Planta (1993) 190:88–96

Planta

Influence of the tobacco mosaic virus 30-kDa movement protein on carbon metabolism and photosynthate partitioning in transgenic tobacco plants

William J. Lucas[1], **Amnon Olesinski**[2], **Richard J. Hull**[1,*], **James S. Haudenshield**[1], **C. Michael Deom**[3], **Roger N. Beachy**[4], **Shmuel Wolf**[2]

[1] Section of Botany, Division of Biological Science, University of California, Davis, CA 95616, USA
[2] Department of Vegetable Crops, Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot 76–100, Israel
[3] Department of Plant Pathology, University of Georgia, Athens, GA 30602, USA
[4] Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037, USA

Received: 12 August 1992 / Accepted: 20 September 1992

Abstract. Transgenic tobacco (*Nicotiana tabacum* L.) plants expressing the 30-kDa movement protein of tobacco mosaic virus (TMV-MP) were employed to investigate the influence of a localized change in mesophyll-bundle sheath plasmodesmal size exclusion limit on photosynthetic performance and on carbon metabolism and allocation. Under conditions of saturating irradiance, tobacco plants expressing the TMV-MP were found to have higher photosynthetic CO_2-response curves compared with vector control plants. However, this difference was significant only in the presence of elevated CO_2 levels. Photosynthetic measurements made in the greenhouse, under endogenous growth conditions, revealed that there was little difference between TMV-MP-expressing and control tobacco plants. However, analysis of carbon metabolites within source leaves where a TMV-MP-induced increase in plasmodesmal size exclusion limit had recently taken place established that the levels of sucrose, glucose, fructose and starch were considerably elevated above those present in equivalent control leaves. Although expression of the TMV-MP did not alter total plant biomass, it reduced carbon allocation to the lower region of the stem and roots. This difference in biomass distribution was clearly evident in the lower root-to-shoot ratios for the TMV-MP transgenic plants. Microinjection (dye-coupling) studies established that the TMV-MP-associated reduction in photosynthate delivery (allocation) to the roots was not due to a direct effect on root cortical plasmodesmata. Rather, this change appeared to result from an alteration in phloem transport from young source leaves in which the TMV-MP had yet to exert its influence over plasmodesmal size exclusion limits. These results are discussed in terms of the rate-limiting steps involved in sucrose movement into the phloem.

* *Permanent address*: Plant Sciences Department, University of Rhode Island, Kingston, RI 02881, USA

Abbreviations: PFD = photon flux density; SEL = size exclusion limit; TMV-MP = tobacco mosaic virus movement protein

Correspondence to: W.J. Lucas; FAX: 1(916)752-5410

Key words: Carbon allocation – Movement protein – *Nicotiana* – Photosynthesis – Plasmodesma – Tobacco mosaic virus – Transgenic tobacco

Introduction

The processes involved in transport of photosynthate from the cytosol of source-leaf mesophyll cells to the site of phloem loading within the vascular bundle require examination to identify likely rate-determining steps. It is presently considered that the symplasmic pathway, involving movement of sugars through plasmodesmata, presents the most likely route for the movement of carbohydrates from the mesophyll (Gamalei and Pakhomova 1981; Erwee et al. 1985; Russin and Evert 1985; Madore et al. 1986; Fisher 1986, 1990; Evert and Mierzwa 1986; Van Bel et al. 1988; Van Kestern et al. 1988; Madore and Lucas 1989; Turgeon and Hepler 1989; Robards and Lucas 1990). Diffusion appears to be the mechanism by which this efflux occurs (Tyree 1970; Weiner et al. 1988; Tucker et al. 1989; Robards and Lucas 1990) through these cytoplasmic bridges and it has been claimed that, as such, this process might impose a major barrier to the overall movement of sugars from the site of synthesis to the point of entry into the special symplasmic compartment of the phloem (Hatch and Osmond 1976; Madore and Lucas 1987; Burnell Hatch 1988; Robards and Lucas 1990).

Recent studies on virus-plasmodesmata interaction have provided the background for the design of experiments aimed at probing the role of plasmodesmata in terms of the physiological function of the tissues of the source leaf. Tobacco mosaic virus (TMV) encodes for a 30-kDa protein that is essential for TMV cell-to-cell movement (Deom et al. 1987; Meshi et al. 1987). During the infection process, as well as in transgenic tobacco plants expressing the gene for this 30-kDa TMV movement protein (TMV–MP), this movement protein becomes localized to the plasmodesmata (Tomenius et al.

1987; Atkins et al. 1991; Ding et al. 1992). Microinjection experiments (dye-coupling) performed on transgenic tobacco plants expressing TMV–MP showed that plasmodesmata within mature leaves had undergone a significant increase in size exclusion limit (SEL) from control values of 800–900 to approx. 15000 Da (Wolf et al. 1989). This effect of the TMV–MP on plasmodesmal SEL was found to be under developmental control (Deom et al. 1990). Although the TMV–MP was present in young leaves (the most recent leaf to achieve a length of 5 cm was defined as leaf 1), the increase in plasmodesmal SEL was first detected in the tip region of leaf 4. This basipetal pattern of change in functional plasmodesmal SEL was found to correlate with the onset of secondary plasmodesmata formation between mesophyll cells within these transgenic tobacco plants (Ding et al. 1992). Immunogold labeling studies using antibodies raised against the TMV–MP revealed that the 30-kDa TMV–MP was localized to secondary plasmodesmata connecting mesophyll and mesophyll-bundle sheath cells (non-vascular tissues). Furthermore, microinjection of fluorescently labeled probes established that the TMV–MP-induced change in plasmodesmal SEL was confined to these same cell types (Ding et al. 1992).

In the present study we used transgenic tobacco plants expressing TMV–MP to investigate the influence of the localized (mesophyll-bundle sheath) change in plasmodesmal SEL on photosynthetic performance and on carbon metabolism and allocation. Although expression of the TMV–MP had no effect on overall plant biomass production, its expression did cause significant changes in both sugar metabolism within and photosynthate translocation from mature tobacco leaf tissue. Furthermore, in comparison with control plants, TMV–MP transgenic tobacco had smaller roots, indicating an alteration in the overall pattern of carbon allocation.

