control plants. As a consequence, plant line 277 had a root-to-shoot ratio that was 38% smaller than the value measured for plant line 306. We have previously established that the decrease in root-to-shoot ratio in TMV-MP-expressing tobacco plants, when compared with control plants, is observed at various developmental stages between the two genotypes (Lucas et al., 1993). Under the water-stress regime employed in the present experiments, in which the turgor within source leaves was allowed to decline to the point where these leaves underwent wilting, overall growth of both plant lines (277 and 306) was reduced to approximately 60% of that observed under well-watered conditions (Table 1). It is interesting that, whereas root biomass of water-stressed control plants decreased by 30%, the value remained unaltered in plant line 277. As a consequence of this differential response to the imposed water stress, the root-to-shoot ratio in plant line 277 increased to a value comparable to that measured in control line 306. These results, confirmed in three separate experiments, established that water stress results in the activation of a signal that can completely override the effect of the TMV-MP in terms of negating its influence over biomass partitioning to the root.

Effect of N Stress on Biomass Partitioning

As expected, growth of plant lines 306 and 277 decreased as N availability was lowered (Table II). Reducing the available N from 8 to 5 mM had little effect on leaf, stem, or root biomass, whereas in the presence of 2.0 or 0.5 mM N, total plant biomass was reduced significantly (Table II). (Low-N plants exhibited chlorotic leaves, and plant height and leaf number were reduced in both lines [data not shown].) Under 0.5 and 2.0 mM N conditions, biomass partitioning to the roots was reduced less than partitioning to above-ground organs, such that root-to-shoot ratios increased in both lines 306 and 277, when compared with plants that received 5 and 8 mM N. The N-deficiency-induced shift in carbon partitioning to the roots of control line 306 resulted in a 70% increase in the root-to-shoot ratio (0.5 or 2 mM compared with 5-mg-grown plants). A similar response was detected for plant line 277, with the increase being 100 and 83% higher under 0.5 and 2.0 mM N, respectively, compared with 5 mM N conditions. The important finding from these studies was that even though N deficiency induced an increase in the root-to-shoot ratio in TMV-MP-expressing plants, in contrast to the situation with water stress, this form of nutrient stress failed to relieve the influence of the TMV-MP over resource allocation to the roots. This can be seen by comparing root-to-shoot ratios for plant lines 306 and 277 measured on plants grown under the four N levels. For the 0.5, 2, 5, and 8 mM N cultures, root biomass was consistently lower in the TMV-MP-expressing line when compared with the vector control line (Fig. 1), and the root-to-shoot ratios for line 277 plants were smaller than those of the equivalent line 306 plants by 30, 40, 35, and 30%, respectively.

Under N-deficient conditions, photosynthetic rates of leaves of control tobacco plants were only 25% of those grown with high-N nutrition (Table III). Previously, it has been observed

that the photosynthetic rates were comparable between the transgenic line expressing the MP and the vector control line under adequate nutrition (Lucas et al., 1993). Our present studies confirm those results and show that even under N-deficient conditions, the photosynthetic rates were similar between the two lines (Table III).

Influence of P Stress on Biomass Partitioning

Under low-P nutrition, plant growth was similarly reduced in tobacco lines 306 and 277 (Table IV). (Low P-grown plants were reduced in height and number of leaves [data not shown], but they did not display signs of chlorosis.) It is important to note that, for both plant lines, root growth under low- and high-P conditions was identical. Consequently, root-to-shoot ratios underwent a considerable increase in response to reduced levels of available P, being 83 and 55% higher in lines 306 and 277, respectively. However, under this imposed P stress, the root-to-shoot ratio for line 277 still remained 36% below that of control line 306. Thus, both N and P deficiencies elicited a relative increase in carbon allocation to the root system, but these responses did not override the effects of the TMV-MP. As in the case of N nutrition studies, under low-P conditions the photosynthetic rates were comparable between both transgenic tobacco lines 277 and 306, being reduced to 60% of the equivalent lines grown at high-P conditions (Table 111).

Influence of K⁺ Deficiency on Biomass Partitioning

Total plant weight, leaf, stem, and root growth were all reduced in tobacco plants grown under 0.1 mM K⁺ (Table V); except for early senescence of lower leaves, deficiency symptoms were not visible on these plants. Biomass partitioning to the roots was more reduced under these low-K⁺ nutrient conditions, than was partitioning to the shoots. Hence, in these experiments, root-to-shoot ratios actually decreased in plant lines 306 and 277. However, as observed under both N and P deficiencies, the root-to-shoot ratio of plant line 277 remained 30% smaller than control line 306 plants grown in 0.1 mM K⁺.

DISCUSSION

Transgenic tobacco lines expressing the TMV-MP have been extensively characterized with respect to biomass allocation, carbon export from source leaves, and sugar and starch accumulation in leaves of various ages (Lucas et al., 1993; Balachandran et al., 1995; Clesinski et al., 1995). The reduction in root-to-shoot ratio in TMV-MP-expressing tobacco plants, when compared with vector control plants, has been observed at different developmental stages of the plant (Lucas et al., 1993). The aim of the present study was to determine whether the activation of stress-related signals could influence the effects of the TMV-MP on resource allocation in transgenic tobacco plants. The discussion will be confined to this context, with due recognition of the extensive literature pertaining to the interaction between water/nutrient stress and the matrix of effects on both physiological and developmental processes, including adjustments to the set-point for control over the root-to-shoot ratio of such stressed plants (Thornley, 1972; Radin and Eidenbock, 1984; Wilson, 1988; Fredeen et al., 1989; Davies et al., 1990; Mooney and Winner, 1991; Qiu and Israel, 1992; Bano et al., 1993).

Based on the data presented in this study, we have established that the imposition of water stress on TMV-MP-expressing tobacco plants can alleviate the influence exerted by the TMV-MP over carbon allocation to the roots. Under the water-stress regime employed in the present experiments, total biomass production was reduced equally (to 60% of the well-watered controls) in TMV-MP-expressing and vector control plants (Table I). However, the pattern of biomass partitioning was altered significantly in water-stressed tobacco plants expressing the TMV-MP. The increase in the root-to-shoot ratio that occurred in these water-stress experiments was achieved by a shift in resources from the stems to the root system. This can be seen by comparing the percentage of dry matter partitioned to the stems and roots of these plants under control (well-watered) and water stress growth conditions. Under control conditions, 38% of the total plant dry weight was present in the stems, with only 7% in the roots; stem and root values for plant line 306 were 41 and 12%, respectively. In contrast, under water-stress conditions, only 29% of the total plant dry weight was present in the stems, whereas 13% was present in the roots; stem and root values for plant line 306 were 37 and 14%, respectively.

It is important to note that, although water stress caused the anticipated reduction in total leaf biomass, both lines of stressed plants retained approximately the same level of biomass partitioning in the leaves (shoots) as occurred in the well-watered treatments. This suggests that the tobacco plant may utilize two carbon pools: one that delivers resources to the vegetative meristem and leaves (shoot associated pool), and a second that serves the stem and root systems. Although we have established that the imposition of water stress activates (or alters) a signal transduction cascade that can override the influence of the TMV-MP over resource allocation to the roots, the components of this pathway have yet to be elucidated.

The complexity of the interaction between the TMV-MP and the endogenous resource control network(s) is further illustrated by our finding that water stress failed to override the TMV-MP-induced increase in carbon allocation to the shoot-associated pool. For example, of the total plant biomass produced under well-watered conditions, plant line 306 allocated 47% to this pool, whereas the value for plant line 277 was 57% (see leaf dry matter in Table 1); under water-stress conditions the values were 50 and 60%, respectively. The earlier studies that we performed on transgenic tobacco lines expressing mutant forms of the TMV-MP established that the TMV-MP is responsible for the observed changes in allocation to these two pools (Balachandran et al., 1995). Deletion of the C-terminal 10 amino acids from the TMV-MP had no effect on its ability to increase plasmodesmal SEL or perform its other viral related functions; however, biomass partitioning to leaves, stems, and roots in plants expressing this mutant MP was identical to plant line 306.

Imposed N and P deficiencies also elicited a shift in resource allocation that gave rise to an increase in the root-to-shoot ratio (Tables 11 and IV). As observed for water-stressed tobacco plants, this increase was achieved by a shift in the allocation of resources from the stem to the roots. In the N experiments this shift in resource allocation appeared to involve a threshold level of available N, in that plant lines 306 and 277 grown under 5 or 8 mM N partitioned 9 and 6% of their total biomass to their roots, respectively, whereas under 0.5 or 2 mM N the values were 14 and 9%, respectively (Table 11). It is interesting that under P-stress conditions, root growth was maintained at the same level observed in plants supplied with sufficient P, whereas leaf and stem growth were reduced. This suggests that the factors regulating biomass partitioning to roots are either spatially or functionally separated from those involved in biomass partitioning to the vegetative meristem and leaves (shoot system). The influence of the TMV-MP, in terms of increasing the proportion of biomass allocation to the vegetative meristem and leaves, was similarly unaffected by a nutrient deficiency of either

N or P (Tables 11 and IV). The physiological limitations imposed by growing plants under 0.5 compared with 8 mm N is illustrated by the significant reduction in total plant weight for the low-N situation (30% of 8 mm N-grown plants). The constancy of the effect of the TMV-MP on the allocation between the two carbon pools may reflect an endogenous target site that serves a crucial role in the resource signaling cascade. Clearly, the input from this signal must be at a higher level than signals produced in response to nutrient deficiency. On this same theme, the inability of line 277 N- and P-stressed plants to reset their root-to-shoot ratios to control values likely reflects the hierarchical position of the TMV-MP-associated signal relative to nutrient-stress-induced signals.

The reduction in biomass partitioning to roots in TMVMP-expressing plants is not due to an alteration in carbon allocation into starch that is then re-allocated preferentially to the young, growing leaves (Huber, 1983), because the levels of sugars and starch in young, growing leaves were comparable between the transgenic line expressing the MP and the control line (Lucas et al., 1993). During the onset of N deficiency, new leaf initiation and expansion were much more sensitive to leaf N than was photosynthesis, and therefore more carbohydrates would have become available for export to the roots (Tolley-Henry and Raper, 1986, 1991). Photosynthetic rates of source leaves of transgenic tobacco plants expressing the TMV-MP and vector control plants were comparable under nutrient-deficient (N and P) conditions (Table 111). Similarly, under high-nutrient conditions, photosynthetic rates were not significantly different between lines 277 and 306 (Lucas et al., 1993). Although the root-to-shoot ratio is increased in MP-expressing transgenic plants grown under low-N, compared with high-N grown plants, biomass partitioning to the roots was still lower than that of low-N-grown control plants. Therefore, the mechanisms through which carbon supply to the roots was reduced in TMV-MP-expressing plants supplied with high N must still prevail during growth under deficient N conditions.

