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Pleiotropic effects of tobacco-mosaic-virus movement protein on carbon metabolism in transgenic tobacco plants

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Abstract. Transgenic tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants expressing wild-type or mutant forms of the 30-kDa movement protein of tobacco mosaic virus (TMV-MP) were employed to study the effects of the TMV-MP on carbon metabolism in source leaves. Fully expanded source leaves of transgenic plants expressing the TMV-MP were found to retain more newly fixed ^{14}C compared with control plants. Analysis of ^{14}C-export from young leaves of TMV-MP plants, where the MP is yet to influence plasmodesmal size exclusion limit, indicated a similar pattern, in that daytime ^{14}C export was slower in TMV-MP plants as compared to equivalent-aged leaves on control plants. Pulse-chase experiments were used to monitor radioactivity present in the different carbohydrate fractions, at specified intervals following $^{14}CO_2$ labeling. These studies established that the TMV-MP can cause a significant adjustment in short-term ^{14}C-photosynthate storage and export. That these effects of the TMV-MP on carbon metabolism and phloem function were not attributable to the effect of this protein on plasmodesmal size exclusion limits, per se, was established using transgenic tobacco plants expressing temperature-sensitive and C-terminal deletion mutant forms of the TMV-MP. Collectively, these studies establish the pleiotropic nature of the TMV-MP in transgenic tobacco, and the results are discussed in terms of potential sites of interaction between the TMV-MP and endogenous processes involved in regulating carbon metabolism and export.

Key words: Carbon (allocation, metabolism) – Movement protein – *Nicotiana* – Plasmodesma – Tobacco mosaic virus – Transgenic tobacco

Introduction

Sucrose synthesis occurs within the cytosol of tobacco mesophyll cells, but the pathway followed by sucrose during its movement from the site of synthesis to the cells of the phloem remains equivocal. The prevailing view is that solute movement between mesophyll cells occurs via a symplasmic route through plasmodesmata (Giaquinta 1983; Tucker et al. 1989; Robards and Lucas 1990). In many species, however, the actual process involved in loading into the sieve element-companion cell complex may involve an apoplasmic step (van Bel 1992). Furthermore, it remains to be elucidated whether the loading process constitutes the rate-determining step, or major control site, in the export of recently fixed photosynthate.

Experimental control over plasmodesmal size exclusion limit (SEL) has recently been achieved using expression of viral movement proteins (MPs) in transgenic plants. In transgenic tobacco expressing the MP of tobacco mosaic virus (TMV-MP), this movement protein becomes localized to mesophyll plasmodesmata (Atkins et al. 1991; Ding et al. 1992; Moore et al. 1992) where it causes a significant increase in plasmodesmal SEL from the control value of approx. 800 Da to greater than 9.4 kDa (Wolf et al. 1989). Photosynthesis and carbon-allocation experiments performed on these transgenic tobacco plants revealed that the presence of the TMV-MP resulted in a change in carbon metabolism (Lucas et al. 1993). Although total chlorophyll, net photosynthesis and total leaf proteins were not significantly different between control and TMV-MP plant lines, it was found that, compared with control plants, fully expanded leaves expressing the TMV-MP had unexpectedly high levels of sugars and starch. The level of carbohydrates within these TMV-MP leaves appeared to increase more rapidly during the photoperiod, compared with control leaves, with the converse occurring during the dark period. In addition, there was a significant difference in biomass distribution between the various plant organs, resulting in lower root-to-shoot ratios in TMV-MP transgenic plants, although, under the growth conditions employed in these studies, the *total* biomass remained similar in both plant lines (Lucas et al. 1993).

This complex influence of the TMV-MP on carbon metabolism was unexpected, since it was anticipated that increasing the plasmodesmal SEL would enhance symplasmic transport of sugars from the mesophyll to the site

Abbrevations: MP = movement protein; SEL = size exclusion limit; TMV = tobacco mosaic virus; ts = temperature sensitive

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of phloem loading. If this were the case, sugar levels within the mesophyll tissue of TMV-MP plants should have been lower, not higher, than control values. Furthermore, if plasmodesmal SEL within the mesophyll did not constitute a rate-limiting step on the process of loading, carbon metabolism should have remained unaffected by the TMV-MP. Interpretation of these results was further complicated by the finding that although the TMV-MP was expressed in the vascular tissue, it did not cause an increase in the SEL of the plasmodesmata that interconnect the cells within the vein (Ding et al. 1992).

In the present study, we further explored the influence of wild-type and mutant forms of the TMV-MP on carbohydrate metabolism and export. These experiments established the pleiotropic effects of the TMV-MP in transgenic tobacco plants and are discussed in terms of likely target sites that may effect the observed changes in photosynthate metabolism and carbon export via the phloem.

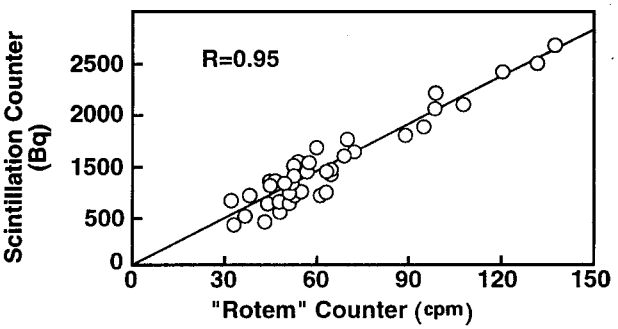

Fig. 1. Comparison between measurements from a scintillation counter and a "Rotem" portable β-counter. Radioactivity within an intact leaf was assayed using the portable β-counter system following $^{14}CO_2$ feeding. Then, three leaf discs were punched from the detection site and dissolved for 3–5 d in Soluene-350. Radioactivity within these discs was then assayed by scintillation spectrometry as described in the *Materials and Methods*