Material and methods

Plant material. Transgenic *Nicotiana tabacum* L. cv. Xanthi, expressing the TMV–MP gene (line 277) as well as a vector control that was transformed but lacked the TMV–MP (line 306), were grown from seeds (R1 progeny) in an insect-free greenhouse. (Some experiments were performed on tobacco line 274 which was identical to plant line 277 except that it was homozygous for the TMV–MP gene.) At four weeks, plants were transplanted into plastic pots (approx. 10 cm diameter). In some experiments, plants were utilized in the greenhouse, while in others tobacco plants were transferred to a controlled-environment chamber for preconditioning prior to use. Temperatures within the greenhouse were maintained at approx. 25° C/18° C (day/night) and a similar regime was used in the controlled-environment chamber: 16-h photoperiod; photon flux density (PFD) level of 260 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photosynthetically active radiation provided by a combination of Sylvania (Danvers, Mass., USA) VHO Cool White fluorescent and incandescent lamps. No attempt was made to control the relative humidity in either the greenhouse or the controlled-environment chamber. Plants were fertilized on a regular basis with Hoagland solution (Hoagland and Arnon 1938).

Prior to using plants of line 277 the nopaline-synthase bioassay was performed on a sample of leaf tissue to ensure that the experimental material was transgenic for the TMV–MP gene (Wolf et al. 1991).

In experiments in which root biomass was to be determined, tobacco plants were grown under controlled-environment conditions in 1-l opaque glass jars. Seeds were germinated in fine sandy loam and then after 30 d the root system was washed free of oil and the plantlets were supported by cork stoppers such that their roots grew into aerated Hoagland solution. Roots from these hydroponically-grown plants were also used in microinjection experiments.

Gas exchange. Measurements of gas exchange were performed on leaves 5 and 6 of control and TMV–MP transgenic tobacco plants, where leaf 1 was defined as the last expanding leaf to achieve a laminar length (index) of 5 cm. The open gas-exchange system described by Kirschbaum and Pearcy (1988) was used for these experiments. In brief, a single attached tobacco leaf was enclosed in a special whole-leaf chamber and was exposed to a programmed change in either CO_2 concentration (under a constant light and humidity regime) or PFD (CO_2 and humidity held constant). Changes in PFD were achieved by screening the output from a 2.5-kW water-cooled Xenon-arc lamp; PFD values were measured using a quantum flux sensor (model LI–190 sR; LiCor, Lincoln, Neb., USA). Details of the computer-controlled gas regulation and analysis system is as described by Kirschbaum and Pearcy (1988). All gas-exchange parameters were calculated on the basis of the equations of Von Caemmerer and Farquhar (1981).

Gas-exchange measurements were also performed on six-week-old tobacco plants in either the greenhouse, under natural conditions, or in the controlled-environment chamber. Uptake of CO_2 was determined using a portable, closed infra-red gas-exchange system (model LI–6000; LiCor). The appropriate leaf (still attached to the plant) was placed in a 1-l lexan chamber such that a 10-cm^2 area was illuminated. Incident PFD, air and leaf temperature, as well as relative humidity inside the lexan chamber were measured concurrently with CO_2 uptake. Experiments were performed on plants that had been grown under bright sunny conditions (greenhouse) or under the constant light regime of our controlled-environment chamber. During these gas-exchange measurements the ambient CO_2 was 3.30 ± 0.1 Pa, relative humidity ranged between 30 and 40% and the PFD was approx. 1500 and 250 $\mu mol\ m^{-2}\ s^{-1}$ for greenhouse and controlled-environment-chamber experiments, respectively. Net photosynthesis, transpiration, stomatal conductance and sub-stomatal CO_2 concentrations were computed from these data.

Mass and photosynthate partitioning. Plants used for biomass distribution were dissected into leaves, stems and roots before being dried, at 70° C, in a forced-air oven (2 d). Dry-weight data were used to compute percentage mass distribution between the three organs, and for the determination of root-to-shoot ratios.

Partitioning of recently fixed photosynthate was examined by exposing specific tobacco leaves to $^{14}CO_2$. All labeling experiments were commenced near the middle of the photoperiod and were performed using the system previously described by Grusak and Lucas (1984). In brief, attached tobacco leaf No. 3 or 7 was sealed into the center of a specially constructed Plexiglas photosynthesis chamber, where it was held flat between two layers of nylon monofilament. Under a regulated stream of CO_2 (pCO_2 held at 3.30 Pa) and a PFD of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (provided by a quartz-iodide lamp), $^{14}CO_2$ was metered into the chamber to yield a constant specific activity of $1.35 \cdot 10^5$ Bq · mg^{-1} carbon. After 10 min, injection of $^{14}CO_2$ was terminated (approx. 4 MBq of ^{14}C fixed) and the plant was returned to the controlled-environment chamber.

Following a 24-h chase period, radiolabeled plants were dissected into six component parts: the treated leaf, leaves and stem above the treated leaf, leaves and stem below the treated leaf, and roots. The plant parts were dried at 70° C in a forced-air oven, weighed, and ground in a mortar and pestle. A sample (150 mg, when available) of each plant component was oxidized in a Tri-Carb Sample Oxidizer (model 306; Packard Instruments CO., Downers Grove, Ill., USA) and the $^{14}CO_2$ captured in Carbo-Sorb to which was then added PermaFluor V scintillation solution. The samples

were assayed for radioactivity using scintillation spectroscopy (model LS 9800; Beckman Instruments, Fullerton, Calif., USA). Specific activity was computed for each sample and this value, multiplied by the total mass of the appropriate plant part, yielded the total radioactivity for that component. The total radioactivity of all components from each plant was summed and used as the basis for calculating the percentage distribution of recently fixed photosynthate within each experimental plant.

Starch and sugar determinations. Carbohydrate content within leaves of differing ages was determined as a function of the day/night cycle. Six-week-old tobacco plants, equivalent to those used in gas-exchange studies, were randomly distributed (lines 306 and 277) to ensure against minor positional effects within the greenhouse or growth chamber. Leaf discs were punched from the appropriate leaves and were then extracted, four times, in 80% ethanol before being washed twice with 1 ml H_2O. Following each extraction, the supernatants were combined and evaporated to dryness. Sugars were then resolubilized in 1 ml of H_2O and the pH was adjusted to pH 8–9, by addition of 1 M NH_4OH, before passage through 1-ml beds of BioRex (BioRad, Richmond, Calif., USA) anion-exchange resin (100–200 mesh, chloride form) to remove organic acids. The resultant solution was then filtered through 0.2-μm nylon membrane high-performance liquid chromatography (HPLC) filters. Soluble sugars were separated on an LDC (LDC Anal., Riviera Beach, Fla., USA) Analytical HPLC system, fitted with a Sugar-Pak I column (20 cm long; Waters Associates, Milford, Mass., USA) and an LDC Analytical RefractoMonitor IV, using water as the mobile phase at a flow rate of 0.5 ml · min^{-1}.