Our K'-deficiency experiments yielded interesting results. Under limiting K' conditions, biomass partitioning to the roots was reduced, with a concomitant decrease in root-to-shoot ratios compared with the same plant lines grown under a high level of K'. A reduced root-to-shoot ratio in low-K'-grown plants has been reported previously (Cakmak et al., 1994a). This effect might be related to the enhancement of photoassimilate export, which occurs when high levels of K' are present in source leaves (Conti and Geiger, 1982; Cakmak et al., 1994b). Plants grown under low-K' levels would likely have reduced K' concentrations in their source leaves, which could retard the rate of phloem loading, thereby leading to the observed reduction in export in K'-stressed tobacco plant lines 306 and 277. The data presented in Table V indicate that such a change in export must have occurred in a preferential manner, since a reduction in carbon partitioning was detected only in the roots. Finally, the root-to-shoot ratio of TMV-MP-expressing plants remained approximately 30% below that of low-K'-grown vector control plants. Because similar results were obtained in N- and P-deficiency experiments, it is probably safe to assume that all nutrient deficiency signals involved in readjusting resource allocation at the whole-plant level act at a level below that of the TMV-MP.

Plant Control over Resource Allocation

Clearly, plants have evolved efficient signaling systems that operate between shoots and roots to control developmental and physiological processes occurring within these physically separated organs. Although much is known in terms of the manner in which this signaling network permits the plant to interact with and respond to environmental input signals, very

little is known in terms of the specific molecular constituents that comprise this "long-range" signaling pathway. Obviously, certain hormones that are synthesized in the root and then translocated via the xylem translocation stream to their site of action in the shoot (Vaadia and Itai, 1969; Wilson 1988; Farrar, 1992) represent one form of input signal (for review, see Jackson [1997]). Likewise, hormones moving in the phloem could establish a communication link between source leaves and developing plant organs. However, the established complexity of the resource allocation system of the plant, including recent findings that source leaves in some way control direction and levels of allocation to specific tissues (Minchin et al., 1994; Balachandran et al., 1995), implicate the involvement of novel signaling molecules.

We recently advanced the hypothesis that macromolecules (proteins or ribonucleo-protein complexes) may act as signal molecules in controlling biomass partitioning in plants (Balachandran et al., 1995; Lucas et al., 1996; Wolf and Lucas, 1997). This model was based on the recent findings that specific viral and plant proteins have the ability to move from cell to cell via plasmodesmata (Lucas and Gilbertson, 1994; Lucas et al., 1995). Now, it has also been established that the 100 or more proteins present in the enucleate sieve elements of the higher plant phloem (Fisher et al., 1992) have the capacity to interact with plasmodesmata to mediate their own cell-to-cell transport (S. Balachandran, Y. Xiang, W.J. Lucas, unpublished results). These findings support the hypothesis that plasmodesmal trafficking of macromolecules could establish a dual-level information network, which functions between the companion cell-sieve element system and the neighboring mesophyll (local regulation) and distant organs (long-distance regulation via the phloem translocation stream) (for review, see Lucas et al. [1996]; Wolf and Lucas, 1996). Such a system, in conjunction with the normal array of plant hormones, may permit the plant to control its developmental and physiological processes at a supracellular level. The challenge will be to develop appropriate methods to identify the components of the signaling pathway involved in regulating resource partitioning at the whole-plant level (Stitt, 1996).

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Table I. Influence of water stress on biomass partitioning in transgenic tobacco plant expressing the plasmid vector control (line 306) and the TMV-MP (line 277).

Data represent means $\pm$ SE (n = 6).

Treatment	Genotype	Dry Matter			Root-to-Shoot Ratio	
		Total plant wt	Leaves	Stems		
g						
Well-watered ^b	306	25.8±0.8	12.1±0.3	10.6±0.4	3.1±0.1	0.13±0.003 ^c
Well-watered	277	18.8±0.4	10.8±0.3	7.1±0.4	1.3±0.1	0.08±0.003 ^c
Water-stressed ^d	306	16.0±0.5	8.1±0.3	5.7±0.2	2.2±0.1	0.16±0.002 ^e
Water-stressed	277	10.8±0.6	6.4±0.4	3.1±0.2	1.4±0.1	0.15±0.005 ^e

^aPlants were 65 to 68 d old at the time of harvest.

^bPlants were supplied with 0.5X Hoagland nutrientent solution twice daily.

^cSignificantly different at P = 0.01.

^dPlants were supplied with 2X Hoagland solution on alternate morning.

^eNot significantly different at P = 0.01.

Table II. Influence of N deficiency on biomass partitioning in transgenic tobacco expressing the plasmid vector control (line 306) and the TMV-MP (line 277).

Data represent means $\pm$ SE (n = 6).

Treatment	Genotype	Dry Matter				Root-to-Shoot Ratio
		Total plant wt	Leaves	Stems	Roots	
g						
8 mM Nitrate	306	15.8±1.1	8.7±0.7	5.8±0.5	1.4±0.1	0.10±0.005 ^b
8 mM Nitrate	277	9.5±0.5	6.2±0.4	2.7±0.2	0.6±0.06	0.07±0.004 ^b
5 mM Nitrate	306	13.2±0.5	7.4±0.4	4.7±0.5	1.2±0.1	0.10±0.006 ^b
5 mM Nitrate	277	7.9±0.7	5.4±0.5	1.2±0.2	0.5±0.07	0.06±0.005 ^b
2 mM Nitrate	306	6.5±0.7	4.1±0.5	1.5±0.2	0.9±0.1	0.17±0.005 ^c
2 mM Nitrate	277	5.5±0.8	3.6±0.4	1.3±0.2	0.5±0.06	0.11±0.01 ^c
0.5 mM Nitrate	306	4.9±0.4	2.8±0.4	1.3±0.3	0.7±0.06	0.17±0.008 ^c
0.5 mM Nitrate	277	2.7±0.4	1.7±0.3	0.7±0.13	0.3±0.04	0.12±0.007 ^c

^aPlants were 60 d old at the time of harvest. ^bSignificantly different at P = 0.05.

^cSignificantly different at P = 0.001.

Table III. Photosynthetic rates of mature source leaves of high N- and high-P, low N-, and low P grown transgenic tobacco plants expressing the plasmid vector control (line 306) and the TMV-MP (line 277).

Data represent means $\pm$ SE (n = 4-8).

Genotype	Photosynthetic Rate ^a		
	High nitrate and High phosphate ^b	Low nitrate ^c	Low phosphate ^d
	$\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$		
306	12.8 $\pm$ 0.2	3.3 $\pm$ 0.1	8.3 $\pm$ 0.4
277	12.3 $\pm$ 0.3	3.5 $\pm$ 0.1	7.1 $\pm$ 0.5

^aPhotosynthetic rate was measured 2 to 4 d before harvesting the plants for biomass partitioning studies.

^bPlasnts were supplied with 8 mM nitrate and 1 mM phosphate.

^{c,d}Plants were supplied with 0.5 mM nitrate and 0.05 mM phosphate, respectively.

Table IV. Influence of P deficiency on biomass partitioning in transgenic tobacco plant expressing the plasmid vector control (line 306) and the TMV-MP (line 277).

Data represent means $\pm$ SE (n = 6).

Treatment	Genotype	Dry Matter				Root-to-Shoot Ratio
		Total plant wt	Leaves	Stems	Roots	
				g		
High phosphate ^b	306	15.3 $\pm$ 0.9	8.9 $\pm$ 0.6	4.7 $\pm$ 0.3	1.7 $\pm$ 0.08	0.12 $\pm$ 0.004 ^c
High phosphate	277	13.4 $\pm$ 1.4	8.0 $\pm$ 0.9	3.7 $\pm$ 0.5	1.0 $\pm$ 0.1	0.09 $\pm$ 0.001 ^c
Low phosphate ^d	306	8.8 $\pm$ 0.5	5.0 $\pm$ 0.3	2.2 $\pm$ 0.1	1.6 $\pm$ 0.09	0.22 $\pm$ 0.001 ^c
Low phosphate	277	8.5 $\pm$ 0.9	5.4 $\pm$ 0.6	2.1 $\pm$ 0.3	1.0 $\pm$ 0.08	0.14 $\pm$ 0.01 ^c

^aPlants were 60 to 62 d old at the time of harvest.

^bPlants were supplied with 1 mM and 0.05 mM phosphate, respectively

^cSignificantly different at P = 0.05 and 0.01, respectively.

Table V. Influence of K⁺ deficiency on biomass partitioning in transgenic tobacco expressing the plasmid vector control (line 306) and the TMV-MP (line 277).

Data represent means $\pm$ SE (n = 6).

Treatment	Genotype	Dry Matter			Root-to-Shoot Ratio	
		Total plant wt	Leaves	Stems		
				g		
High K ^{+b}	306	14.2±0.6	7.2±0.4	5.3±0.3	1.7±0.04	0.14±0.003 ^b
High K ⁺	277	12.3±0.7	6.7±0.5	4.6±0.3	1.0±0.05	0.09±0.004 ^b
Low K ^{+b}	306	9.8±0.4	5.3±0.3	3.7±0.1	0.8±0.06	0.09±0.005 ^c
Low K ⁺	277	8.2±0.4	4.6±0.3	3.1±0.1	0.5±0.03	0.06±0.004 ^c

^aPlants were 60 to 62 d old at the time of harvest.

^bPlants were supplied with 7 mM and 0.01 mM K⁺, respectively.

^cSignificantly different at P = 0.001 and 0.01, respectively.

Figure 1. Relationship between root biomass and N supply for experiments performed using transgenic tobacco plants expressing the TMV-MP (line 277) and vector control plants (line 306).

Effect of Tobacco Mosaic Virus Movement Protein on Photosynthesis in Transgenic Tobacco Plants

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Numerous studies have demonstrated the negative influence of viral infection on leaf photosynthetic activity (Goodman et al., 1986; Fraser, 1987). Systemic viral infection often causes mosaic symptoms and/or necrotic lesions, indicative of structural changes in the chloroplasts, altered carbon metabolism and the accumulation of starch grains. Higher chlorophyllase activity has been demonstrated after infection with the tobacco mosaic virus (TMV) resulting in significant reductions in chlorophyll content (Goodman et al., 1986).

A direct interaction between virally encoded protein and the photosynthetic system was first demonstrated in the case of the TMV coat protein (CP). In these studies the TMV-CP was found to be associated with thylakoids in tobacco plants infected with TMV (Hodgson et al., 1989; Reiner and Beachy, 1989). These infected plants showed inhibition of photosynthetic electron transport through PS II, and the authors suggested that this virally-imposed inhibition of photosynthetic activity resulted from the association between the viral CP and the PS II complex.

