

Expression of the Movement Protein of Tobacco Mosaic Virus in the Cyanobacterium *Anabaena* sp. Strain PCC 7120

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The movement protein (MP) of tobacco mosaic virus (TMV) alters the structure and function of the plasmodesmata, the intercellular communication channels in plants. cDNA encoding the TMV MP was cloned in a cyanobacterial shuttle vector and transferred to *Anabaena* sp. strain PCC 7120. MP was synthesized in *Anabaena* sp. strain PCC 7120 from the *tac* promoter and was induced severalfold by addition of the *lac* inducer IPTG. Immunoblot analysis of subcellular fractions obtained from IPTG-induced vegetative cells indicated that MP was highly enriched in the cell wall and the cytoplasmic membrane fractions. High levels of MP expression prevented diazotrophic growth and new heterocyst differentiation in cultures of *Anabaena* sp. strain PCC 7120. Although expression of MP in undifferentiated filaments did not inhibit growth, these filaments could not form heterocysts when subsequently deprived of combined nitrogen. Wild-type cells or cells containing the shuttle vector lacking the MP gene grew normally and differentiated heterocysts in the presence of IPTG. These results are consistent with the idea that interaction of MP with components in the cells of *Anabaena* sp. strain PCC 7120 perturbs normal cell-to-cell communication in the filaments, thereby preventing heterocyst differentiation and diazotrophic growth.

Additional keywords: cyanobacteria, differentiation, heterocyst, plasmodesmata.

Cyanobacteria are photosynthetic prokaryotes that have oxygen-evolving photosynthetic reaction centers that are essentially the same as those of higher plants (Bryant 1987). Many strains, including those in the genus *Anabaena*, are capable of aerobic nitrogen fixation in morphologically and biochemically distinct cells called heterocysts, which comprise 5 to 10% of cells in a filament (Fleming and Haselkorn 1973; Peterson and Wolk 1978). Heterocysts have active nitrogenase, lack photosystem II activity, and have additional envelope layers that probably function in oxygen protection of nitrogenase. Thus, the differentiation of a vegetative cell into a heterocyst involves major changes in both cell structure and biochemistry (reviewed Haselkorn 1978; Wolk 1982, 1991; Buikema and Haselkorn 1993).

In the presence of a source of fixed nitrogen, *Anabaena* sp. grows as a filament of apparently identical vegetative cells,

but within hours of removal of fixed nitrogen from the medium, heterocysts appear in a semiregular pattern. During growth with N₂ new heterocysts differentiate from vegetative cells positioned about midway between existing heterocysts (Wilcox et al. 1973), forming a predictable pattern that suggests communication between cells in the filament (Wolk 1991). Ultrastructural studies of cyanobacterial filaments show channels of 20 nm diameter intersecting the cell wall septa of adjacent vegetative cells as well as vegetative-heterocyst cell junctions (Lang and Fay 1971; Giddings and Staehelin 1978). These intercellular connections are believed to be functionally equivalent to plasmodesmata and were named microplasmodesmata (Lang and Fay 1971). However, there is no evidence that microplasmodesmata represent a continuity of plasma membrane. Instead, they may be separate protein channel-forming elements (Giddings and Staehelin 1978). Nevertheless, the microplasmodesmata are potential conduits for the exchange of metabolites and for the movement of molecules that may serve as regulatory signals to control cell differentiation in a filament.