Starch content was determined on the ethanol/water-extracted leaf discs. Leaf discs in 1 ml of H_2O were boiled for 1 h before being allowed to cool to room temperature. One half volume of 0.5% (w/v) amyloglucosidase (Cat. No. A–7255; Sigma Chemical Co., St. Louis, Mo., USA) in 15 mM sodium acetate (pH 4.5, 0.45-μm-filtered) was then added to each sample, followed by a 3-h incubation at 55° C. Starch content, as glucose equivalents, was determined using the Sigma (H K) quantitative glucose determination kit.

Protein and chlorophyll determinations. Leaf discs punched from the appropriate 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). A second set of discs was processed for determination of total soluble protein using the procedures detailed by Bradford (1976).

Microinjection experiments. The SEL values of plasmodesmata in control (line 306) and transgenic (lines 274 and 277) tobacco plants were established using microinjection and dye-coupling procedures as previously described (see Wolf et al. 1989; Ding et al. 1992). Preliminary dye-coupling experiments were performed to ensure that mesophyll plasmodesmata within plants grown under greenhouse, growth chamber and hydroponic conditions had SEL values consistent with our previous findings. In addition, SEL experiments were also performed on root cortical cells. Roots from hydroponically-grown tobacco plants were excised into 3-cm lengths and suspended in a reservoir of 0.5 mM $CaSO_4$ created by drawing, on a glass microscope slide, an outline around the segment with a wax pencil. The ends of the root segment were secured using silicone grease which provided sufficient support to enable insertion of a micropipette. The root segment was placed on the stage of a Leitz Orthoplan microscope (Ernst Leitz, Wetzler, FRG) and an hydraulic micromanipulator (model No. MO–104N; Narishige Scientific Instrument Laboratory, Greenvale, N.Y. USA), in combination with epiillumination, was used to insert a micropipette into a cortical cell. Fluorescent probes having a range of M_f values (see Wolf et al. 1989) were pressure-injected into the target cell using a pneumatic PicoPump (model PV830; World Precision Instruments, Sarasota, Fla., USA) and dye movement was then monitored using the epifluorescence mode (excitation filter No. BP 390–490) on a Leitz Orthoplan-video intensified microscopy system (model No. C1966–20; Hamamatsu Photonics K.K., Hamamatsu City, Japan).

Statistical analyses. All quantitative data were subjected to an analysis of variance procedure unless the means being compared were based on different numbers of observations; in such situations, the statistical significance was assessed using the student *t* test.

Results

Photosynthetic performance of TMV–MP transgenic plants. The results presented in Fig. 1 indicate that, under conditions of saturating PFD, tobacco plants expressing the TMV–MP have a higher photosynthetic CO_2-response curve compared with control plants. Significant differences in the photosynthetic performance of tobacco lines 277 (transgenic for TMV–MP) and 306 (transformed vector control) were observed only under elevated CO_2 conditions. Photosynthetic rates measured on plants in the greenhouse, under endogenous growth conditions, revealed that there was little difference between the TMV–MP-expressing and control tobacco plants. Table 1 represents a typical set of data collected on such greenhouse-grown tobacco in which photosynthetic rates were measured as a function of leaf development (age). In these experiments, leaf 1 was undergoing the transition from sink to source and was therefore not included in this study. For leaves 2 and 3, and 9 and 10 there was no significant difference between the photosynthetic values measured on control (line 306) and TMV–MP (line 277) plants. However, in this set of experiments, leaves 5 and 6 of the control line had slightly higher photosynthetic rates compared with the same leaves on the TMV–MP tobacco plants.

Although under some conditions TMV–MP tobacco plants had the appearance of uneven chlorophyll distribution (leaf mottling, see Wolf et al. 1991), the data presented in Table 1 indicate that there were no differ-

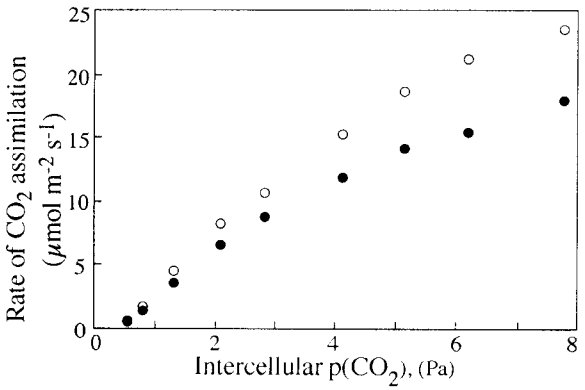

Fig. 1. Photosynthetic CO_2-response curves measured on mature leaves (Nos. 5 and 6) of six-week-old TMV–MP transgenic (line 277; ○) and vector control (line 306; ●) tobacco plants. Experiments were performed using the open gas-exchange system described by Kirschbaum and Pearcy (1988); leaf temperature maintained at 24 ± 1° C, PFD = 1600 μmol · m^{-2} · s^{-1}, relative humidity approx. 40%

Table 1. Relationship between leaf development and the physiological parameters of photosynthetic rate, chlorophyll content and total soluble protein in TMV-MP transgenic (line 277) and control (line 306) tobacco plants. Greenhouse-grown tobacco plants (five to six weeks old) were used (five plants per experiment); experimental protocols are detailed in the *Material and methods*

Plant type	Photosynthesis ($\mu mol \cdot m^{-2} \cdot s^{-1}$) Leaf numbers			Total chlorophyll content ($mg \cdot m^{-2}$) Leaf numbers			Total soluble protein ($g \cdot m^{-2}$) Leaf numbers		
	2–3	5–6	9–10	2–3	5–6	9–10	2–3	5–6	9–10
Control	9.8±0.6	12.5±0.6	6.3±0.5	340±10	320±10	200±10	48.0±1.5	30.6±0.8	12.6±1.6
Transgenic (TMV-MP)	9.8±0.6	10.3±0.9	7.0±0.8	350±10	320±10	200±10	53.8±2.6	32.4±0.6	12.9±0.8
Significance[a]	ns	*	ns	ns	ns	ns	ns	ns	ns

[a] * and ns = significant at $P = 0.05$ and not significantly different, respectively

ences in chlorophyll (a + b) or total soluble protein content between control and TMV–MP transgenic plants over all leaf developmental stages examined. Similar experiments performed on tobacco plants grown under controlled-environment conditions (low PFD) yielded essentially the same results (data not shown).