Evidence accumulated in recent years suggests that, in addition to its direct effect on the photosynthetic system, viral infection influences the process of sucrose transport and carbon partitioning within the source leaves (Tecsı et al., 1995). Interestingly, infection of pea plants with red mottle comovirus caused a significant increase in the starch content of leaves far from the infection sites, where viral particles were still absent (Tecsı et al., 1992). These results indicate that the viral infection's influence on carbon metabolism is not due to simple interference in the biosynthesis pathway, but rather to a process mediated by information perceived by distant tissues.

It is now well established that most plant viruses encode for protein(s) essential for the traffic of viral particles from the site of infection to surrounding cells. Expression of these movement proteins (MP) in transgenic plants and direct microinjection of bacterially produced MP established that these proteins can alter the plasmodesmal function (Wolf et al., 1989; Waigmann et al., 1994). Further studies with transgenic plants expressing viral MPs demonstrated that in addition to its effect on plasmodesmal size exclusion limit, TMV-MP significantly alters the rate of sucrose export and the partitioning of carbohydrates in source leaves (Wolf and Lucas, 1997). Analyses of carbon metabolism in source leaves expressing TMV-MP showed the levels of sucrose, glucose, fructose and starch to be significantly higher than those present in equivalent control leaves (Lucas et al., 1993). Moreover, analyses of ^{14}C export following pulse-chase experiments indicated a lower rate of sucrose export during the photoperiod in TMV-MP-expressing source leaves than in control leaves (Olesinski et al., 1995).

In view of the close interaction between the photosynthetic apparatus and carbohydrate metabolism, the first aim of this study was to determine the effect of TMV-MP on the photosynthetic activity of source leaves. Sucrose-accumulation-mediated reduction in leaf Pi level has been suggested as a key factor controlling the photosynthetic rate (Foyer, 1988). The second aim of this study was to verify whether differences in Pi level could explain the TMV-MP-mediated influence on photosynthetic rate.

Materials and Methods

Plant material

Transgenic tobacco plants (*Nicotiana tabacum* L. cv. Xanthi) expressing the TMV-MP were kindly provided by Dr. Roger Beachy (Scripps Institute, La Jolla, CA). Transgenic lines 274, 277 (Xanthi nn) 2004 and 2005 (Xanthi NN) expressed the TMV-MP, while lines 306 (Xanthi nn) and 3001 (Xanthi NN) contained only the plasmid vector (Deom et al., 1987, 1991). Three-week-old seedlings were transferred to plastic pots (15 cm diameter) and grown in an

insect-free temperature-controlled greenhouse (approx. 25 °C day, 18 °C night). Plants were grown under natural sunlight with a midday average photon flux density (PFD) of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and fertilized every other day with a 0.5 % solution of 20:20:20 fertilizer (containing 20 % each of N, P and K). Transgenic plant lines 274 and 277 were homozygous for the TMV-MP (Deom et al., 1990), while lines 2004 and 2005 were heterozygous (Deom et al., 1991). A nopaline-synthase bioassay was performed on a sample of leaf tissues of lines 2004 and 2005 to select the positive plants expressing the TMV-MP gene (Wolf et al., 1991).

Photosynthesis measurements

All measurements were performed on leaves 5 and 6 of 8-week-old plants (12-15 nodes), where leaf 1 was defined as the last expanding leaf to achieve a laminar length of 5 cm. CO_2 uptake was determined under natural conditions using a portable, closed infra-red gas-exchange system (model LI-6200; Li-Cor, Lincoln, NE, USA) as described previously (Lucas et al., 1993).

Fluorescence measurements

Chlorophyll fluorescence was measured using a PAM-200 fluorometer (Waltz, GmbH, Germany). Minimal fluorescence (F_0) was determined after incubating the leaf in the dark for 15 min. The leaf was then exposed to a pulse of saturating light to determine maximal fluorescence (F_m). Variable fluorescence (F_v) was calculated as the difference between these two parameters.

The quantum yield (Y) was calculated as: $Y = (F_m' - F_t) / F_m'$, where F_m' and F_t are maximal and actual fluorescence at a given time point, respectively.

Non-photochemical quenching (Q_n) was calculated as:

$Q_n = (F_m - F_m') / F_m - F_0'$, where F_0' is the minimal fluorescence at a given time point (determined after exposing the leaves to far-red light).

The electron transfer rate (ETR) was calculated as: $\text{ETR} = Y * 0.5 * 0.84$, based on the assumption that 84 % of the photons are absorbed by the leaf and two photons are required to transfer one electron through the two photosystems.

In intact leaves, ETR was determined on the youngest fully expanded leaf. Leaves were detached under water and immediately inserted into test tubes containing 50 mL of either 33 mM KH_2PO_4 or H_2O as a control. Measurements were performed once the leaves had reached a steady state under a PFD of 950 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Sensitivity to photoinhibition was determined after exposing the leaves to a PFD of 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 5 min. After 10 min of incubation in the dark, F_m' was measured and the F_m' / F_m ratio was calculated. These measurements were performed using a MFMS fluorometer (Hansatech, Norfolk, UK).

Chlorophyll and Pi content

Leaf discs punched from the selected leaves were weighed and then ground in a glass tissue homogenizer in 10 mL of N,N-dimethylformamide. After storage for 48 h in the dark at 5 °C, the absorbance of the supernatant was measured at 647 and 664 nm, and chlorophyll content computed according to Moran (1982).

Pi content was determined in leaf samples after removing the veins. Dried samples (100 mg) were dissolved in sulfuric acid (0.8 M) and Pi concentration was measured using an atomic specflame (Spectro Kleve, Germany).

Results

The photosynthetic rate of TMV-MP expressing lines was significantly ($P=0.05$) lower than the control lines (Fig. 1). This phenomenon was evident in several plant lines expressing the TMV-MP under two genetic backgrounds, indicating that the effect of the TMV-MP on the photosynthetic system is not due to a position effect of the insert in the genome of the transgenic plants.

Chlorophyll content was similar in the examined leaves of all plant lines. Similar values were obtained for chlorophyll a and b (Table 1). Chlorophyllids content was less than 1% of the total chlorophyll with no differences among the different plant lines (data not shown). Further analyses included the minimal and maximal fluorescence of leaves after 15 min in the dark, and the variable-to-maximal fluorescence ratio (F_v/F_m). All of these parameters were found to be similar for all four examined lines (Table 1).

The size of the chlorophyll's antenna in PS II was estimated by exposing leaves to pulses of light of increasing intensity. As indicated in Fig. 2, the distance between the point at which the lines cross the Y axis and the origin was similar for plant lines 306 and 277 (Fig. 2A) and for plant lines 3001 and 2005 (Fig. 2B). These results indicated that the chlorophyll's antenna size is not affected by TMV-MP expression. Susceptibility to photoinhibition was also similar for TMV-MP- expressing and control plant lines (Table 2). The maximal chlorophyll fluorescence, following exposure of the leaves to high light intensity ($2000 \mu\text{mol m}^{-2} \text{s}^{-1}$), was reduced to about 60 % (of the original values) in both plant lines.

Fluorescence quenching analysis is a common method of estimating the relative effects of photochemical and non-photochemical factors on the quenching of chlorophyll fluorescence. Non-photochemical quenching (Q_n) can provide a measure of the potential use of ATP and, indirectly, activity of the Calvin Cycle. This parameter was significantly higher ($P=0.05$) in plant line 277 than in control plant line 306 (Table 2). The photosynthetic yield, however, of plant line 277 was significantly lower as compared with plant line 306 (Table 2). Collectively, these results indicate that the TMV-MP-imposed inhibition of photosynthetic rate is due to an alteration in the biochemical, and not the photochemical, processes of the photosynthetic system.

Effect of increased Pi supply

The Pi concentration is one of the major factors involved in controlling the Calvin cycle and the partitioning of carbon between sucrose and starch synthesis (Dietz and Foyer, 1986; Foyer et al., 1987; Stitt et al., 1990). As indicated in Table 3, Pi content in leaves of transgenic plants expressing the TMV-MP (lines 277 and 2004) was significantly lower than in the control lines. Incubation of detached leaves in 33 mM KH_2PO_4 solution caused a significant increase in Pi levels in all plant lines, resulting in similar values for TMV-MP expressing and the respective control lines (Table 3). To verify whether the differences in Pi content could explain the inhibition of the photosynthetic rate, the ETR was measured in intact leaves after external application of Pi. After reaching a steady state in water, ETR values of control plants were higher than those of the TMV-MP-expressing plants (Fig. 3A,B). However, when incubated in Pi solution, a transient increase in ETR levels was observed in TMV-MP expressing lines up to values even higher than those observed for control plants (Fig. 3C,D). It is important to note that the same pattern was observed in different sets of experiments including transgenic plants expressing the TMV-MP under two different genetic backgrounds.

Discussion

Expression of the TMV-MP in transgenic tobacco plants inhibited photosynthetic rate (Fig. 1). A recent study has indicated a 20-50 % lower photosynthetic rate in transgenic tobacco plants expressing the potato leafroll virus MP (MP17) as compared to control plants (Herbers et al., 1997). A higher photosynthetic rate in the youngest fully expanded control leaf relative to TMV-MP-expressing plants has also been observed in our previous studies (Lucas et al., 1993). In both our current and previous studies, the inhibition of photosynthetic rate due to TMV-MP expression ranged from 10 to 20 %. Interestingly, comparisons between the total dry weights of TMV-MP- expressing and control tobacco plants also indicated differences of about 20 % (Balachandran et al., 1995). One could expect that a 10-20 % reduction in photosynthetic rate to cause much higher differences in the final dry weight, if these differences were reflected in the whole photosynthetic area. However, analyses of the photosynthetic rate of leaves of different ages have indicated that the TMV-MP- imposed reduction is reflected only in young mature leaves (Lucas et al., 1993). These results suggest that the differences in total CO₂ assimilation per plant are lower than 10 %, making the differences in final dry weight rather small.

Earlier studies demonstrated a TMV-MP effect on carbon metabolism and on sucrose export from source leaves (Lucas et al., 1993; Balachandran et al., 1995; Olesinski et al., 1995). These studies established that expression of the TMV-MP in transgenic tobacco plants under the control of the CaMV 35S promoter caused the accumulation of sugars and starch during the photoperiod to significantly higher levels than those accumulated in source leaves of control plants. This phenomenon was associated with a lower rate of sucrose export from those leaves. Obviously, the lower photosynthetic rate of TMV-MP-expressing plants vs. controls further supports the conclusion that the accumulation of sugars and starch must be associated with an inhibition in export rate.