Material and methods

Plant material. Transgenic tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants expressing wild-type (plant line 277, Deom et al. 1987; line 2004, Deom et al. 1991), temperature-sensitive mutant (ts, MPP 154A) (line 2-72, Wolf et al. 1991) and C-terminal (10 amino acids) deletion mutant (line MP1, Berna et al. 1991) forms of TMV-MP, as well as vector control tobacco plants (lines 306, 3001; transformed but without the *MP* gene), were used in the present experiments. Three-week-old seedlings were transferred into plastic pots (15 cm diameter) and plants were grown in an insect-free, temperature-controlled greenhouse (approx. 25°C day/18°C night). In some experiments, the temperature within this same greenhouse was raised to 34°C/32°C (day/night, respectively). Plants were grown under natural sunlight having a midday average photon flux density of 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Gas-exchange measurements. Net photosynthesis (measured as CO_2 uptake) was determined using a closed-portable infrared gas-exchange system (LI-6200, LICOR Inc., Lincoln, Neb., USA). An attached leaf was placed in a 1 l lexan chamber such that a 10-cm^2 area was exposed to light and gas flow. The youngest fully expanded leaf (#5 or #6, with leaf #1 being the last leaf to achieve a length of 5.0 cm) was used in these experiments. All measurements (photosynthesis and respiration) were carried out on well-watered plants during the late morning h (10–12 noon) on bright, sunny days. The initial CO_2 concentration in the chamber was 340 $\pm$ 10 $\mu l \cdot l^{-1}$, and a 30-s measurement was started immediately after a reduction in CO_2 concentration was detected. Dark respiration was measured by covering the leaf chamber with a black cloth and measurements were started immediately after an increase in CO_2 concentration was observed. Negative values of photosynthesis were interpreted as dark respiration.

Sugar and starch determinations. Carbohydrate content within leaves was determined as described by Lucas et al. (1993). In brief, soluble sugars were extracted in 80% ethanol from leaf discs (1.5 cm^2). After evaporating the supernatant to dryness, sugars were redissolved in H_2O and then filtered through a 0.45-μm membrane (Whatman, Clifton, N. J., USA). Sugar separation was carried out on an analytical HPLC system (LDC Anal., Reviera Beach, Fla, USA), fitted with a Sugar-Pak I column (6.5 mm i.d., 300 mm long; Waters Associates, Milford, Mass., USA) using an LDC refractive-index detector (Refractor Monitor IV). Starch content was determined on the ethanol-water extracted leaf discs following starch conversion by amyloglucosidase (Cat. No. A-7255; Sigma Chemical Co., St. Louis, Mo., USA). Starch content, as glucose equivalents, was measured using the Sigma (HK) quantitative glucose determination kit.

Radioactive labeling with $^{14}CO_2$. Tobacco leaves were labeled with $^{14}CO_2$ by using a pulse-labeling system. Experiments were carried out in the greenhouse, during the late morning hours on bright days, under optimal photosynthetic conditions. An attached tobacco leaf was sealed into a 4-l Plexiglas chamber where it was held between two layers of nylon monofilament. A volume of 60 cm^3 of $^{14}CO_2$ was then released into the chamber (40 s) to give an initial specific activity of $2 \cdot 10^5$ $Bq \cdot mg^{-1}$ carbon. It took less than 20 min for the CO_2 concentration in this chamber to be reduced to the compensation point, by which time the $^{14}CO_2$ would have been assimilated into photosynthetic products. At this point, the leaf was released from the chamber and was used for analysis of ^{14}C-photosynthate export.

Measurements of radioactive ^{14}C. Rate of reduction in radioactivity of either total leaf ^{14}C or ^{14}C-labeled carbohydrates was determined from time-course measurements that commenced immediately after a leaf was released from the $^{14}CO_2$-labeling chamber. Total radioactive content within each ^{14}C-labeled leaf was determined using one of the following two methods. First, at various time intervals, leaf discs were punched from an attached leaf and were then dissolved in tissue solubilizer (Soluen-350; Packard Instrument Co., Inc., Downers Grove, Ill., USA). After 3–5 d the radioactivity was measured on a Betamatic liquid scintillation counter (Kontron, Zürich, Switzerland) using Ultima-gold scintillation cocktail (Packard Instrument Co.). Second, a portable, Geiger-Müller tube (RAM-DA; Rotem Industries, Be'er Sheva, Israel) containing a circled β-counter, (Model GM-10, diam. 4 cm) was placed on the adaxial surface, to the side of the main vein, in the mid-region of an intact ^{14}C-labeled leaf; data were collected (100 s sampling period) at appropriate times over a 24-h experimental period. A comparison between *in-planta* β-radiation detection (Geiger-Müller) and scintillation-based ^{14}C analysis demonstrated that the two methods yielded data that were highly correlated over the range of radioactivity levels employed in the present experiments (Fig. 1). In view of this finding, the portable β-radiation detector was used to perform non-destructive time-course experiments on $^{14}CO_2$ pulse-labeled source leaves of TMV-MP transgenic (lines 277, 2004 and 2-72) and control (lines 306 and 3001) tobacco plants.