Effect of TMV–MP on photosynthate levels. Greenhouse- and controlled-environment-grown tobacco plants were employed to investigate whether the expression of the TMV–MP had any effect on leaf carbohydrate levels. Previously we established that the TMV–MP effects its change on plasmodesmal SEL in a basipetal pattern, commencing with leaf 4 (Deom et al. 1990; Ding et al. 1992). The data presented in Fig. 2 are consistent with this finding, in that the sucrose, glucose, fructose and starch levels were found to be similar in leaves 2 and 3 from TMV–MP and control tobacco plants. Within leaves 5 and 6, where the SEL of the mesophyll plasmodesmata would have been increased significantly in the TMV–MP transgenic plants, there was a large increase in the levels of sucrose, glucose, fructose and starch. Interestingly, this difference in the levels of sugars and starch between the TMV–MP and control plants was absent in the older, senescing leaves (leaves 9 and 10; see also Table 1).

The data presented in Fig. 2 illustrate that in leaves of TMV–MP transgenic tobacco the base levels of sucrose, glucose, fructose and starch, at the beginning of the photoperiod, were elevated above those present in equivalent leaves of vector control plants. It is interesting to note that over the ensuing photoperiod the sugar levels within leaves 5 and 6 of the two lines increased more or less in parallel, but with the TMV–MP tissue maintaining the higher concentrations. A similar pattern was observed with respect to changes in the starch levels over the photoperiod. Here, the change in starch content of the leaves followed the normal pattern of an increase late in the photoperiod (Fig. 2D).

Similar carbohydrate analyses were performed on leaves 5 and 6 taken from transgenic tobacco plants (lines 274 and 277) that were grown under controlled-environment conditions. Identical results were obtained, in that the sucrose, glucose, fructose and starch levels were also approximately double those found in control leaves (data not shown). However, as the irradiance changed in a stepwise manner, the sugar levels did not display the same peak in the time course as detected under greenhouse conditions (see Fig. 2).

To ensure that the detected alteration in carbohydrate levels was due to the expression of the TMV–MP gene, rather than the site of gene insertion, somatic variation that could have arisen during plant regeneration, etc., a set of similar experiments was performed on an additional line of control and TMV–MP-expressing tobacco plants. For these experiments a different control line of tobacco (*N. tabacum* cv. Xanthi-nc) was employed to produce TMV–MP-expressing plants (line 1N–16). The fact that the same basic results were obtained with these independently generated TMV–MP plants, in that the levels of sugars and starch in line 1N–16 were approximately twice those present in the control plants (data not shown), confirmed that expression of the TMV–MP is responsible for the alteration in carbon metabolism.

Biomass distribution. The above-ground parts of greenhouse- and controlled-environment-grown transformed tobacco plants (lines 274, 277 and 306) appeared to be very similar in terms of leaf shape and size, as well as overall plant height. In view of the complex effects of nutrient (especially nitrogen) and water availability on carbon allocation and biomass distribution (Reynolds and Thornley 1982; Huber 1983; Schulze 1983; Hunt and Nicholls 1986; Ågren and Ingestad 1987; Wilson 1988; Kachi and Rorison 1989; Levin and Mooney 1989), we utilized hydroponically-grown plants to investigate the influence of the TMV–MP on root development. The data presented in Table 2 establish that although there was no significant difference in total plant dry weight for either five- or ten-week-old plants, the biomass distribution differed between plant lines 274 and 306. The TMV–MP-expressing tobacco plants consistently retained more mass in their leaves and partitioned less to stems (older plants) and roots. Five- and 10-week-old TMV–MP plants produced root masses that were 43 and 45% smaller, respectively, than equivalently aged control plants. This difference in biomass distribution is clearly evident when the data are expressed in terms of root-to-shoot ratios (Table 2). It is important to stress

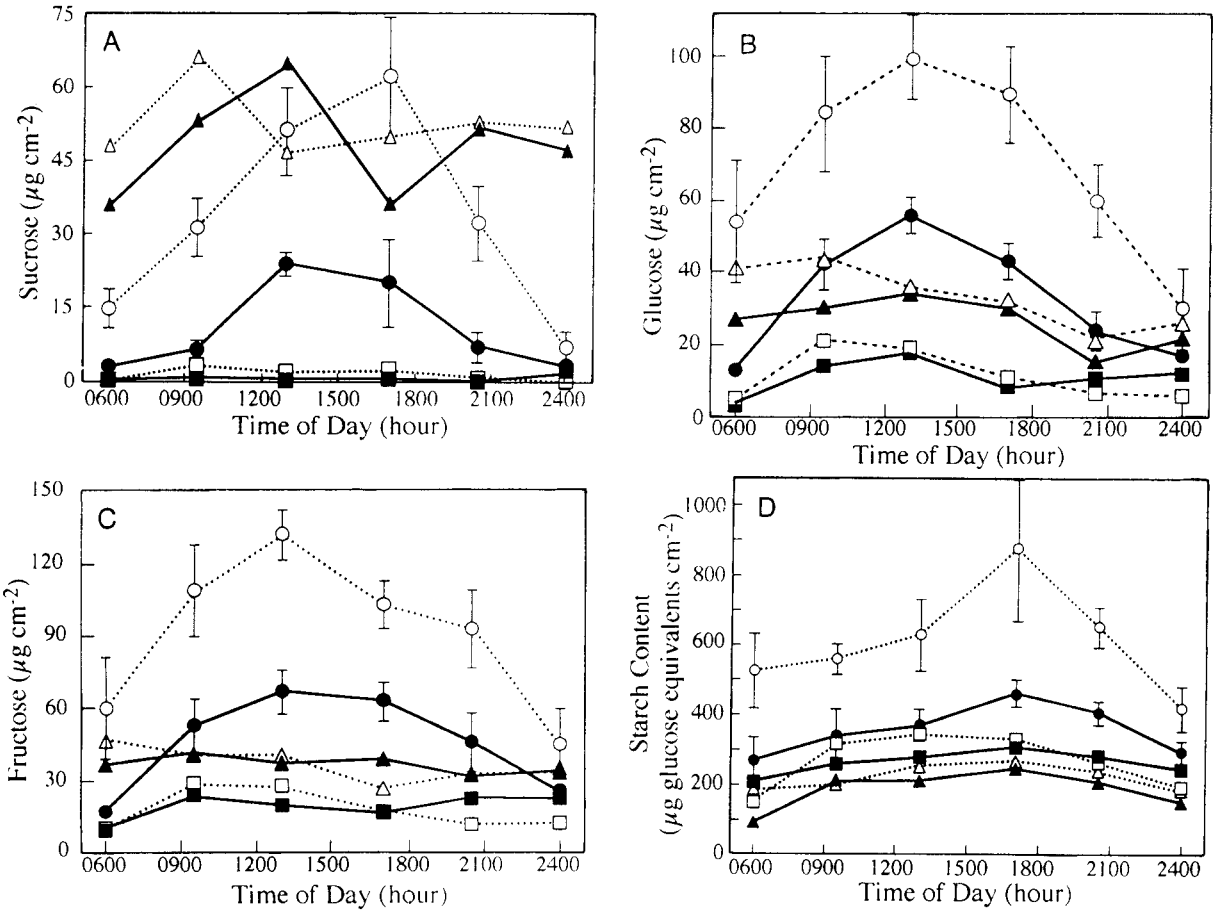

Fig. 2A–D. Carbohydrate contents within leaves of differing ages from TMV–MP transgenic (line 277) and vector control (line 306) tobacco plants determined as a function of the day/night cycle. Plants were grown in a greenhouse under natural sunlight, with sunrise and sunset being at approx. 0630 and 1700, respectively. Average midday PFD was 1500 μmol · m^{-2} · s^{-1}. Leaf discs were analyzed for sucrose (**A**), glucose (**B**), fructose (**C**) and starch (**D**) levels. Symbols are as follows: △ and ▲, leaves 2, 3; ○ and ●, leaves 5, 6; □ and ■, leaves 9, 10, for TMV–MP and control plants, respectively