Several reports have already documented an interaction between the TMV-CP and the photosynthetic system (Hodgson et al., 1989; Reinero and Beachy, 1989). One of the earliest indications of an interaction between a TMV component and the host chloroplasts in tissues that ultimately become chlorotic was found in chlorophyll fluorescence (Balachandran et al., 1994). These authors suggested that photoinhibition and photooxidation result from the interaction between the TMV-CP and components of photosystem II. The ample data obtained from our chlorophyll fluorescence analyses (Fv/Fm ratio, chlorophyll antenna size, the kinetics of chlorophyll fluorescence and the susceptibility to photoinhibition) indicate that the TMV-MP does not have a direct effect on any of the steps in the electron-transfer chain.

Fluorescence-quenching analysis was employed to estimate the relative roles of the photochemical and non-photochemical factors involved in fluorescence quenching. These studies revealed that the TMV-MP causes a lower photosynthetic yield and higher Qn (Table 2). Collectively, the results presented in this study indicate that the TMV-MP-imposed inhibition of photosynthetic rate is due to an alteration in the biochemical, and not the photochemical, processes of the photosynthetic system.

Earlier studies indicated that Pi can play a key role in the control of photosynthetic activity (Foyer et al., 1987; Stitt et al., 1990). Inhibition of the photosynthetic rate due to sucrose formation was observed following an increase in photoperiod. This inhibition was associated with a Pi deficiency (Foyer, 1988). Based on these results, the author concluded that the decreased photosynthetic rate was caused by a progressive limitation of Pi during the photoperiod. Pi content in leaves of TMV-MP-expressing plants was significantly lower than that in control plants (Table 3). To verify whether these differences could explain the inhibition of the photosynthetic rate, the ETR was measured in intact leaves after external

application of Pi. This set of experiments demonstrated an elevated Pi level in source leaves after external application of Pi. Furthermore, the supplemented Pi alleviated the TMV-MP-imposed reduction in ETR values for two independent transgenic lines in two different experiments (Fig. 3). The transiently alleviation of the TMV-MP imposed reduction in ETR values indicated that Pi shortage may be one of the key factors limiting the photosynthetic rate of TMV-MP expressing plants.

Inhibited photosynthetic rate, increased leaf carbohydrate level and altered root-to-shoot ratio, have been demonstrated in sugar beet plants subjected to phosphate stress (Rao and Terry, 1995). The authors suggested that the main cause for the reduction in photosynthetic rate under phosphate shortage was an inhibition in the regeneration of RuBP, which resulted in reduced sucrose synthesis and starch accumulation. RuBP content was not measured in our study, however, the fact that TMV-MP-expressing plants accumulate both sucrose and starch to high levels (Lucas et al., 1993; Olesinski et al., 1995) indicates that the content/activity of RuBP is not a limiting factor in these plants.

It is not yet clear, why the phosphate level in TMV-MP-expressing plants is lower than that in the controls. The root mass of TMV-MP-expressing plants is known to be significantly lower than that of controls (Balachandran et al., 1995). The limited root mass may result in the inhibition of nutrient uptake, causing low phosphate levels. On the other hand, one should remember that our plants were fertilized regularly and nitrogen levels were similar in the examined leaves of all plant lines (data not shown). These observations suggest that root volume is not a limiting factor for nutrients, including phosphate, uptake. In this case, the shortage of phosphate in the leaves must result from specific inhibition of phosphate uptake.

We have recently advanced the hypothesis that the TMV-MP interferes with elements of a supracellular communication network, which operates to transport signals between the various plant organs (Lucas et al., 1996; Wolf and Lucas, 1997). This "long-range" signaling pathway is essential for the efficient control of development and physiological processes occurring in distant organs. One of the outcomes of this interference may be the inhibition of phosphate uptake and/or transport to source leaves.

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Table 1. Chlorophyll content and chlorophyll fluorescence of TMV-MP-expressing (277, 2005) and vector control (306, 3001) lines of tobacco plants. Experiments were performed using 8-week-old plants grown in a temperature-controlled greenhouse (leaf temperature of 24 ± 1 °C and PFD of $1500\ \mu\text{mol m}^{-2}\text{s}^{-1}$).

Plant line	Chlorophyll content (mg m^{-2})			Chlorophyll fluorescence		
	Chl a	Chl b	Total Chl	F_0	Fm	Fv/Fm
306	230 $\pm$ 84	60 $\pm$ 22	290 $\pm$ 104	0.21 $\pm$ 0.01	1.33 $\pm$ 0.02	0.843 $\pm$ 0.002
277	235 $\pm$ 48	59 $\pm$ 11	294 $\pm$ 58	0.21 $\pm$ 0.01	1.33 $\pm$ 0.05	0.842 $\pm$ 0.002
3001	241 $\pm$ 71	62 $\pm$ 15	303 $\pm$ 86	0.20 $\pm$ 0.01	1.30 $\pm$ 0.06	0.845 $\pm$ 0.003
2005	205 $\pm$ 57	52 $\pm$ 13	257 $\pm$ 70	0.23 $\pm$ 0.01	1.40 $\pm$ 0.03	0.835 $\pm$ 0.003

Table 2. Susceptibility to photoinhibition as the ratio between a leaf's maximal fluorescence values before and after exposure to a PFD of $2000\ \mu\text{mol m}^{-2}\text{s}^{-1}$ for 5 min (F_m'/F_m), quantum yield (Y) and non-photochemical quenching (Qn) in TMV-MP-expressing (277) and vector control (306) transgenic tobacco lines. Measurements were performed on mature leaves (nos. 5 and 6) of 8-week-old plants grown in a temperature-controlled greenhouse. F_m'/F_m was measured where leaf temperature was maintained at 24 ± 1 °C and a PFD of at least $1200\ \mu\text{mol m}^{-2}\text{s}^{-1}$. Y and Qn were measured in the evening hours following a 15-min incubation in the dark and then a 6-min exposure to far-red light. Values indicate mean $\pm$ S.E. of 5 replications. Numbers within lines followed by the same letter are not significantly different from another at the 5 % probability level as determined by the Student's t test.

Plant line	306	277
F_m'/F_m	0.59 $\pm$ 0.03a	0.61 $\pm$ 0.03a
Quantum yield	0.56 $\pm$ 0.01a	0.50 $\pm$ 0.02b
Non-photochemical quenching	0.38 $\pm$ 0.02b	0.44 $\pm$ 0.02a

Table 3. Effect of Pi supply on the Pi content (mg g^{-1}) in source leaves of transgenic tobacco plants expressing the TMV-MP (lines 277,2005) as compared to control leaves (lines 306,3001). Petioles were placed in a 33 mM KH_2PO_4 solution and Pi content was determined before and after 1 h incubation in this solution. Values indicate mean $\pm$ S.E. of 5 replications. Numbers within columns followed by the same letter are not significantly different from another at the 5 % probability level as determined by the Student's t test.

Plant line	Before Pi feeding	After 1 h of Pi feeding
Xanthi nn		
306	6.09 $\pm$ 0.45a	7.93 $\pm$ 0.15a
277	4.15 $\pm$ 0.33b	5.72 $\pm$ 0.72a
Xanthi NN		
3001	5.20 $\pm$ 0.25a	6.29 $\pm$ 0.57a
2005	3.56 $\pm$ 0.49b	5.66 $\pm$ 0.52a

Figure Legends

Fig. 1: Photosynthetic rate of several TMV-MP-expressing (plant lines 2005, 2004, 277 and 274) and control (3001, 306) tobacco plants with different genetic backgrounds. Plant lines 3001, 2004 and 2005 are Xanthi NN, while plant lines 306, 277 and 274 are Xanthi nn. Experiments were performed on mature leaves (nos. 5 and 6) of 8-week-old plants grown in a temperature-controlled greenhouse. Leaf temperature was maintained at 24 ± 1 °C and a PFD of at least $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Fig. 2: Estimation of the chlorophyll antenna size in Xanthi nn tobacco plant lines 277 and 306 (A) and Xanthi NN lines 2005 and 3001 (B). Plant lines 277 and 2005 expressed the TMV-MP while lines 306 and 3001 were their respective controls. Excitation PFDs were 500, 1000 and $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Experiments were performed on mature leaves (nos. 5 and 6) of 8-week-old plants grown in a temperature-controlled greenhouse. Leaf temperature was maintained at 24 ± 1 °C and a PFD of at least $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Fig. 3: Electron transfer rate (ETR) of Xanthi nn tobacco plant lines 277 and 306 (A,C) and Xanthi NN lines 2005 and 3001 (B,D). Plant lines 277 and 2005 expressed the TMV-MP while lines 306 and 3001 were their respective controls. Measurements were performed on intact leaves after feeding with water (A,B) or 33 mM KH_2PO_4 (C,D). Experiments were begun after leaves had reached a steady state under a PFD of $950 \mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were grown in a temperature-controlled greenhouse at 25/18 °C (Day/night) and an average daily PFD of $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$, and were fertilized every other day with 0.5 % 20:20:20 fertilizer (containing 20 % each of nitrogen, potassium and phosphorus).

Figure 1.

Figure 2.

Figure 3.

Interaction between phloem proteins and viral movement proteins

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Evidence accumulated over recent years supports the hypothesis that long-distance signals are involved in the regulation of newly fixed carbon metabolism and resource allocation among the various plant organs. According to this model, trafficking of regulatory (information) molecules through plasmodesmata (PD) establishes a supracellular communication network between the companion and mesophyll cells, which operate to regulate the process of sugar transport and carbon partitioning (Lucas and Wolf 1999; Oparka and Turgeon 1999). First experimental support for this concept was provided by studies with virally infected plants. Analysis of the carbon metabolism of cucumber mosaic virus (CMV)-infected plants revealed altered starch synthesis in tissues far from the cells in which virus particles were present (Tecsı *et al.* 1996). These studies established that CMV-imposed alterations in carbon metabolism are independent of viral replication and accumulation in a specific tissue.

Molecular studies established that most plant viruses encode a protein (movement protein [MP]) that is essential for their movement through PD from the site of replication to surrounding, uninfected cells (Gilbertson and Lucas 1996; Lazarowitz and Beachy 1999). These MPs can bind viral nucleic acids and increase the PD size exclusion limit (SEL), a mechanism thought to control transfer of the viral genome to the adjoining cell (Citovsky *et al.* 1992). Constitutive expression of the tobacco mosaic virus (TMV)-MP in transgenic tobacco plants resulted in accumulation of soluble sugars and starch in source leaves during the light hours (Olesinski *et al.* 1995). An even more significant change in phenotype was a reduction in root mass, resulting in a twofold decrease in root-to-shoot ratio (Balachandran *et al.* 1995). Grafting experiments and analyses of transgenic plants expressing various mutants of the TMV-MP indicated that its influence on carbon allocation is independent of its alteration of the PD SEL (Olesinski *et al.* 1995). Furthermore, alteration in sugar levels of source leaves was also evident in transgenic potato plants expressing the TMV-MP in specific tissues (Olesinski *et al.* 1996; Almon *et al.* 1997). When expressed under the control of a phloem-specific (*rolC*) promoter, the TMV-MP elicited a daily accumulation of carbohydrates in source leaves and an alteration in the ratio between tuber and shoot dry weight (Almon *et al.* 1997). The fact that virally encoded proteins can exert a profound effect on sugar metabolism and photoassimilate allocation, at sites distant from their expression, indicates that information molecules can traffic between source leaves and distant sink organs. Based on this assumption, we proposed that interaction of the viral MP with plant endogenous factors interferes with the routine communication network operating at the whole plant level (Lucas *et al.* 1996; Wolf and Lucas 1997).