Partitioning of newly fixed carbon within leaf carbohydrates was determined in a manner that gave data on both concentration and radioactivity of each component. Sugars (sucrose, glucose and fructose) were identified and fractionated by HPLC. Radioactivity that eluted at the sample-front peak was collected separately and termed the liquid residue (comprised of carboxylic acids, phosphorylated carboxylic acids and phosphorylated carbohydrates). Radioactivity in the starch fraction was measured after enzymatic conversion to glucose residues. Following carbohydrate extraction, the remaining

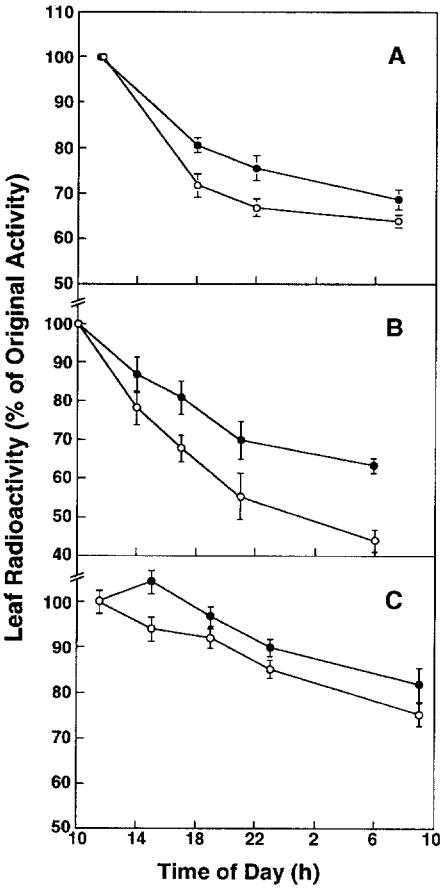

Fig. 2A–C. Diurnal changes in ^{14}C-photosynthates in leaves of TMV-MP transgenic (●) and vector control (○) tobacco plants. Plants were grown in a greenhouse (25°/18°C day/night) under natural sunlight with a midday average photon flux density of 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Radioactivity within intact leaves was assayed non-destructively with a "Rotem" portable β-counter. Experiments were performed on either fully expanded source leaves (#5 and 6) (**A, B**) as well as on younger, expanding source leaves (#2 and 3) (**C**). Plant lines 277 and 306 were used for experiments in **A** and **C**, while plant lines 2004 and 3001 were employed in the experiments presented in **B**. Values represent mean ± SE ($n = 5$)

leaf tissue was solubilized (Soluen-350) for 3–5 d prior to assaying for radioactivity (this fraction was termed the solid residue).

Results

Pulse-labeling of source leaves. In a recent study, we established that TMV-MP transgenic plants (line 277) accumulated much higher amounts of carbohydrate during the day as compared to control tobacco plants (line 306). However, over the dark period, the level of all carbohydrates appeared to decline more rapidly in 277 than in 306 plants, often resulting in the establishment of comparable levels in both lines by the next morning (Lucas et al. 1993). The time-course data presented in Fig. 2A indicated that the rate of reduction in radioactivity was significantly lower in *TMV-MP*-expressing tobacco leaves as compared to control plants. To further confirm the effect of the TMV-MP on the rate of reduction of ^{14}C-labeled photosynthate, we next performed a similar series of experiments on transgenic tobacco plants that expressed the TMV-MP in a slightly different genetic background (*N. tabacum* Xanthi NN). Plants homozygous for the *TMV-MP* gene and which had levels of TMV-MP within leaf tissues that were comparable to those measured in TMV infected plants (plant line 2004, Deom et al. 1991), were chosen for these studies. As illustrated in Fig. 2B, plant line 2004 also exhibited a slower rate of reduction in the level of radioactivity that remained in the source leaves compared with equivalent leaves on the respective vector control plants (line 3001).

Pulse-labeling experiments, performed on young leaves (leaf #2) in which the TMV-MP would not yet have caused an increase in plasmodesmal SEL (Deom et al. 1990; Ding et al. 1992), demonstrated lower rates of reduction in the level of residual leaf radioactivity compared to fully expanded leaves (Fig. 2C). Although the rate of reduction of ^{14}C was similar between the two plant lines, line 277 consistently retained more ^{14}C photosynthate over a diurnal cycle (statistically significant at $P = 0.05$). These findings are in full agreement with an earlier study performed on a completely independent *TMV-MP* transformant (line 274; see Lucas et al. 1993).

Distribution and turnover of ^{14}C-photosynthate within the source leaf. Analysis of ^{14}C-labeled and total photoassimilates revealed a significant difference between 277 and 306 plant lines (Table 1). During the daylight hours the sucrose levels in TMV-MP transgenic plants were almost double those of control plants (Table 1, see also Lucas et al. 1993). In control plants, ^{14}C-sucrose declined during the daylight hours to approximately 22% of the value measured shortly after the $^{14}CO_2$ labeling treatment; while for plant line 277, this value decreased to only 50% over the same period. The decrease in [^{14}C]sucrose in line 277 represented the major portion of the total loss of radioactivity, whereas in control plants it represented about half of the total loss. The other fraction contributing to the total decline of radioactivity in control leaves (306) was the liquid residue (Table 1).

Interestingly, [^{14}C]glucose decreased by only 4% in line 277 compared with 30% in control plants, while in both lines [^{14}C]fructose increased during the period after $^{14}CO_2$ labeling, with a subsequent decrease being detected only in control plants. As expected, ^{14}C activity in the starch fraction remained relatively constant, in both lines, over the ensuing photoperiod. The solid-residue fraction consisted of non-extractable metabolites and structural carbohydrates. Radioactivity in this component increased in both lines, probably due to turnover of cell wall components.