Table 2. Mass distribution within TMV-MP transgenic (line 274) and control (line 306) tobacco plants. Plants were grown hydroponically in aerated Hoagland solution under controlled-environmental conditions (PFD = 260 μmol · m^{-2} · s^{-1}, 16-h photoperiod and 25° C day/18° C night)

Plant type	Plant age (weeks)	Plant DW (gm)	Mass distribution (%)			Root-to-shoot ratio
			Leaves	Stems	Roots	
Control	5[a]	5.0	54.8	31.5	13.7	0.16
Transgenic (TMV-MP)	5	5.0	62.4	29.5	8.0	0.09
Significance		ns[c]	**	ns	**	**
Control	10[b]	13.4	52.1	33.2	14.7	0.17
Transgenic (TMV-MP)	10	11.1	62.5	27.7	9.7	0.11
Significance		ns	**	*	**	**

[a] Four plants of each line were employed in these experiments
[b] Twelve plants of each line were employed in these experiments
[c] *, **, and ns = significant at $P = 0.05$, 0.01 and not significantly different, respectively

that as both plant lines employed in the root biomass experiments were grown under *identical* physiological conditions (optimal nutrient and water supply), the smaller root system of plant line 274 must reflect the effect of the TMV–MP on some aspect of photosynthate partitioning.

Current photosynthate partitioning. The distribution of newly fixed photosynthate 24 h following exposure to $^{14}CO_2$ is summarized in Table 3. These experiments were designed to investigate the influence of the TMV–MP on photosynthate movement out of the source leaves. To this end, we applied $^{14}CO_2$ to leaves 3 (near full expan-

Table 3. Distribution of ^{14}C-photosynthate in TMV-MP transgenic (line 274) and control (line 306) tobacco plants 24 h after specific leaves were exposed for 10 min to $^{14}CO_2$

Plant type	Leaf No. exposed	Plant parts[a]					
		TL	UL	LL	US	LS	R
		Percentage of ^{14}C recovered					
Control	3	64.0	4.0	0.8	2.4	11.3	17.6
Transgenic (TMV-MP)	3	75.8	3.9	0.6	3.2	7.6	8.9
Significance		*[b]	ns	ns	ns	*	*
Control	7	61.2	6.9	0.8	8.6	8.6	13.9
Transgenic (TMV-MP)	7	57.3	5.5	0.4	8.5	13.0	15.3
Significance		ns[b]	ns	ns	ns	*	ns

[a] TL, treated leaf; UL, leaves above treated leaf; LL, leaves below treated leaf; US, stem above treated leaf; LS, stem below treated leaf; R, roots
[b] * and ns = significant at $P = 0.05$ and not significantly differently, respectively

sion) and 7 (fully expanded), since both would be functioning as source tissues, while the mesophyll plasmodesmata in leaf 3 would have normal SEL, but those within leaf 7 would, in the TMV–MP plants, have undergone the TMV–MP-induced increase in SEL. Application of $^{14}CO_2$ to leaf 3 revealed that even though the TMV–MP had not affected the plasmodesmal SEL its expression did affect photosynthate distribution. In comparison with control line 306, TMV–MP plants (line 274) retained more of the newly fixed carbon within leaf 3 with less being translocated to the lower stem and root tissues (Table 3). However, little difference was detected between TMV–MP and control plants in the distribution of recently fixed ^{14}C-photosynthate when leaf 7 was exposed to $^{14}CO_2$. The slight increase in photosynthate translocation to the lower stems in the TMV–MP plants may have offset, to some extent, the reduction in carbon allocation from the upper leaves of these transgenic plants (Table 3).

Dye-coupling in tobacco root cortical cells. In a recent study, we established that within leaves the TMV–MP only modified the SEL of secondary plasmodesmata between the juncture of mesophyll and mesophyll-bundle sheath cells (Ding et al. 1992). In view of the pronounced effect of the TMV–MP on long-term carbon allocation to the roots of TMV–MP-expressing plants, we performed microinjection experiments to ascertain whether plasmodesmata within the tobacco root were also influenced by the TMV–MP. For these experiments, root cortical cells were impaled at the point where the nucleus was adjacent to the outer lateral wall. In this way, reliable cytoplasmic injections could be achieved and pressure injection of fluorescent probes (see Wolf et al. 1989) enabled us to establish that the TMV–MP did not influence the SEL of root cortical cell plasmodesmata (data not shown).

Discussion

The experiments performed in this study were aimed at testing the hypothesis that an increase in mesophyll plasmodesmal SEL would result in a modification in both photosynthetic performance and carbon allocation. The results presented establish that the expression of the TMV–MP in transgenic tobacco plants does result in a change in both carbon metabolism and photosynthate partitioning. Mature leaves of TMV–MP transgenic tobacco plants had an higher CO_2-response curve compared with the vector control line (Fig. 1). However, this difference in photosynthetic performance was not detected when measurements were made under endogenous growth conditions (Table 1). Indeed, under natural sunlight, young and old leaves of control and TMV–MP tobacco plants had comparable photosynthetic rates. Only with leaves 5 and 6 was there any significant difference, and unexpectedly, here the photosynthetic rate was higher in control (line 306) rather than TMV–MP transgenic (line 277) plants (Table 1).

The underlying basis for this apparent discrepancy between the data presented in Fig. 1 and Table 1 may indeed be complex. However, the data on soluble sugars within these two plant lines (Fig. 2) provides one possible explanation. A TMV–MP-associated enhancement in the levels of sucrose, glucose and fructose within leaves 5 and 6 would result in feedback inhibition of the chloroplast triosephosphate translocator. A logical outcome of such a feedback inhibition would be an increase in starch synthesis, and therefore storage, as was indeed observed in leaves 5 and 6 of the TMV–MP plants. A related effect of reducing the flow of triosephosphate out of the chloroplast would be a slight reduction in net photosynthesis (see Table 1).