Several attempts were made to identify plant endogenous proteins that interact with the TMV-MP. Some were aimed at identifying plasmodesmal proteins and therefore the MPs were extracted with the cell wall fraction (Deom *et al.* 1990). The fact that the TMV-MP is insoluble may partially explain the lack of success in attempts to characterize protein-protein interactions using common methods, such as the yeast two-hybrid system or a reaction with expression libraries.

The far-western method has been employed to identify diverse protein-protein interactions such as transcriptional regulators with histones (Edmondson and Roth 1998), subunits of eukaryotic initiation factors (Kimball *et al.* 1998) and keratin intermediate filaments with desmosomal proteins (Kouklis *et al.* 1994).

In the present study, we used several unique approaches to identify interaction between TMV- or CMV-MPs and endogenous proteins of cucurbit plants. We identified, for the first time, several phloem proteins that specifically interact with viral MPs. This reaction may be

one of the causes of symptom development in virally infected plants and/or may play a role in the MP-induced alteration of carbon metabolism and resource allocation in transgenic plants.

Materials and methods

Plant material

Cucurbit plants: melon (c.v. Hales Best Jumbo (HB)) and squash (c.v. B26), were grown in a coconut mixture in 15-cm diameter plastic pots in a controlled greenhouse with a temperature of 25-28°/18-20°C (day/night). The melons inoculated with CMV were grown in a temperature-controlled chamber (Model F15 Conviron, Asheville, NC, USA) with a 12-h photoperiod and 25/18°C day/night temperatures.

CMV inoculation

When the plants were 3 weeks old and had four developed true leaves, the oldest (fourth) leaf was inoculated with CMV (strain Fny) using carborundum as an abrasive and 500 µl inoculum containing 40 µg virus. Control plants were subjected to a similar inoculation procedure with H₂O. To verify the presence of virus particles in specific leaves, leaf discs were collected for either coat protein or RNA analyses.

Purification and blotting of the cucurbit phloem-sap proteins

Phloem exudate (sap) was collected from scalpel-cut petioles using 20 µl microcapillary pipettes. The collected sap was immediately transferred to a micro-tube containing 0.5% β-mercaptoethanol and 50 mM Tris/HCl pH 7.5. Sample buffer was added to the extract and was boiled for 3 min followed by separation by 12% SDS-PAGE. The proteins were partially renatured by washing the gel three times for a total of 30 min in transfer buffer without SDS and blotted onto nitrocellulose (Towbin *et al.* 1979).

Far-western procedure and in vitro translation

The CMV- and TMV-MPs were cloned in a pET vector and expressed in *Escherichia coli* BL-21 using IPTG. Purified inclusion bodies from the expressed bacteria were denatured and renatured using standard protocols. The MPs were used to react with the phloem-sap proteins (PSPs), which had been blotted onto nitrocellulose using a custom-made strip-blotter to separate between lanes, followed by a specific anti-MP antibody. Goat anti-rabbit horseradish peroxidase conjugate was used as a secondary antibody and detection was performed with an ECL detection kit (Amersham, England).

In vitro translation of the CMV-MP and ΔrepA (as a control) was carried out in the same pET vector using the TnT-T₇ coupled transcription/translation system (Promega, USA) and ³⁵S-Met. The translated proteins were reacted with PSPs using the above-described protocol. The interaction was detected by either phosphor-imager or exposure to X-ray film.

Binding of naturally folded phloem proteins to fused TMV-MP

The TMV-MP was fused downstream of the maltose-binding protein (MBP) in a pMAL-c2 vector (New England Biolabs, MA USA) at the BamHI-HindIII sites, thus creating the fused protein MBP-TMV-MP by expression in bacteria. The overexpressed fused protein was bound to amylose resin following the manufacturer's protocol. Melon phloem sap was collected in a capillary tube, transferred to column buffer (20 mM Tris-HCl pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM DTT) at a ratio of 1:10, respectively and centrifuged for 10 min (15,000 g) at 4°C. PSPs were passed through the MBP-TMV-MP amylose resin, followed by

10 column-volume washes with column buffer. The resin was then analyzed by SDS-PAGE using silver staining.

Results

CMV-MP is localized in the phloem sap of infected melon plants

The fact that sugar metabolism and resource allocation are altered in transgenic potato plants expressing the TMV-MP under a phloem-specific promoter suggests that this virally encoded protein may interact with plant proteins localized in the phloem. To verify the generality of MP expression in the phloem sap of virally infected plants, melon plants were infected with CMV by mechanical inoculation. CMV-MP was identified within the phloem sap 3 weeks post-inoculation using immunoblotting with anti-CMV-MP antibodies (Fig. 1). It is important to note that the sap taken from the petiole was not contaminated with other tissues' proteins (see also Ruiz-Mederano *et al.* 1999). As an additional control, the CMV-MP was expressed after insertion of the gene inside the genome of mutated zucchini yellow mosaic virus (ZYMV) vector. Analyses of CMV-MP expression in melon plants infected with this mutated ZYMV indicated massive expression in leaf discs, but no detectable levels in the phloem sap (unpublished data). Collectively, these results established that the CMV-MP is localized within the phloem sap of CMV-infected melon plants and may interact with one or more PSPs.

Interaction between the CMV-MP and PSPs

The far-western procedure was employed as a first means of identifying interactions between CMV-MP overexpressed in bacteria and proteins localized in the phloem sap of melon and squash plants. As indicated in Fig. 2, the CMV-MP interacted with several PSPs. Three specific bands (8, 25 and 47 kDa) were observed after interaction of the CMV-MP with squash sap (lane 1). Reaction of CMV-MP with melon PSP indicated more than three interactions (as compared with the respective control), with five major bands corresponding to proteins with molecular masses of 8, 23, 35, 40 and 45 kDa (lane 3).

Another approach to characterizing protein-protein interactions included *in vitro* translation of CMV-MP with ^{35}S -Met. This method eliminated the possibility of nonspecific cross-reactions, such as between the antibody against the CMV-MP and bacterial proteins or phloem proteins that do not actually react with the CMV-MP, or between the secondary antibody and either protein. The first experiment included phloem sap collected from melon and squash plants. Three squash and four melon PSPs (molecular masses of 8, 47, 65 kDa and 8, 23, 40 and 65 kDa, respectively) were found to interact with the *in vitro*-translated CMV-MP (Fig. 3A). To verify the specificity of these interactions, a second experiment was designed to include several controls. Again, similar protein-protein interactions were observed between PSPs collected from the respective cucurbit plants and the *in vitro* translated CMV-MP (Fig. 3B). In this experiment, an additional melon protein (65 kDa) was found to interact with CMV-MP. A nonrelated viral protein (ΔrepA) was translated *in vitro* as a negative control. As indicated in Fig. 3B, no specific interaction was observed when the *in vitro*-translated ΔrepA was reacted with phloem sap collected from either squash or melon plants. Moreover, no protein-protein interaction was observed after reaction of CMV-MP with bacterial proteins. An additional control included phloem sap collected from wheat (kindly provided by Prof. D. Fisher, Washington State University, Pullman, WA USA). Interestingly, no interaction was observed between CMV-MP and proteins originating from the wheat phloem sap.

Interaction between the TMV-MP and PSPs from melon

Although TMV does not infect cucurbits in nature, we tested the interaction of TMV-MP with phloem sap collected from melon. Interestingly, TMV-MP reacted specifically with three putative melon PSPs with deduced sizes of 8, 23 and 47 kDa (Fig. 4, lane 2). The two controls on the same membrane included reaction of melon phloem sap with bacterial proteins (Fig. 4, lane 1) and with overexpressed CMV-MP (Fig. 4, lane 3). The whole membrane was reacted with anti-TMV-MP secondary antibody.

One limitation of the aforementioned methods of determining protein-protein interactions results from the unnatural structure of the target protein(s). Although the SDS-polyacrylamid gel was washed several times to reduce the amount of the denatured protein, one can assume that this procedure did not completely renature the PSPs. An alternative method was based on interacting natural melon PSPs with the TMV-MP. The TMV-MP was fused to MBP and bound to amylose column. Figure 5 presents the silver-stained proteins bound to either MPB-TMV-MP or MPB amylose columns. Most of the stained proteins probably originated from the bacteria and reflect some degree of adhesion to the amylose resin (lanes 2 and 4). A comparison between lanes 1 and 3 (presenting phloem sap eluted through either MPB-TMV-MP or MPB amylose columns, respectively) indicated that two melon PSPs interact specifically with the TMV-MP. These two proteins had molecular masses of 8 and 23 kDa, similar to the putatively interacting proteins found by the far-western procedure (Fig. 4).

Discussion

It is now established that most, if not all, plant viruses encode for MP(s) that facilitate the cell-to-cell transfer of viral particles (Gilbertson and Lucas 1996). Based on their cellular localization in infected as well as transgenic plants, and their significant modification of plasmodesmal function (for a review see Lazarowitz and Beachy 1999), it is logical to assume that these proteins interact with one or more endogenous plasmodesmal constituents. Identification and characterization of these endogenous factors (proteins) is clearly of major importance; however, to date there has been no report of a plant protein interacting with any viral MP.

Recent studies have indicated the possible involvement of MPs in long-distance viral transport. Long-distance traffic of MP-mutated TMV from the stock to the scion was evident in double-grafted plants only if the wild-type TMV-MP was expressed in the center graft under the phloem-specific (*rolC*) promoter (Arce-Johnson *et al.* 1997). Localization of CMV-MP to the parietal layer of mature sieve elements of infected *Nicotiana clevelandii* (Blackman *et al.* 1998) may also indicate this protein's role in the long-distance movement of the virus. Our finding that CMV-MP is present in the phloem sap of infected melon plants (Fig. 1) further supports the assumption that viral MPs can function within the phloem and interact with PSPs.