Effects of temperature on carbon partitioning in tobacco expressing ts mutant and wild-type TMV-MP. A temperature-sensitive (ts) TMV-MP mutant, MPP154A, was generated by changing the proline residue at amino acid 154 of the TMV-MP to alanine (Wolf et al. 1991; see also Leonard and Zaitlin 1982; Ohno et al. 1983; Deom et al. 1987; Meshi et al. 1987). Studies on plasmodesmal SEL in transgenic tobacco plants expressing this ts mutant TMV-MP (line 2-72) indicated that, under permissive

Table 1. ^{14}C-carbohydrates and total carbohydrate content within leaves of TMV-MP transgenic (line 277) and vector control (line 306) tobacco plants. Plants were grown in a greenhouse under natural sunlight with average midday photon flux density of 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Six plants were used from each line. Leaf disks were punched from fully expanded leaves (leaf #5–6), and were analyzed for radioactivity and carbohydrate content (values in parentheses) of each compound

Plant line	Time of day	Radioactivity[a] and content											
		SC[b]		GL		FR		STR		LR	SR	TOT	
306	10:30	368	(28.7)	161	(38.0)	138	(54.2)	506	(462)	533	123	1829	
	14:00	120	(37.7)	158	(43.7)	183	(54.3)	546	(577)	319	133	1459	
	17:30	81	(31.5)	117	(36.4)	136	(52.5)	505	(659)	255	152	1244	
Significance[c]		*	*	ns	ns	ns	ns	ns	*	*	*	*	
277	10:30	479	(47.6)	176	(92.4)	148	(114.5)	631	(1002)	564	128	2127	
	14:00	293	(61.4)	171	(101.6)	173	(125.8)	579	(1041)	535	151	1902	
	17:30	227	(59.0)	168	(95.4)	185	(118.5)	573	(1076)	519	169	1841	
Significance[b]		*	*	ns	ns	ns	ns	ns	ns	ns	*	*	

[a] Radioactivity and content (values in parenthesis) presented as dpm and $\mu g \cdot cm^{-2}$, respectively
[b] SC, sucrose; GL, glucose; FR, fructose; STR, starch; LR, liquid residue; SR, solid residue; TOT, total radioactivity
[c] * and ns represent significant and not significantly different, respectively, at $P = 0.05$

Table 2. Temperature effects on photosynthesis and dark respiration in wild-type TMV-MP transgenic (line 277), ts mutant TMV-MP transgenic (line 2-72) and control (line 306) tobacco plants. Mature leaves (leaf #5–6 on 12 to 14-leaf plants) were measured using a closed infrared gas-exchange system. (Six plants of each line were used per experiment and value presented represent mean ± SE)

Plant line	Temperature (°C)	Plasmodesmal[a] SEL (kDa)	Photosynthesis $\mu mol\ CO_2 \cdot m^{-2} \cdot s^{-1}$	Dark respiration $\mu mol\ CO_2 \cdot m^{-2} \cdot s^{-1}$
306	25	0.8	14.4 ± 0.9	2.6 ± 0.2
277	25	> 9.4	12.5 ± 1.0	2.4 ± 0.2
2-72	25	> 9.4	14.1 ± 3.3	2.2 ± 0.2
306	32-34	0.8	23.8 ± 1.3	6.1 ± 0.4
277	32–34	> 9.4	24.6 ± 0.2	5.9 ± 0.4
2-72	32–34	0.8	22.3 ± 0.9	6.2 ± 0.7

[a] Data from Wolf et al. (1991)

temperatures (24°C; i.e. temperatures that permitted infection of these plants by an MP − strain of TMV), the SEL was elevated to levels identical to those measured in wild-type TMV-MP transgenic plants (line 277). However, under non-permissive temperatures (32°C), the SEL in plant line 2-72 was similar to that detected in control plants (line 306) (Wolf et al. 1991). Furthermore, this plant line was selected for the present study as homozygous plants were shown to express the ts-form of the TMV-MP to yield levels that were equivalent to those present in wild-type TMV-MP plants (lines 277, 274, etc.). Finally, our previous studies on plant line 2-72 established that the ts TMV-MP did not undergo accelerated degradation under elevated temperatures (Wolf et al. 1991).

Before we could examine the effects of temperature-induced alteration in TMV-MP-mediated increase in plasmodesmal SEL, it was necessary to ensure that temperature, per se, did not cause differential effects on photosynthesis or respiration in plant lines 277, 2-72 and 306. Photosynthesis and dark-respiration measurements, performed on plants grown at 25°C daytime temperature, indicated that all plant lines examined yielded statistically identical parameters (Table 2). A similar situation was observed when plants were maintained under non-permissive temperatures (32–34°C, day and night), in that values for net photosynthesis and dark respiration were equivalent between plant lines 277, 2-72 and 306. However, the data presented in Table 2 show that the absolute rates of photosynthesis and dark respiration were considerably higher in the plants exposed to 32–34°C conditions. These control studies established that neither photosynthetic production nor local consumption differences could provide a basis to explain the MP-induced changes in carbon metabolism (Lucas et al. 1993).

As shown in Fig. 3A, during the first hours after ^{14}C-labeling, the rate of reduction in radioactivity in source leaves of plants maintained at 25°C was identical in transgenic plants expressing either the ts mutant or wild-type TMV-MP, but was slower compared to control plants (line 306). Interestingly, during the night, the rate of reduction in radioactivity within these source leaves was slower in the ts mutant TMV-MP transgenic plants compared to wild-type TMV-MP transgenic plants. Transgenic plants expressing either the ts mutant or the wild-type TMV-MP were also found to have similar sugar levels when the experiments were performed at 25°C (Fig. 3 E, G, I). Consistent with our previous findings (Lucas et al. 1993), these sugar levels were always significantly higher in the TMV-MP plants compared with control plants. Interestingly, the starch levels in line 2-72 were intermediate between lines 277 and 306 (Fig. 3C).