Although the above explanation can account for the observed response, it does not address the fundamental question of why the sugar levels underwent such an increase in leaves 5 and 6, while no such change occurred in leaves 9 and 10, even though the TMV–MP would have increased the plasmodesmal SEL in leaves at both positions. Given that metabolite movement between mesophyll cells occurs through plasmodesmata, via diffusion, an increase in plasmodesmal SEL should translate into a reduced resistance to metabolite flow between these cells. (It should be noted that this statement presumes that there is no change in the absolute fre-

quency of mesophyll secondary plasmodesmata in TMV–MP transgenic versus control tobacco plants. This aspect is discussed in a later section.) However, sucrose transport out of the mesophyll into the minor veins, for eventual loading into the sieve elements, involves a number of steps any one of which may set the limit on the overall process of "phloem loading".

Under the influence of the TMV–MP, the mesophyll plasmodesmata within leaves 5 and 6 undergo a significant increase in SEL (Wolf et al. 1989; Deom et al. 1990; Ding et al. 1992) resulting in an enhanced exchange of both small (including sugars and sugar phosphates, etc.) and larger (proteins?) molecules between mesophyll cells. If this part of the symplasmic component of the pathway for "phloem loading" had constituted a limiting step (Madore and Lucas 1987; Robards and Lucas 1990), then the TMV–MP-induced reduction in the resistance to the diffusion of sucrose should have been reflected in a decrease in sucrose concentration in the transgenic TMV–MP leaves as compared with the control line. Since the opposite condition was observed (Fig. 2), it can be deduced that the rate-limiting step for sucrose loading into the phloem must be located within the interior of the vascular tissues, and logically in the phloem. A corollary would be that TMV–MP-mediated enhancement in the exchange of metabolites and-or larger molecules would alter the intercellular coordination of leaf biochemistry which could have resulted in the observed increase in sugar levels within the TMV–MP tobacco plants (see also Sharkey et al. 1992).

Analysis of the data presented in Fig. 2 reveals that from the onset of the photoperiod the levels of sucrose, glucose and fructose increased more rapidly in leaves 5 and 6 of the TMV–MP plant than in equivalent leaves of control plants. Differences in the rate of starch accumulation were less pronounced during the early part of the photoperiod, but from 14:00 to 17:00 hours the level increased much more rapidly in leaves 5 and 6 of the TMV–MP plants compared with leaves of plant line 306. By the end of the photoperiod, the absolute levels of carbohydrate were always much higher in leaves 5 and 6 of the TMV–MP plants, yet by the end of the dark period the levels had fallen to values near those of the previous morning (Fig. 2). Considering that the photosynthetic rates (net carbon exchange) within the leaves of these two lines would have been almost identical (see Table 1) these results, in conjunction with the ^{14}C-photosynthate data presented in Table 3, indicate that the rate of carbon export from the TMV–MP leaves must be reduced below that of control plants during the day, but then rises above the control rate during the night. Since the only difference between the two plant lines is the presence of the TMV–MP, it is clear that the constituitive expression of the TMV–MP gene within the tobacco plant influences, by some as yet unknown mechanism, the process(es) of carbon export via the phloem.

In both control and TMV–MP tobacco plants, leaves 9 and 10 had begun senescing, as evidenced by the 43 and 77% decrease in chlorophyll and soluble-protein levels, respectively (Table 1). Photosynthesis within these leaves had also declined and consequently the levels of sucrose, glucose and fructose were below those of the other leaves examined. Thus, the effect of the TMV–MP-mediated increase in plasmodesmal SEL may have been offset by a decline in the level of the enzymes involved in sugar metabolism. The pleiotropic effect of the TMV–MP on carbon export might also have been reduced in these older leaves. However, the carbohydrate data presented in Fig. 2 indicate that although the overall levels were low, the amount of sucrose, glucose, fructose and starch was higher in the light and lower in the dark for TMV–MP leaves compared with control leaves 9 and 10.

An alternative explanation for the observed influence of the TMV–MP on sugar levels and carbon export is that expression of the TMV–MP gene increases the SEL, but simultaneously alters the development (number and-or length of the newly formed protoplasmic bridges) of the secondary plasmodesmata at the mesophyll-mesophyll and mesophyll-bundle sheath interfaces. Both Ding et al. (1992) and Moore et al. (1992) reported no significant differences for tobacco lines 306 and 274, in terms of the secondary plasmodesmata that form in these leaf tissues, except for the presence of a filamentous material (TMV–MP) that filled the central cavity of the secondary plasmodesmata in mature TMV–MP leaves. The presence of this filamentous material may have inhibited the movement of sucrose from the mesophyll into the bundle-sheath cells. Such an inhibition in sucrose export would result in an increase in the level of sugars which would cause a shift from triose-phosphate export to starch accumulation within the chloroplasts. Experiments using a range of transgenic tobacco plants that have very different levels of TMV–MP will be employed to investigate this possibility.

As the effect of the TMV–MP on plasmodesmal SEL is under developmental control (Deom et al. 1990), being reflected in leaves 4–12, the change in carbon export from leaf 3 (Table 3) must have resulted from an indirect effect of the TMV–MP on the processes involved in phloem loading and-or long-distance transport. This change in carbon allocation was specific, in that the TMV–MP transgenic leaf 3 delivered only 16.5% of the recently fixed ^{14}C-photosynthate to the lower stem and roots, in comparison with 28.9% for the control plants. Results of this nature highlight the likely complexity of the interaction between the TMV–MP and the physiological controls involved in the coordinated functioning of the long-distance transport processes of the phloem. Collectively, the results presented in the present study support the hypothesis that the TMV–MP gene has a pleiotropic effect on the physiological functioning of transgenic tobacco plants. Ongoing experiments in our laboratory are aimed at further testing the hypothesis that the presence of the TMV–MP causes changes in diurnal translocation of photosynthate from the source leaf. Such studies will involve a detailed characterization of the export of recently fixed carbon as a function of both leaf position and progression through the normal day/night cycle.