Daily accumulation of carbohydrates, as well as often necrotic symptoms, were evident in transgenic tobacco plants expressing MP17, a MP of PLRV, a phloem-restricted luteovirus (Herbers *et al.* 1997). These results, together with the fact that TMV-MP exerts an effect on the physiological function of transgenic potato plants when expressed under a phloem-specific promoter (Almon *et al.* 1997), may postulate an interaction between this protein and plant endogenous molecules that traffic within the phloem stream.

A specific interaction between either CMV-MP or TMV-MP and cucurbit PSPs was established in this study by several independent approaches. An evaluation of phloem sap collected from two cucurbit species revealed that various PSPs interact with CMV-MP.

However, only two squash proteins (8 and 47 kDa) and three melon proteins (8, 23 and 40 kDa) were found to interact specifically, based on both far-western and *in vitro* translation procedures (Figs. 2 and 3, respectively). Three melon PSPs (8, 23 and 47 kDa) were also found to interact with the TMV-MP, despite the fact that melon is not a natural host for TMV (Fig. 4). Moreover, two out of the three proteins (8 and 23 kDa) interacted with the TMV-MP using the more elegant system, in which the phloem proteins do not undergo denaturation. It is important to note that two proteins originating from melon phloem sap (8 and 23 kDa) interacted with both CMV- and TMV-MPs.

Based on the findings that plant endogenous proteins are also involved in plasmodesmal trafficking, it has been suggested that through evolution viruses have acquired specific plant genome sequences encoding different MPs. The first plant paralog to viral MP was recently characterized (Xoconostle-Cazares *et al.* 1999), providing experimental support for this hypothesis. If both the CMV- and TMV-MPs originated from the higher plant endogenous genome, it may very well be that they still share some similar functions, including interaction with the same PSPs. If such a property were preserved in the MP, it would not have to be associated with the virus's ability to infect a specific plant.

It is still unclear how an interaction of phloem proteins with viral MPs can alter the physiological function of the whole plant. Nevertheless, that the phloem sap contains a large number of proteins is now a well-established fact (Fisher *et al.* 1992; Balachandran *et al.* 1997).

As indicated earlier, a *Cucurbita maxima* phloem protein (cmPP16) was characterized as a paralog to viral MP (Xoconostle-Cazares *et al.* 1999). This protein is able to bind its own RNA, as well as other ssRNA, and can modify plasmodesmal function, suggesting its role in the control of RNA transport through the phloem. Assuming that such transport is of importance in terms of plant development and/or function, an interaction of viral MP with a protein(s) such as cmPP16 would clearly alter the physiological function of the whole plant.

It is now evident that higher plant phloem contains a large number of functioning proteins such as thioredoxin, sucrose transporter, PP2, and others (for a review see Schulz 1998). At this stage, we cannot rule out the possibility that MP-imposed alterations in whole-plant functioning are due to disturbance in the activity of such a protein (e.g. transporter) after an interaction with the viral MP.

The current challenge is to characterize those endogenous PSPs and to verify their putative role in the process of phloem transport and resource allocation.

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Figure legends

Figure 1.

Immunolocalization of the CMV-MP in CMV-infected melon plants 3 weeks post-inoculation. **A.** Leaf immunoblot; **B.** phloem immunoblot. **I**-infected; **H**-healthy. Arrows indicate the 30 kDa CMV-MP.

Figure 2.

Interaction between phloem sap proteins of squash (cv. B26) and melon (cv. Hales Best Jumbo (HB)) with CMV-MP expressed in bacteria (lanes 1 and 3, respectively). The control lanes (2 and 4) represent interaction of the respective PSPs with bacterial proteins. Arrows indicate squash PSPs (8, 25 and 47 kDa) and melon PSPs (8 and 23 kDa) that specifically interacted with the CMV-MP.

Figure 3.

Interaction of PSPs with *in vitro*-translated CMV-MP. B26, Squash PSPs; HB, Hales Best Jumbo melon PSPs; Wheat-wheat phloem proteins; Bact-bacterial proteins; B26 and HB in the two right lanes in B indicate interaction of the respective PSPs with *in vitro*-translated Δ repA as an additional control for the left two lanes. Arrows indicate squash PSPs (62 and 47 kDa) and melon PSPs (8 and 23 kDa) that specifically interacted with the CMV-MP.

Figure 4.

Interaction between phloem sap proteins of melon (cv. Hales Best Jumbo (HB)) with the TMV-MP expressed in bacteria (lane 2) as compared with overexpressed bacterial proteins (lane 1) and CMV-MP (lane 3), followed by exposure to anti-TMV-MP antibodies. Arrows indicate PSPs that specifically interacted with the TMV-MP (8, 23 and 47 kDa).

Figure 5.

Interaction between melon (cv. Hales Best Jumbo (HB)) PSPs and TMV-MP fused to maltose-binding protein (MBP). Arrows indicating two PSPs (8 and 23 kDa) that specifically interacted with the TMV-MP-MBP fused protein. Lanes 1 and 3 present phloem sap eluted through MPB-TMV-MP and MPB amylose columns, respectively. Lanes 2 and 4 present silver staining of the resins without elution of phloem sap.

Figure 1. **Figure 2.**

Figure 3.

Figure 4.

Figure 5.

**Cucumber mosaic virus infection affects sugar transport in melon
(*Cucumis melo* L.) plants**

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One of the key factors controlling carbohydrate allocation to the various plant organs is the process of phloem loading. Photosynthetic carbon assimilation and sucrose synthesis take place within mesophyll cells of mature source leaves. The flow of sucrose is governed by cell-to-cell transport through mesophyll cells and vascular tissues, after which it is loaded into the companion cell-sieve element (CC-SE) complex (van Bel, 1993). Phloem loading is defined by Geiger (1975) as "the process by which the major translocated substances are selectively and actively delivered to the sieve tubes in the source region prior to translocation." Structural studies have suggested two possible mechanisms for phloem loading. In most agronomically important crops, the transport of sucrose to the loading region is via the symplasmic pathway, followed by an apoplasmic step to transfer sucrose into the CC-SE complex (van Bel, 1993; Turgeon, 1996). Cucurbits, however, are characterized by the presence of numerous plasmodesmata (PD) connecting the minor veins with the surrounding bundle sheath (BS), suggesting a symplasmic pathway along the entire route from the mesophyll to the CC-SE complex. Based on these structural observations and the fact that the main translocated sugar in cucurbits is stachyose, an alternative model for phloem loading was developed (Turgeon, 1991; Grusak et al., 1996). According to this model, sucrose is synthesized in the mesophyll and then diffuses through the BS into the intermediary cells (IC: a specific type of companion cell in minor veins of cucurbit plants) through the abundant PD that connect the two cell types. Raffinose and stachyose are synthesized from sucrose in the IC (to maintain the diffusion gradient for sucrose). However, these sugar molecules are thought to not be able to diffuse back into the mesophyll because they are larger than the size exclusion limit (SEL) of the connecting PD and as a result, the concentration of raffinose and stachyose rises in the phloem. This hypothesis is based on the assumption that PD between the bundle sheath and IC must be somewhat smaller (400-500 Da) than is common for PD at other interfaces (800-1000 Da) in order to "trap" the oligosaccharides in the IC.

Numerous reports have indicated that carbohydrate metabolism in the source leaf is influenced by viral infection (Tecsi et al., 1994a,b, 1996). Infected source leaves are usually characterized by reduced photosynthetic rate, a decrease in the concentration of soluble sugars, and often starch accumulation (Goodman et al., 1986; Fraser, 1987).

Detailed analysis of the host response to cucumber mosaic virus (CMV) infection was performed in cotyledons of marrow (*Cucurbita pepo* L.) plants (Tecsi et al., 1994a,b). An increase in starch and a decrease in sucrose contents were observed during the first few days after infection. However later on, the infected cotyledons incorporated less of the recently fixed carbon into structural carbohydrates and more into soluble sugars (Tecsi et al., 1994b). Fine-mapping of the lesions formed by CMV in cotyledons of marrow plants was used to define the relationships between viral accumulation, starch content and photosynthetic activity (Tecsi et al., 1994a). This study indicated that viral replication within specific cells initiates a series of metabolic events in the neighboring cells over several days following infection. Interestingly, the alteration in photosynthetic activity and starch accumulation was not found to be a direct consequence of viral replication.

The effect of viral movement proteins (MPs) on PD function is well documented (Carrington et al., 1996; Gilbertson and Lucas, 1996). It is therefore logical to assume that alteration of plasmodesmal function in virally infected plants will affect the process of phloem loading particularly in plants that are determined to be symplasmic loaders. In this paper, we examined the effect of CMV infection on carbon assimilation and transport in melon (*Cucumis melo* L.) plants. We show that viral infection causes a significant

increase in the proportion of sucrose within the phloem. Moreover, this effect was also evident in the phloem sap of leaves in which virus was absent, indicating that the influence over the loading of sugars into the phloem cannot be attributed to a simple interference of virus particles with this process.

MATERIALS AND METHODS

Plant Material

Melon plants (*Cucumis melo* L. cv. Hale's Best Jumbo) were grown in coconut mixture in 15-cm diameter plastic pots. Two-weeks-old seedlings were transferred to a temperature-controlled chamber (Model F15 Conviron, Asheville, NC) with a 12 h photoperiod and 25/18°C day/night temperature. Photon flux density was 200-300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at canopy level. Auxiliary buds were removed as they appeared such that all plants had one main stem. In some experiments the seedlings were transferred to an insect-free, temperature controlled greenhouse (approx. 25/18°C day/night temperature, respectively), with a natural sunlight (midday average photon flux density of 1200-1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

CMV Inoculation

When the plants were 3 weeks old and had four developed true leaves (leaf no. 1 was defined as the last expanding leaf to achieve a laminar length of 5 cm), the oldest (fourth) leaf was inoculated with CMV (strain Fny) using carborundum as an abrasive and 500 μl inoculum containing 40 μg virus. Control plants were subjected to a similar inoculation procedure with H_2O .

To verify the presence of virus particles in specific leaves, leaf discs were collected for either protein or RNA analyses. Proteins were extracted from 0.5 g (fresh weight) of tissue using denaturing buffer (75 mM Tris pH 6.8, 9 M urea, 4.5% SDS, 7.5% mercaptoethanol) at a 1:2 ratio. Proteins were then separated by 12% SDS-PAGE, and blotted onto nitrocellulose paper (Towbin et al., 1979). To detect the CMV coat protein (CP) we used rabbit polyclonal antibodies (kindly provided by Dr. Garcia-Luque, CIB-CSIC, Madrid, Spain) diluted 1:2,000. Goat anti-rabbit horseradish peroxidase conjugate was used as a second antibody, and detection was performed with an ECL detection kit (Amersham, England).