Pretreatment of the three plant lines at non-permissive temperatures (32–34°C) for 72 h caused an increase in the overall rate of reduction of ^{14}C remaining in the source

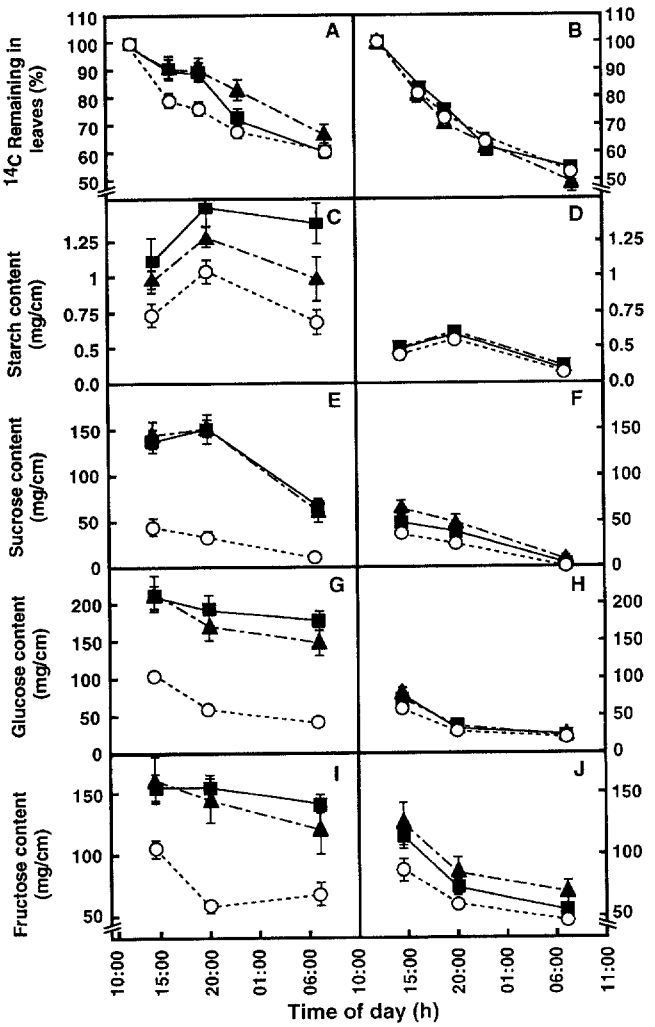

Fig. 3A–J. Carbohydrate content and ^{14}C-radioactivity detected within source leaves of transgenic tobacco plants expressing wild-type TMV-MP (line 277; ■), temperature-sensitive TMV-MP mutant (line 2–72; ▲) and vector control (line 306; ○). Plants were grown in the greenhouse under natural sunlight with a midday average photon flux density of 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$, under two temperature regimes of 25°/18°C day/night (left side) and 34°/32°C day/night (right side). Radioactivity was detected using a "Rotem" β-counter (**A, B**) and leaf discs were analyzed for starch (**C, D**), sucrose (**E, F**), glucose (**G, H**) and fructose (**I, J**). (Five plants from each line and for each temperature regime were used in these experiments, and values represent mean ± SE)

leaves of all plants tested (cf. Fig. 3A, B). The interesting point to note in these experiments is that the rate of ^{14}C reduction in source leaves of plant lines 2-72 and 277 increased to such an extent that their values converged with those obtained on control plants (Fig. 3B). Under elevated temperatures, carbohydrate levels declined significantly in all plant lines and, as with ^{14}C-carbon export, the actual diurnal change in the levels of the individual sugars and starch was similar between the three plant lines (Fig. 3D, F, H, J). However, sucrose, glucose and fructose levels still exhibited significant differences between lines 2-72, 277 and the control line 306.

Influence of TMV-MP C-terminal deletion on carbohydrate metabolism. A mutant form of the TMV-MP, in which the C-terminal 10 amino acids were deleted, retained full wild-type function, supporting viral infection and causing an increase in plasmodesmal SEL (Berna et al. 1991; Gafny et al. 1992). Experiments performed on tobacco plants expressing this mutant form of the TMV-MP provided unequivocal support for the hypothesis that this viral protein functions in a pleiotropic manner in transgenic tobacco plants (see also Balachandran et al. 1995). As shown in Table 3, deletion of the terminal 10 amino acids appeared to eliminate the influence of the TMV-MP on carbohydrate metabolism within mature source leaves. Comparable levels of sucrose, glucose and fructose were detected in plant lines MP1 and 306, while, as expected, plant line 277 (expressing the wild-type *TMV-MP* gene) had elevated sugar levels. A similar situation was observed with respect to starch metabolism, with plant lines MP1 and 306 having near equivalent values for accumulation and hydrolysis over the experimental period (Table 3). It should be noted that, in plant line 277, the level of starch was higher than in either MP1 or 306 plants and that, in contrast to these plants, little hydrolysis occurred during the night (see also Fig. 3C where a similar situation is reported).

Again, to guard against the effects of positional insertion, somoclonal variation, etc., a second experiment was performed with a different, independent, transgenic tobacco line expressing a TMV-MP in which the C-terminal 10 amino acids were deleted (plant line MP1-1). For this experiment, we also examined the influence of the TMV-MP on sugar metabolism in plant line 2004 and 277 (compared with their respective control lines 3001 and 306). As illustrated in Table 4, carbohydrate levels in transgenic tobacco plants expressing the C-terminal 10-amino-acid deletion form of the TMV-MP were again remarkably similar to the values measured on the relevant control line. Note that these similarities held over the early afternoon, evening and morning periods. Data obtained on plant lines 2004 and 3001 further confirmed that the elevated sugar and starch levels in TMV-MP transgenic plants can be attributed to the presence of the TMV-MP, rather than being due to unrelated events associated with plant transformation.