Expression of the TMV–MP within the cells of the root could have influenced the sink strength of this tissue by altering plasmodesmal SEL and, thus, the resistance to the flow of sucrose as it moves out from the phloem

into the cortical tissues. Although the absolute level of the TMV–MP in the roots is only about 30% of that found in mature leaves, this amount is still comparable to that found in TMV-infected nontransformed tobacco plants (Deom et al. 1990). However, microinjection studies on TMV–MP transgenic roots revealed that the plasmodesmata connecting the cortical cells retained their normal SEL. Thus, the observed reduction in the root-to-shoot ratio in hydroponically-grown TMV–MP tobacco plants cannot be attributed to a direct effect of the TMV–MP on the coordinated biochemistry of the root symplasm. This TMV–MP-induced alteration in biomass distribution must have been caused either by an indirect effect of the TMV–MP on phloem unloading or by an adjustment in the plant's programmed carbon allocation within the source tissues.

It has long been recognized that resource allocation by the plant involves a form of dynamic "set point" that governs the allocation of carbon and nitrogen within the plant to control the root-to-shoot ratio (see Mooney and Winner 1991, and references therein). Increasing nitrogen availability results in a reduction in carbon allocation to the roots and this causes a decrease in the root-to-shoot ratio (Ågren and Ingestad 1987; Vessey and Layzell 1987; Kachi and Rorison 1989; Mooney and Winner 1991). Imposition of water stress has the opposite effect on the root-to-shoot ratio (Sharpe and Rykiel 1991); presumably an enlarged root biomass (increased surface area) assists in water uptake. Although these responses have been well characterized, there is presently little information on the underlying mechanism(s) utilized by the plant to adjust the "set point" and thereby alter the root-to-shoot ratio (Mooney and Winner 1991). In the present study we have established that expression of the TMV–MP alters not only carbon translocation and allocation but also the "set point" involved in governing the root-to-shoot ratio. These TMV–MP-induced changes could be explained on the basis of the alteration in photosynthate partitioning into starch, as Huber (1983) has shown that starch storage within the leaves is translocated, preferentially, to young developing leaves over lower parts of the plant including the roots. That this is not the basis for the TMV–MP-induced alteration in partitioning to the roots is clearly established by the fact that the starch levels in leaves 2 and 3 of both lines (306 and 277) are not significantly different (Fig. 2D).

Although biomass experiments were not performed on soil-grown TMV–MP transgenic tobacco plants, a reduction in root biomass would account for the phenotype observed when these plants are grown under conditions of limiting water availability and elevated temperatures. Plant lines 277 and 274 often show symptoms of water stress and develop accelerated senescence of their lower leaves, whereas plant line 306 does not display these symptoms. In ongoing experiments we are exploring the mechanism by which expression of the TMV–MP gene effects the observed change in root-to-shoot ratio. Graft experiments involving TMV–MP transgenic and control roots and shoots are being used to provide information on tissue-specific aspects of the pleiotropic nature of the TMV–MP. Finally, TMV–MP tobacco plants are being grown under different levels of available nitrogen to determine whether the TMV–MP-induced reduction in root-to-shoot ratio can be overridden by imposing a nitrogen stress. These studies should provide valuable information on the operation of the regulatory components involved in resource allocation within the plant.

Future studies will explore the mechanism by which the TMV–MP interacts to modify the SEL of the mesophyll and bundle-sheath plasmodesmata. This knowledge may potentiate the development of a wide range of transgenic plants in which the functional properties of specific plasmodesmata may be altered in a controlled manner. Such a system should enable us to dissect further the influence of the TMV–MP on both carbon metabolism and carbohydrate allocation by the phloem, as well as provide a valuable tool for the study of the rate-determining step(s) in the overall process of phloem loading.

This work was supported by National Science Foundation grant No. DCB–9016756 (W.J.L.) and United States-Israel Binational Science Foundation grant No. 90–00070 (S.W. and W.J.L.). Special thanks are due to Bryce Falk for the use of pathogen-free greenhouse space at the University of California, Davis, Plant Pathology Greenhouse Facility, and to Robert Pearcy, for the use of his gas-exchange system. R.J.H. was on sabbatical leave from the University of Rhode Island, Kingston, RI.

References

Ågren, G.I., Ingestad, T. (1987) Root: shoot ratio as a balance between nitrogen productivity and photosynthesis. Plant Cell Environ. **10,** 579–586

Atkins, D., Hull, R., Wells, B., Roberts, K., Moore, P., Beachy, R.N. (1991) The tobacco mosaic virus 30K movement protein in transgenic tobacco plants is localized to plasmodesmata. J. Gen. Virol. **72,** 209–211

Burnell, J.N. (1988) An enzymic method for measuring the molecular weight exclusion limit of plasmodesmata of bundle sheath cells of C_4 plants. J. Exp. Bot. **39,** 1575–1580

Bradford, M.M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254

Deom, C.M., Oliver, M.J., Beachy, R.N. (1987) The 30-kilodalton gene product of tobacco mosaic virus potentiates virus movement. Science **337,** 389–394

Deom, C.M., Schubert, K., Wolf, S., Holt, C., Lucas, W.J., Beachy, R.N. (1990) Molecular characterization and biological function of the movement protein of tobacco mosaic virus in transgenic plants. Proc. Natl. Acad. Sci. USA **87,** 3284–3288

Ding, B., Haudenshield, J.S., Hull, R.J., Wolf, S., Beachy, R.N., Lucas, W.J. (1992) Secondary plasmodesmata are specific sites of localization of the tobacco mosaic virus movement protein in transgenic tobacco plants. Plant Cell **4,** 915–928

Erwee, M.G., Goodwin, P.B., Van Bel A.J.E. (1985) Cell-cell communication in the leaves of *Commelina cyanea* and other plants. Plant Cell Environ. **8,** 173–178

Evert, R.F., Mierzwa, R.J. (1986) Pathway(s) of assimilate movement from mesophyll cells to sieve tubes in the *Beta vulgaris* leaf. In: Phloem transport, pp. 419–432, Cronshaw, J., Lucas, W.J., Giaquinta, R.T., eds. Alan Liss, New York

Fisher, D.G. (1986) Ultrastructure, plasmodesmatal frequency, and solute concentration in green areas of variegated *Coleus blumei* Benth. leaves. Planta **169,** 141–152

Fisher, D.G. (1990) Distribution of plasmodesmata in leaves. A comparison of *Cananga odorata* with other species using dif-

ferent measures of plasmodesmatal frequency. In: Parallels in cell-to-cell junctions in plants and animals, pp. 199–221, Robards, A.W., Lucas, W.J., Pitts, J.D., Jongsma, H.J., Spray, D.C., eds. Springer-Verlag Berlin

Gamalei, Y.V., Pakhomova, M.V. (1981) Distribution of plasmodesmata and parenchyma transport of assimilates in the leaves of several dicots. Soviet Plant Physiol. **28,** 649–661

Grusak, M.A., Lucas, W.J. (1984) Recovery of cold-inhibited phloem translocation in sugar beet. I. Experimental analysis of an existing mathematical recovery model. J. Exp. Bot. **35,** 389–402