Viral RNA was detected by RT-PCR analysis. Total RNA was extracted from leaf samples (200 mg) with Tri-Reagent (Molecular Research Inc., Cincinnati, OH) following the manufacturer's instructions. cDNA was prepared with specific antisense primer based on the CMV-MP 3' primer (5' GACCGTTAACCACCTGCG 3') followed by PCR analysis with the addition of a CMV-MP 5' primer (5' CCCGAGGCATGGCTTTCC 3'). The PCR product was detected on a 1.2% agarose gel stained with ethidium bromide.

Gas-Exchange Measurements

Net photosynthesis (measured as CO_2 uptake) was determined using a closed, portable, IR gas-exchange system (LI 6200, Li-Cor, Lincoln, NE) as described by Olesinski et al. (1996). All measurements were carried out on the youngest fully expanded leaf (no. 4 or 5) at noon with an initial CO_2 concentration in the chamber of 350 ± 10 ppm; a 30-s measurement was begun immediately after a reduction in CO_2 concentration was detected. For respiration analyses, the chamber was covered with black cloth for 2 min and measurements were begun immediately after an increase in CO_2 concentration was detected. Gross photosynthesis was calculated as the sum of the net photosynthesis

and respiration rates.

Starch and Sugar Determination

Leaf carbohydrate content was determined as described earlier (Olesinski et al., 1996). Briefly, four leaf discs (1 cm² each) were sampled between the small ribs of each leaf. Soluble sugars were extracted in 80% ethanol, and after evaporating the supernatant, sugars were redissolved in H₂O and filtered through a 0.45-μm membrane HPLC filter (Whatman, Maidstone, England). Sugars were separated in an analytical HPLC system (Pump System 320, Kontron, Switzerland) fitted with a Sugar-Pak I column (6.5 mm x 300 mm; Waters, Milford, MA) using an LDC refractive-index detector. Starch content was determined in the ethanol-water-extracted leaf discs following starch conversion by amyloglucosidase (Sigma Chemicals Co. St. Louis, MI). Starch content as glc equivalents was determined using the Sigma (HK) quantitative glc determination kit.

Phloem exudate (sap) was collected from cut petioles using microcapillary pipettes calibrated to 20 μl. The first drops were discarded and the collected sap (5-10 μl) was immediately transferred to an Eppendorf tube containing 300 μl of ice-cold 50 μg ml⁻¹ EDTA in HPLC-grade H₂O. The solution was then centrifuged at 4°C, 10,000 rpm for 5 min and stored at -20°C. Sugars in the sap were determined using the above described HPLC system.

Radioactive Labeling with ¹⁴CO₂

Pulse-chase experiments were conducted according to the procedure described by Olesinski et al. (1995). In short, an attached leaf (no. 5) was sealed into a 4-l Plexiglas chamber where it was held between two layers of nylon monofilament. ¹⁴CO₂ was released into the chamber to give an initial specific activity of about 2•10⁵ Bq mg⁻¹ carbon. After 15 chase min the leaf was released from the chamber and was used for analysis of ¹⁴C-photosynthate export. Total radioactivity of each ¹⁴C-labeled leaf was monitored, for 120 min, using a portable Geiger-Muller tube (RAM-DA; Rotem Model GM-10) that was placed on the adaxial surface of the leaf.

Partitioning of newly fixed carbon within phloem sap sugars was determined 30 and 60 min post-labeling. Sugars were identified and fractionated by HPLC and radioactivity of each fraction was measured in a liquid scintillation counter (Tri-Carb, Packard, Model 1600 TR, Groningen, The Netherlands).

RESULTS

CMV infection alters assimilation rate and carbohydrate metabolism in source leaves

Typical CMV symptoms were observed 8 to 12 days post-infection (DPI). First symptoms were evident in the uppermost leaves, with the systemic infection moving gradually to the lower leaves. It is important to note that in all experiments, the first leaf above the infected one did not exhibit any symptoms even 30 DPI.

Daytime changes in carbohydrate level were analyzed in 4 to 5 week-old plants, 2 weeks after inoculation with CMV. Although discs were taken from the leaf area between the minor veins, it was impossible to completely eliminate the presence of minor ribs in the samples. As indicated in Fig 1, levels of primary sugars (glucose and fructose) were significantly higher in CMV-infected plants than in controls. Similar results were obtained in the morning and afternoon hours. Interestingly, starch levels were lower in the infected plants at both sampling hours, though the differences were not

statistically significant (Fig. 2).

Analysis of photosynthetic activity in leaf no. 4/5 (exhibiting typical mosaic symptoms) indicated no differences in gross photosynthesis between infected and control plants (Table I). However, the values of respiration rate for the infected leaves were almost twice than those for the control plants, resulting in a significant CMV-imposed reduction in net photosynthesis, with values close to zero, in the infected leaves (Table I).

Effect of CMV infection on phloem sap sugar composition

Phloem sap was collected from the petiole of leaf no. 4/5 before the beginning of the light period and after 8 h of photoperiod. Stachyose was found to be the major sugar present in the phloem sap of healthy plants (Fig. 3). Only low levels of sucrose were detected in these petioles, about 20-25% of the stachyose levels. A significant increase in the concentration of sucrose in the phloem sap was evident in petioles of CMV-infected plants. Both at the beginning of the light period and in the afternoon, sucrose concentration was about sixfold that of stachyose. The sucrose to stachyose ratio in the petioles of infected plants was about 20 times higher than that of controls.

To further explore the effect of CMV infection on phloem sap composition, plants were infected at an early stage, just after cotyledon expansion (to ensure infection of the whole plant). Phloem sap was collected from young-mature and old leaves 5 weeks after inoculation. The selected old leaves were the oldest in each plant (nos. 9/10). No significant differences were observed in stachyose or raffinose levels of phloem sap collected from young vs. old petioles (Table II). All sugars were more concentrated (though not statistically significant) in the phloem sap of infected vs. control leaves. However again, the most pronounced effect of the CMV infection was on the level of sucrose, which was over 10 times higher in young infected petioles relative to their respective controls. Interestingly, the rise in sucrose concentration due to CMV infection was relatively small in petioles of old leaves, with no significant differences between the infected and control plants.

A pulse-labeling experiment was performed as an alternative approach to verifying the effect of CMV infection on phloem-sugars composition. This experiment was conducted in a temperature-controlled greenhouse under natural irradiation regimes. Net photosynthetic rate under these conditions was $14.8 \mu\text{mole CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for the control plants, significantly higher than the value obtained for the CMV-infected plants ($7.7 \mu\text{mole CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). Analysis of total radioactivity in the labeled leaves during the first 2h post-labeling indicated a higher rate of reduction in radioactivity in CMV-infected vs. control plants (Fig. 4). It is important to note that the data is presented on the basis of leaf area and that the analyzed source leaves of the infected plants were smaller than noninfected leaves. Therefore, one cannot conclude that the rate of export is higher in CMV-infected plants at the whole plant level.

To better explore the origin of the various phloem sugars, sap was collected from petioles of the labeled leaves 30 and 60 min after labeling and the radioactivity of each sugar was determined. As indicated in Fig. 5, radioactivity level of phloem sugars nearly doubled from 30 to 60 min post-labeling. In control plants, this increase was solely due to the increase in the radioactivity of stachyose and raffinose, while the radioactivity of sucrose remained constant at a low level. However, in CMV-infected leaves, the elevated radioactivity of the phloem sugars was made up of an increase in the radioactivity of stachyose + raffinose and a significant increase (more than double) in the radioactivity of sucrose. These results indicated alterations in the process of newly fixed carbon metabolism and transport in CMV-infected leaves.

The effect of CMV infection on phloem sap sugar composition is independent of the presence of virus particles

Long-distance movement of virus particles is known to occur via the phloem, following the stream of sugar transport (Maule, 1991; Gilbertson and Lucas, 1996). The nonsignificant effect of CMV on phloem sucrose concentration in old leaves (Table II) could have been due to the fact that these specific leaves were too old to be sources. However, it is also possible that they did not contain high levels of virus particles.

Further study was aimed at exploring the relationship between progression of the systemic infection and alteration in sucrose levels in the phloem sap of different leaves. Phloem sap was collected from different leaves at different times post-infection. First symptoms were observed in leaf no. 1/2 10 DPI, at which stage the plants had five to six true leaves. As indicated in Fig 6A, sucrose concentration in the phloem sap collected from petioles of leaf no. 2 was 15 times higher than values determined in control leaves. However, a significant elevation in sucrose concentration was also evident in leaf no. 4, which did not show any typical mosaic symptoms. Protein as well as RNA analyses were employed to verify the presence of virus particles in the latter leaves and, according to both analyses no virus particles were detected. A similar phenomenon was observed when leaves were sampled 16 DPI (Fig. 6B). On this date, the plants had seven to eight true leaves and symptoms were observed in all leaves except for leaf no. 6/7 (located above the infected leaf). Again, a significant increase in sucrose concentration was found in petioles of leaf no. 6, although virus particles could not be detected by either western blot or RT-PCR.

To further investigate whether CMV infection causes other functional alterations in the noninfected leaf (no. 6/7), photosynthetic rate and leaf sugar content were determined in an additional experiment. As indicated in Table III, similar photosynthetic and respiration rates were obtained for leaves of infected and noninfected plants. It is important to note that the differences in the rate of net photosynthesis and respiration in leaf no. 4 (exhibiting typical symptoms) from the same set of plants were similar to those presented in Table II. Moreover, the levels of soluble sugars extracted from infected and control leaves (no. 7) were almost identical (Table IV). Nevertheless, once again, phloem collected from petioles of leaves from the infected plants contained significantly higher levels of sucrose (Fig. 7). The sucrose to stachyose ratio in the sap collected from the infected plant (leaf no. 7) was about 40 times higher than in its respective control.

DISCUSSION

Systemic viral infection often causes mosaic symptoms and/or necrotic lesions, indicative of structural changes in the chloroplasts, altered carbon metabolism and the accumulation of starch grains (Goodman et al., 1986; Fraser, 1987). A gradual decline in net photosynthesis has been observed in cotyledons of marrow plants during the first week after infection with CMV; however, the most pronounced effect observed in the infected cotyledons was a sharp increase in respiration rate (Tecsı et al., 1994b). A significant increase in respiration rate after CMV infection was observed in our study as well (Table I). Interestingly, the gross photosynthetic rate, two weeks post infection, was similar for control and infected source leaves. Significantly higher net photosynthetic rate in control as compared with CMV-infected leaves was observed under natural sunlight in

greenhouse grown plants. Nevertheless, the differences in gross photosynthetic rate were about 20% only. These results indicate that, despite the severe mosaic symptoms observed at this stage of plant development, the potential photosynthetic activity of CMV-infected leaves is relatively high.