Discussion

Short-term storage of carbohydrate and ^{14}C export are altered by TMV-MP. In the present study we further characterize the influence of the TMV-MP on carbon metabolism and photosynthate export within source leaves of transgenic tobacco plants. Earlier studies showed significantly higher levels of carbohydrates in mature source leaves of TMV-MP transgenic plants compared to control plants (Lucas et al. 1993). Our $^{14}CO_2$-labeling experiments were aimed at determining whether the reason for this high accumulation of sugars and starch was due to an alteration in export of newly fixed carbon from the source leaves of *TMV-MP*-expressing plants. Our results indicate that during the photoperiod, fully-expanded source leaves of plant lines 277 and 2004 exported

Table 3. Carbohydrate content within source leaves of transgenic tobacco plants expressing wild-type TMV-MP (line 277), a 10-amino acid C-terminal deletion mutant of the TMV-MP (line MP1), and the vector control (line 306). Experimental details as in Table 1 (mean ± SE, $n = 5$)

Time of day	Plant line	Sucrose[a]	Glucose	Fructose	Starch[b]
13:00	277	125.6 ± 12.7 d	236.7 ± 46.4 d	172.5 ± 33.6	817 ± 51 d
	MP1	61.7 ± 16.8 e	143.3 ± 26.8 e	126.5 ± 23.7	521 ± 40 e
	306	65.7 ± 11.5 e	129.4 ± 12.9 e	130.4 ± 12.9	528 ± 54 e
Significance[c]		**	*	ns	**
19:00	277	177.1 ± 8.7 d	194.3 ± 45.9 d	162.6 ± 36.2 d	979 ± 146
	MP1	61.9 ± 14.4 f	105.1 ± 20.5 e	102.5 ± 15.6 e	854 ± 103
	306	115.9 ± 7.3 e	106.9 ± 14.6 e	126.3 ± 14.5 de	758 ± 74
Significance		**	*	*	ns
07:00	277	52.5 ± 9.3 d	169.9 ± 37.4 d	129.4 ± 21.4 d	954 ± 151 d
	MP1	5.5 ± 4.3 e	64.7 ± 7.8 e	72.3 ± 13.5 e	445 ± 72 e
	306	11.2 ± 5.0 e	59.8 ± 10.0 e	92.4 ± 14.0 de	304 ± 33 e
Significance		**	**	*	**

[a] Sugars presented as $\mu g \cdot cm^{-2}$
[b] Starch content presented as μg glucose equivalents $\cdot cm^{-2}$
[c] *, **, and ns represent significant ($P = 0.05$ and $P = 0.01$) and not significantly different, respectively (d, e, f indicate significant differences between values, using the Student-Newman-Keuls multiple range test; ns, no significant difference)

Table 4. Carbohydrate content within source leaves of transgenic tobacco plants expressing wild-type TMV-MP (lines 277, 2004), a 10 amino acid C-terminal deletion mutant of the TMV-MP (second, independent transformant, line MP1-1), and the vector controls (lines 306 and 3001). Experimental details as in Table 1 (mean ± SE, $n = 5$)

Time of day	Plant line	Sucrose[a]	Glucose	Fructose	Starch[b]
13:00	277	194.6 ± 23.7 d	311.1 ± 24.7 d	182.0 ± 18.0	1129 ± 98 d
	MP1-1	106.9 ± 11.6 e	175.4 ± 25.2 e	143.8 ± 16.9	500 ± 15 e
	306	112.1 ± 20.0 e	173.4 ± 32.9 e	143.9 ± 23.5	588 ± 80 e
Significance[c]		**	**	ns	**
	2004	158.1 ± 20.9 f	317.9 ± 25.3 f	182.5 ± 32.1 f	956 ± 141 f
	3001	62.1 ± 8.9 g	69.5 ± 16.7 g	75.3 ± 16.5 g	301 ± 29 g
Significance		**	**	**	**
19:00	277	208.2 ± 21.0 d	277.8 ± 21.3 d	165.3 ± 14.3 d	1438 ± 146 d
	MP1-1	99.3 ± 7.1 e	128.7 ± 12.6 e	104.7 ± 6.9 e	584 ± 103 e
	306	119.7 ± 21.7 e	146.3 ± 28.8 e	111.7 ± 16.8 e	822 ± 123 e
Significance		**	**	*	**
	2004	187.3 ± 19.6 f	271.7 ± 11.0 f	150.2 ± 15.8 f	1091 ± 59 f
	3001	64.8 ± 3.8 g	54.2 ± 8.2 g	74.0 ± 7.2 g	396 ± 39 g
Significance		**	**	**	**
07:00	277	140.3 ± 13.4 d	286.1 ± 13.5 d	172.2 ± 15.6 d	1048 ± 89 d
	MP1-1	46.0 ± 6.0 e	86.7 ± 19.0 e	96.2 ± 11.7 e	424 ± 66 e
	306	49.3 ± 8.5 e	92.0 ± 24.4 e	98.8 ± 15.2 e	488 ± 135 e
Significance		**	**	**	**
	2004	110.6 ± 18.2 f	247.5 ± 31.2 f	154.8 ± 21.9 f	1132 ± 129 f
	3001	38.6 ± 1.2 g	54.3 ± 19.2 g	54.4 ± 6.2 g	189 ± 41 g
Significance		**	**	**	**

[a] Sugars presented as $\mu g \cdot cm^{-2}$
[b] Starch content presented as μg glucose equivalents $\cdot cm^{-2}$
[c] *, **, and ns represent significant ($P = 0.05$ and $P = 0.01$) and not significantly different, respectively (d, e, f, g indicate significant differences between values, using the Student-Newman-Keuls multiple range test)

^{14}C at a lower rate than equivalently-aged control leaves (Fig. 2A, B). In addition, preliminary experiments performed on transgenic tobacco plants expressing both the TMV-MP and the TMV-coat protein yielded identical results, in that export was reduced during the day in the presence of the TMV-MP, but not in the presence of the TMV-coat protein (data not shown).