Hatch, M.D., Osmond, C.B. (1976) Compartmentation and transport in C_4 photosynthesis. In: Encyclopedia of plant physiology, N. S., vol. 3: Transport in plants III, pp. 144–184, Stocking, C.R., Heber, U., eds. Springer-Verlag, Berlin

Hoagland, D.R., Arnon, D.I. (1938) The water-culture method for growing plants without soil. Calif. Agricult. Exp. Stu. Cir. **357,** 1–39

Huber, S.C. (1983) Relation between photosynthetic starch formation and dry-weight partitioning between the shoot and root. Can. J. Bot. **61,** 2709–2716

Hunt, R., Nicholls, A.O. (1986) Stress and the course control of growth and root-shoot partitioning in herbaceous plants. Oikos **47,** 149–158

Kachi, N, Rorison, I.H. (1989) Optimal partitioning between root and shoot in plants with contrasted growth rates in response to nitrogen availability and temperature. Func. Ecol. **3,** 549–559

Kirschbaum, M.U.I., Pearcy, R.W. (1988) Gas exchange analysis of the relative importance of stomatal and biochemical factors in photosynthetic induction in *Alocasia macrorrhiza*. Plant Physiol. **86,** 782–785

Levin, S.A., Mooney, H.A. (1989) The dependence of plant root:-shoot ratios on internal concentration of nitrogen concentration. Ann. Bot. **64,** 71–75

Madore, M.A., Lucas, W.J. (1987) Control of photosynthate movement in source-leaf tissues of *Ipomoea tricolor* Cav. Planta **171,** 197–204

Madore, M.A., Lucas, W.J. (1989) Transport of photoassimilates between leaf cells. In: Transport of photoassimilates, pp. 49–78, Baker, D.A., Milburn, J., eds. Longman, London

Madore, M.A., Oross, J.W., Lucas, W.J. (1986) Symplastic transport in *Ipomoea tricolor* source leaves: Demonstration of functional symplastic connections from mesophyll to minor veins by a novel dye-tracer method. Plant Physiol. **82,** 432–442

Meshi, T., Watanabe, Y., Saito, T., Sugimoto, A., Maeda, T., Okada, Y. (1987) Function of the 30 kD protein of tobacco mosaic virus: Involvement in cell-to-cell movement and dispensability for replication. EMBO J. **6,** 2557–2563

Mooney, H.A., Winner, W.E. (1991) Partitioning response of plants to stress. In: Responses of plants to multiple stresses, pp. 129–141, Mooney, H.A., Winner, W.E., Pell, E.J., eds. Academic Press, New York

Moore, P.J., Fenczik, C.A., Deom, C.M., Beachy, R.N. (1992) Developmental changes in the structure of plasmodesmata in transgenic tobacco expressing the movement protein of tobacco mosaic virus. Protoplasma **170**, 115–127

Moran, R. (1982) Formulae for determination of chlorophyllous pigments extracted with N,N-dimethylformamide. Plant Physiol. **69,** 1376–1381

Reynolds, J.F., Thornley, J.H.M. (1982) A shoot:root partitioning model. Ann. Bot. **49,** 585–597

Robards, A.W., Lucas, W.J. (1990) Plasmodesmata. Annu. Rev. Plant Physiol. Plant Mol. Biol. **41,** 369–419

Russin, W.A., Evert, R.F. (1985) Studies on the leaf of *Populus deltoides* (Salicaceae): ultrastructure, plasmodesmatal frequency, and solute concentrations. Am J. Bot. **72,** 1232–1247

Sharkey, T.D., Savitch, L.V., Vanderveer, P.J., Micallef, B.J. (1992) Carbon partitioning in a *Flaveria linearis* mutant with reduced cytosolic fructose bisphosphatase. Plant Physiol. **100,** 210–215

Sharpe, P.J.H., Rykiel, E.J. (Jr.) (1991) Modelling integrated responses of plants to multiple stresses. In: Responses of plants to multiple stresses, pp. 205–224, Mooney, H.A., Winner, W.E., Pell, E.J., eds. Academic Press, New York

Schulze, E.-D. (1983) Root-shoot interactions and plant life forms. Neth. J. Agric. Sci. **31,** 291–303

Tomenius, K., Clapham, D., Meshi, T. (1987) Localization by immunogold cytochemistry of the virus-coded 30K protein in plasmodesmata of leaves infected with tobacco mosaic virus. Virology **160,** 363–371

Tucker, J.E., Mauzerall, D., Tucker, E.D. (1989) Symplastic transport of carboxyfluorescein in staminal hairs of *Setcreasea purpurea* is diffusive and includes loss to the vacuole. Plant Physiol. **90,** 1143–1147

Turgeon, R., Hepler, P.K. (1989) Symplastic continuity between mesophyll and companion cells in minor veins of mature *Cucurbita pepo* L. leaves. Planta **179,** 24–31

Tyree, M.T. (1970) The symplast concept. A general theory of symplastic transport according to the thermodynamics of irreversible processes. J. Theor. Biol. **26,** 181–214

Van Bel, A.J.E., Van Kestern, W.J.P., Papenhuijzen, C. (1988) Ultrastructural indications for coexistence of symplastic and apoplastic phloem loading in *Commelina benghalensis* leaves. Planta **176,** 159–172

Van Kestern, W.J.P., Van Der Schoot, C., Van Bel, A.J.E. (1988) Symplastic transfer of fluorescent dyes from mesophyll to sieve tube in stripped leaf tissue and partly isolated minor veins of *Commelina benghalensis*. Plant Physiol. **88,** 667–670

Vessey, J.K., Layzell, D.B. (1987) Regulation of assimilate partitioning in soybean: Initial effects following change in nitrate supply. Plant Physiol. **83,** 341–348

Von Caemmerer, S., Farquhar, G.D. (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta **153,** 376–387

Weiner, H., Burnell, J.N., Woodrow, I.E., Heldt, H.W., Hatch, M.D. (1988) Metabolite diffusion into bundle sheath cells from C_4 plants. Plant Physiol. **88,** 815–822

Wilson, J.B. (1988) A review of evidence on the control of shoot: root ratio in relation to models. Ann. Bot. **61,** 433–449

Wolf, S., Deom, C.M., Beachy, R.N., Lucas, W.J. (1989) Movement protein of tobacco mosaic virus modifies plasmodesmatal size exclusion limit. Science **246,** 377–379

Wolf, S., Deom, C.M., Beachy, R.N., Lucas, W.J. (1991) Plasmodesmata function is probed using transgenic tobacco plants that express a virus movement protein. Plant Cell **3,** 593–604