CMV infection significantly altered carbohydrate metabolism. A sharp increase in the concentrations of fructose and particularly glucose was observed in the infected leaves. These changes were associated with a decrease in leaf starch content (Figs. 1 and 2). An earlier study indicated an increase in reducing sugars and a reduction in starch content due to CMV-induced higher starch hydrolase and lower ADP-glucose pyrophosphorylase activities (Tecsı et al., 1994b). The inhibition of starch accumulation and/or starch degradation is probably due to the increased demand for soluble sugars (mainly glucose) required to maintain the high respiration rate.

Perhaps the most important effect of CMV infection observed in the present study was the striking alteration in sugar composition within the phloem sap. Similar to data presented in previous reports (Madore, 1991, Mitchell et al., 1992), stachyose was the predominant sugar in the phloem sap of control plants (Fig. 3). As indicated in Figures 3 and 7, CMV infection caused only small changes in the phloem sap stachyose concentration; in contrast, sucrose concentration increased dramatically. The outcome of these changes was a 15- to 40-fold increase in the sucrose to stachyose ratio. Note that the concentration of sugars in the phloem sap collected from cut petioles may not be identical to their concentration *in vivo*. Dilution of the collected sap by solutes and water from the cut surface and xylem elements could reduce the resultant values. None of our samples contained even traces of monosaccharides, suggesting that the collected solute did not contain detectable contamination from neighboring cells. Nevertheless, water delivered from the xylem elements may have produced lower concentration values. However, even if the collected samples were diluted with water, it is important to note that stachyose concentrations were similar for CMV-infected and control leaves, emphasizing the dramatic changes in sucrose concentration.

The increase in sucrose level might be explained by the initiation of stachyose hydrolysis following CMV infection. If this were the case, however, there would be a parallel increase in galactose concentration in the phloem sap. The fact that the alteration in sucrose concentration was not accompanied by detectable changes in other sugars concentration indicates that CMV infection affects the localization of sucrose rather than its metabolism. An analysis of sugar levels in minor veins using a microdissection procedure has indicated that IC may contain considerable amounts of sucrose; however, the authors suggested that this sucrose is localized in the vacuoles (Haritatos et al., 1996). One could argue that CMV infection may have caused a perturbation in the sugars' localization within the phloem cells. As sucrose can easily diffuse from the IC into the SE, an increase in IC cytosolic sucrose concentration would result in a high concentration of sucrose within the phloem sap as well. Assuming that in melon plants, transport of sucrose from the mesophyll to the IC-SE complex is via diffusion through PD, such an increase in IC sucrose concentration would likely result in sucrose leakage back toward the mesophyll, resulting in the inhibition (or even cessation) of phloem loading. The level of radioactivity in source leaves appeared to decline more rapidly in CMV-infected vs. control plants (Fig. 4). This result, together with the fact that the increased radioactivity in phloem total sugar was similar (about twofold) in infected and control plants (Fig. 5), indicated that the export rate of newly fixed carbon is not inhibited in CMV-infected plants.

An alternative explanation for the CMV-induced alteration in phloem sap sucrose

concentration may relate to the functioning of the CMV-MP. A significant increase in plasmodesmal SEL has been observed in transgenic tobacco plants expressing the CMV 3a protein (Vaquero et al., 1994; Ding et al., 1995), indicating that this is the CMV-MP. Based on the model for phloem loading of sugars in cucurbit plants (Turgeon, 1991; Grusak et al., 1996), an alteration in the SEL of PD at the BS-IC interface could result in diffusion of stachyose from the IC to the BS, thereby impairing the process of phloem loading and sugar transport. However, even if CMV-MP or another viral protein altered plasmodesmal functioning in infected melon plants, the observed increase in sucrose concentration within the phloem sap cannot be simply attributed to such changes. On the contrary, an increase in the plasmodesmal SEL would destroy the sucrose "trap" in the IC, as described by Grusak et al. (1996), and inhibit sucrose traffic into the minor veins. Hence, the basis for the change in sucrose concentration may lie in an alteration of the cellular controls that regulate phloem loading via the symplasmic and apoplastic routes. Recall that, in symplasmic loaders, apoplastic and symplasmic loading mechanisms may function concurrently in the same plant. (van Bel, 1993; Grusak et al., 1996). This hypothesis is founded on structural considerations; e.g., the abaxial phloem of the smallest minor vein is comprised of only IC and SE, whereas in the larger minor veins, ordinary CC are also present. The IC have been suggested to load sucrose symplasmically and convert it to raffinose sugars, while the ordinary CC load sucrose via an apoplastic step (Schmitz et al., 1987; Turgeon et al., 1993). In addition, a transcript highly homologous to a sucrose transporter (SUT1) was recently identified in the phloem sap of *Cucurbita maxima* (Ruiz-Medrano et al., 1999). Clearly, manipulation of plasmodesmal function by viral infection in the source leaves of plants that are defined as symplasmic loaders could have a dramatic effect on plant development, indeed even a lethal one. It is logical to assume that in such a case, the plant response would include an activation of defense mechanisms to inhibit this effect, and such a mechanism could include a shift from symplasmic to apoplastic loading. The outcome of such a shift would be increased sucrose level in the phloem. The rapid elevation radioactive sucrose within the phloem of CMV-infected leaves (Fig. 5) further supports the assumption that high sucrose level in the phloem of these plants is associated with an alteration in the process of phloem loading. In this respect it is interesting to note that other stress responses, such as low temperature and ABA treatment, also induced an increase in melon phloem sap sucrose concentration (Mitchell and Madore, 1992). Moreover, the activation of defense mechanisms includes a process mediated by information perceived by distant tissues. In such a case, the information could also be perceived by leaves in which virus particles are still absent, as seems to have occurred here with the observed sucrose level in the phloem sap of noninfected leaves (Figs. 4 and 5). Further studies are being aimed at elucidating the basis for the CMV-induced change in sugar levels in the phloem of infected melon plants.

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Figure Legends

Figure 1. Effect of CMV infection on sugar content in source leaves of melon plants. Leaf discs were taken at the beginning of the light period (A) and in the afternoon (B). Empty columns – noninfected plants; dotted columns – CMV-infected plants. Data are the means of five measurements. Columns followed by different letters are statistically different at the 5% level.

Figure 2. Effect of CMV infection on starch content (as glucose equivalents) in source leaves of melon plants. Leaf discs were taken at the beginning of the light period (AM) and in the afternoon (PM). Empty columns – noninfected plants; dotted columns – CMV-infected plants. Data are the means of five measurements ($\pm$ SE).

Figure 3. Effect of CMV infection on sugar content of phloem sap collected from petioles of melon source leaves. Sap was collected at the beginning of the light period (AM) and in the afternoon (PM). Empty columns – noninfected plants; dotted columns – CMV-infected plants. Data are the means of five measurements ($\pm$ SE).

Figure 4. Changes in ^{14}C -photosynthates in leaves of CMV-infected (closed circles) and control (open circles) melon plants. Radioactivity within intact leaves was assayed non-destructively with a “Rotem” portable β -counter. Experiments were performed on fully expanded source leaves (no. 5).

Figure 5. ^{14}C -sugars fractionated from phloem sap of melon source leaves. Sap was collected 30 and 60 min after labeling the leaf with $^{14}\text{CO}_2$. Empty columns – noninfected plants; dotted columns – CMV-infected plants. Data are the means of three measurements ($\pm$ SE).

Figure 6. Presence or absence of CMV particles in different leaves of CMV-infected melon plants. Presence of CMV in the various leaves was determined using Western blot of leaf extracts probed with antiserum raised against purified CMV-CP, or by analysis of CMV-MP RNA using RT-PCR. Leaves no. 2 and 4 from two representative plants (no. 5 and 10) were sampled 10 days post-infection (A) and leaves number 5 and 6 from other representative plants (no. 3, 12, 15 and 18) were sampled 16 days post-infection (B). Values indicate the concentration of sucrose (mg ml^{-1}) in the phloem sap of petioles collected from the respective leaves. Data in parentheses indicate the sucrose concentration (mg ml^{-1}) in phloem sap of respective control petioles collected from noninfected plants (ud-undetected).

Figure 7. Effect of CMV infection on sugar content of phloem sap collected from petioles of melon source leaves. Sap was collected 2 weeks after infection from leaf no. 4 displaying typical symptoms and leaf no. 7, which was symptomless. Empty columns – noninfected plants; dotted columns – CMV-infected plants. Data are the means of five measurements ($\pm$ SE).

Table I. Photosynthesis (Pn) and respiration rates of control and CMV-infected melon plants. Plants were grown in a temperature-controlled growth chamber with an average PFD of $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ at the level of the measured (no. 4/5) leaf. Measurements were performed 2 weeks post-inoculation. Values ($\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$) followed by the same letter within a column are not statistically different at the 5% level.

Plant	Gross Pn	Respiration	Net Pn
Control	7.8a	3.6b	4.2a
Infected	6.9a	6.7a	0.2b

Table II. Sugar content in the early afternoon (14:00) in the phloem sap of control and CMV-infected melon plants. Plants were grown in a growth chamber (25/18°C day/night) with a 12-h photoperiod. Sap was collected from petioles of young-mature (no. 3/4) and old (no. 9/10) leaves 5 weeks after infection. Five plants were used for each treatment. Values (mg ml^{-2}) followed by the same letter within a column are not statistically different at the 5% level.

Leaf no.	Stachyose	Raffinose	Sucrose
Leaf 3/4			
Infected	7.5a	1.7a	4.4a
Control	4.0a	0.9a	0.4b
Leaf 9/10			
Infected	6.3a	2.4a	1.1a
Control	3.8a	1.4a	0.4a

Table III. Photosynthesis (Pn) and respiration rates of control and CMV-infected melon plants. Plants were grown in a temperature-controlled growth chamber with an average PFD of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ at the level of the measured (no. 7) leaf. Measured leaves from both control and CMV-infected plants did not exhibit any symptoms. Measurements were performed 2 weeks post-inoculation. Values ($\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$) followed by the same letter within a column are not statistically different at the 5% level.

Plant	Gross Pn	Respiration	Net Pn
Control	4.6a	1.7a	2.9a
Infected	4.0a	1.9a	2.1a

Table IV. Sugar content in leaves of different ages from CMV-infected and control melon plants. Samples were collected in the early afternoon (14:00) from young mature (no. 4) and fully mature (no. 7) leaves 2 weeks after infection. Plants were grown in a temperature-controlled growth chamber (25/18°C day/night) with a 12-h photoperiod. Five plants were used for each treatment. Values ($\mu\text{g cm}^{-2}$) followed by the same letter within a column are not statistically different at the 5% level.

Leaf no.	Sucrose	Glucose	Fructose
Leaf 4			
Infected	9.0a	56.5a	28.8a
Control	5.5a	15.8b	9.3b
Leaf 7			
Infected	4.3a	15.9a	12.3a
Control	4.6a	13.4a	9.0a

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5

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Figure 7.