Given that the sucrose levels are significantly different between TMV-MP-expressing plants (lines 277, 2-72 and 2004) and control plants (lines 306 and 3001), it is possible that the differences in ^{14}C export could be accounted for in terms of differences in sucrose-specific activity. The data presented in Table 1 were used to calculate the rate of [^{14}C]sucrose exported from the leaf. For these calculations we assumed that, during the first hours after ^{14}C-labeling, all reductions in [^{14}C]sucrose were due to export via the phloem. We also assumed that within the mature tobacco leaf sucrose is present in one pool in which newly synthesized [^{14}C]sucrose was able to mix with unlabeled sucrose. Based on these assumptions, the calculated values for [^{14}C]sucrose export from plant lines 277 and 306 were 13.6 and 15.3 $\mu g \cdot cm^{-2} \cdot h^{-1}$, respectively. It should be noted that the rate of reduction in the level of radioactivity in the liquid-residue fraction (Table 1) was much greater in plant line 306 as compared with plant line 277, which suggests that the differences in ^{14}C-export between the two lines would actually have been larger than reflected by the above values. Furthermore, parallel measurements of photosynthesis and respiration established that these processes were occurring at equivalent rates in plant lines 277 and 306 (Table 2). Hence, the TMV-MP-induced alteration in ^{14}C turnover cannot be due to fundamental changes in either of these processes. However, we cannot dismiss the possibility that sucrose is stored within several compartments and that newly fixed [^{14}C]sucrose does not exchange uniformly between such sites. Future experiments will explore this possibility.

Experiments performed on young, expanding source leaves (leaf #2), in which the TMV-MP has yet to influence either plasmodesmal SEL (Deom et al. 1990) or sugar levels (Lucas et al. 1993) established that the presence of the TMV-MP also resulted in a small, but statistically significant, reduction in ^{14}C export in plant line 277 compared to control line 306. As the levels of sucrose would have been similar in these young leaves from lines 277 and 306 (Lucas et al. 1993), the observed reduction in ^{14}C-export from 277 compared with 306 plants could not have been due to differences in the specific activity of [^{14}C]sucrose. These results are consistent with our earlier studies performed on a different TMV-MP transgenic plant line (274), in which ^{14}C-labeling experiments established that, under the influence of the TMV-MP, young expanding source leaves partitioned lower amounts of ^{14}C-photosynthate to the lower stem and root tissues, compared with control plants (Lucas et al. 1993). Although the effect of the TMV-MP on export from these leaves is small, the long-term influence of this change in carbon allocation could well result in the observed phenotype of a reduced root-to-shoot ratio in *TMV-MP*-expressing, compared to control tobacco plants (Lucas et al. 1993).

Sucrose is the major translocated sugar in many plant species, including tobacco (Giaquinta 1983). As demonstrated by analysis of the data presented in Table 1, the actual turnover of ^{14}C in the sucrose pool is retarded by the presence of the TMV-MP. Similar trends were also detected for [^{14}C]glucose and [^{14}C]fructose, in that the absolute levels of these sugars were higher *and* the radiolabel was retained in TMV-MP transgenic, compared with control, plants (see also Table 3 and Fig. 3). Given that sucrose is confined mainly to the cytoplasm in wild-type tobacco mesophyll cells (see Heineke et al. 1994), the TMV-MP may interact at any of the regulatory sites involved in controlling the compartmentation and/or the loading of sucrose into the sieve element-companion cell complex. Reduction in phloem loading would result in a rise in cytosolic sucrose which would lead to an increase in the level of fructose-6-phosphate. This change may then lead to an inhibition of triose-phosphate export from the chloroplast which would result in increased partitioning of photosynthate into starch (Stitt and Quick 1989). Although this hypothesis can account for some of the observed effects of the TMV-MP on carbon metabolism and export, as will be pointed out below, the TMV-MP appears to act at additional target sites within the source leaf.

Studies with TMV-MP mutants confirm its pleiotropic nature. The influence of the TMV-MP on carbon metabolism and export in transgenic tobacco plants was further probed using a ts mutant form of the TMV-MP. First, we should emphasize that the similarity in the results obtained on plant lines 2-72 and 277 (Fig. 3E, G, I), under permissive temperatures, provides further support for the hypothesis that the observed changes in carbon metabolism are caused by the presence of the TMV-MP. Under permissive temperatures (25°C), ^{14}C-export during the photoperiod was identical in plant lines 2-72 and 277 (Fig. 3A). Interestingly, in plants expressing the ts form of the *TMV-MP* gene, the level of starch was intermediate between the levels detected in lines 277 and 306 (Fig. 3C). However, under permissive temperatures, the ts and wild-type TMV-MPs elicited similar effects on sugar accumulation (Fig. 3E, G, I).

Under nonpermissive temperatures (32–34°C), the ts TMV-MP has no effect on plasmodesmal SEL (Wolf et al. 1991). Hence, if the influence of the TMV-MP on carbon metabolism were associated with an increase in plasmodesmal SEL, this effect should be negated in plants exposed to a 32–34°C regime. That this was not the case is established by the data presented in Fig. 3. For example, the level to which sucrose accumulated in the afternoon in plant line 2-72 was twice that measured in plant line 306 (Fig. 3F). The level in plant line 277 was intermediate between 2-72 and 306, which is of interest, as in these plants the plasmodesmata would have remained dilated by the TMV-MP (see also Table 1). Thus, the influence of the ts TMV-MP on sucrose levels strongly supports the hypothesis that the TMV-MP is pleiotropic in its effects within transgenic tobacco plants; i.e., this viral protein contains domains that allow it to interact with secondary plasmodesmata to potentiate the cell-to-cell transport of viral RNA (see Lucas and Gilbertson 1994; Waigmann et al. 1994), as well as a domain(s) that interferes with an endogenous process involved in regulating

photoassimilate storage, translocation and partitioning (Huber et al. 1992; Balachandran et al. 1995). Further evidence in support of the pleiotropic nature of the TMV-MP was provided by the finding that, under nonpermissive temperatures, the levels of glucose and fructose were always highest in plant line 2-72 compared with 277 and 306 (Fig. 3H, J).

Our carbohydrate studies performed on transgenic tobacco plants expressing a mutant form of the *TMV-MP* gene, in which the C-terminal 10 amino acids were deleted, provided strong additional support for the hypothesis that the TMV-MP has pleiotropic effects in source tobacco leaves. Although removal of this C-terminal region of the TMV-MP has no effect on its viral-related functions (Berna et al. 1991; Gafny et al. 1992), expression of this mutant form in tobacco plants (line MP1 and MP1-1) resulted in a return to normal photosynthetic carbon metabolism (Tables 3, 4). Since the SEL of mesophyll secondary plasmodesmata, in these transgenic tobacco plants, would still be elevated to values greater than 9.4 kDa (Berna et al., 1991), the results presented in Tables 3 and 4 clearly demonstrate that the TMV-MP has a second property (function), beyond that associated with dilating plasmodesmata, that allows it to interact with cellular processes involved in carbon metabolism and/or export (Fig. 4).

Sink strength can override the influence of TMV-MP. Under the nonpermissive temperature regime used in the ts TMV-MP mutant studies, all plant lines tested behaved in an identical manner, in terms of net ^{14}C-export and starch metabolism (Fig. 3B, D). This result suggests that the interaction of the TMV-MP on the regulatory pathway(s) of photosynthate compartmentation and/or export is highly complex. By treating plants at high temperatures we clearly induced a change in the source/sink balance. Under these elevated temperatures the vegetative apex entered into a phase of accelerated development, as evidenced by a striking increase in growth rate of all plant lines tested (data not shown). As expected, high temperatures also caused a significant increase in dark respiration (Table 2). These temperature-induced changes in the apical tissues reflect a substantial increase in overall sink strength, which resulted in enhanced sucrose transport from the source leaves, thereby reducing carbohydrate levels in the mesophyll (Fig. 3). Thus, the temperature-induced convergence of net carbon export from lines 2-72, 277 and 306 (see Fig. 3B) may reflect the influence of an hierarchical control site, involving a feedback (or feedforward) mechanism whose input signal can override some of the sites where the TMV-MP interacts with the *endogenous* regulatory pathway(s) that controls photosynthate export and partitioning (see Fig. 4).

In this regard it is interesting to note that, under the high temperature regime, the levels of starch were almost identical within the mature source leaves of plant lines 2-72, 277 and 306. Thus, increased sink demand for photosynthate can completely override the effects of the TMV-MP on altered starch metabolism. However, as the levels of sucrose, glucose and fructose remained elevated in the ts TMV-MP plants (line 2-72), it may well be that there are numerous sites at which the TMV-MP can influence (alter) carbon metabolism.

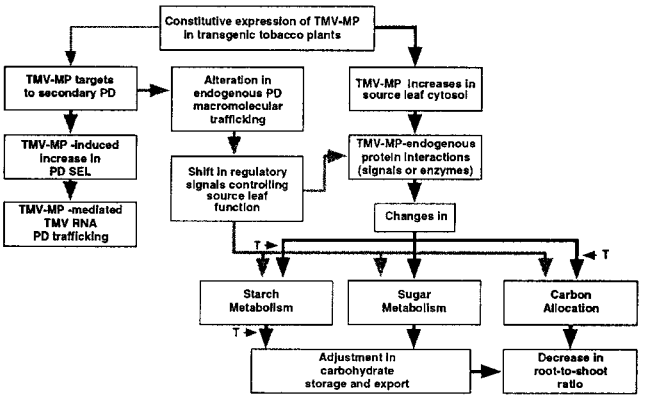

Fig. 4. Schematic diagram illustrating sites where the TMV-MP might interact with the plant's endogenous control network to cause the observed alterations in sugar metabolism and reallocation of photosynthate to yield a reduction of root-to-shoot ratio in transgenic plants expressing the *TMV-MP* gene. T → indicates control sites where the effect of the TMV-MP is overridden when sink strength is altered by raising the growth temperature from 24° to 32°C: PD; plasmodesmata

Future studies will focus on the identification and characterization of the domain(s) within the TMV-MP that causes the observed alterations in carbon metabolism and export. It is anticipated that such studies will provide important insights into the molecular processes involved in the orchestration of photosynthetic carbon metabolism, within the source leaf, in terms of product export and allocation, via the long-distance transport system of the phloem.

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