In plants, plasmodesmata maintain continuity between the plasma membrane of adjacent cells and mediate cell-to-cell transport (reviewed Lucas and Wolf 1993). The permeable spaces of plasmodesma that allow transport have gaps of 1.5 to 3.0 nm and a size exclusion limit of about 1,000 Da (reviewed Robards and Lucas 1990; Citovsky and Zambryski 1991); thus, plant viruses or even viral RNA would normally be restricted to the initially infected cell. Many plant viruses overcome these limitations by producing proteins that facilitate the spread of the infection. In the case of tobacco mosaic virus (TMV), a virus-coded movement protein (MP) facilitates cell-to-cell infectivity (Deom et al. 1992). Evidence that MP is required for viral movement includes: (i) Mutations in the MP gene prevent viral movement between cells (Ohno et al. 1983; Zimmern and Hunter 1983; Gafny et al. 1992; Lapidot et al. 1993; Wolf et al. 1991). (ii) The TMV MP binds to plasmodesmata early during infection (Tomenius et al. 1987). (iii) Transgenic plants expressing the TMV MP in the absence of other TMV genes complemented the cell-to-cell movement defect of TMV strains with a mutation in the MP gene (Deom et al. 1987) that are normally unable to spread the infection. Moreover, these transgenic plants demonstrate altered plasmodesmata structure (Atkins et al. 1991; Ding et al. 1992). Mesophyll cells of transgenic plants allowed the passage of

Table 1. Plasmids used in this study

Plasmid	Source or reference	Resistance	Relevant characteristics
pT7-7:MP	Deom, gift	Ap ^r	Source of MP cDNA
pRL443	Elhai 1989	Ap ^r , Tc ^r	Conjugative plasmid (Km ^s mutant of RP4)
pRL502	Elhai gift	Km ^r /Nm ^r	Shuttle vector ^a expressing <i>luxAB</i> from <i>tac</i> promoter
pRL528	Elhai 1988	Cm ^r	Helper plasmid containing <i>M. Aval</i> and <i>M. Eco 4711</i>
pMZ1	This work	Ap ^r	1-kb MP cDNA subcloned in pUC118
pMZ2	This work	Km ^r /Nm ^r	1-kb MP cDNA subcloned in pRL502, replacing <i>luxAB</i>
pMZ11	This work	Km ^r /Nm ^r Sm ^r /Sp ^r	pMZ2 containing Sm ^r /Sp ^r (<i>aad</i>) inserted at <i>Bgl</i> III site of MP cDNA

^a Replicates in *E. coli* and in *Anabaena* sp. strain PCC 7120.

fluorescent dye molecules having a mass as large as 9,400 Da (Wolf et al. 1989, Deom et al. 1990). It was proposed that increasing the molecular size exclusion limit of the plasmodesma is essential for virus spread (Wolf et al. 1989, 1991; Ding et al. 1992). Citovsky et al. (1990, 1992) demonstrated that MP produced in *Escherichia coli* could, *in vitro*, bind and unfold single-stranded nucleic acid into a thin, elongated molecule and suggested that such molecules pass between cells. It has recently been shown that TMV MP obtained by expression in *E. coli* and injected into single cells of wild-type tobacco plants increases the size exclusion limit of plasmodesmata allowing movement of 20 kDa dextrans between cells. MP affected not only the microinjected cells, but also cells distant from the injections site (Waigmen et al. 1994).

Heterocyst-forming cyanobacteria appear to function as a multicellular organism, manifesting a dependency on intercellular communication for patterned differentiation of heterocysts and for growth of the filament. Therefore, we wanted to determine the effect of TMV MP on cell-to-cell communication in filamentous cyanobacteria. The characterization of TMV MP action in transgenic plants suggested that MP might interact with cell structures involved in the transport of molecules in filamentous cyanobacteria. We demonstrate that a cloned cDNA of the TMV MP, transferred to *Anabaena* sp. strain PCC 7120 via a replicating shuttle vector, was maintained during growth and MP was expressed. The expression of TMV MP had a profound effect on differentiated filaments that require cell-to-cell transport of nutrients and intercellular communication.

RESULTS

Transfer of the MP gene to *Anabaena* sp. strain PCC 7120.

The TMV MP cDNA was cloned in a cyanobacterial shuttle vector under the control of the *tac* promoter (pMZ2; Table 1) and was transferred by conjugation to *Anabaena* sp. strain PCC 7120 from *E. coli* HB101. DNA from this axenic Nm^r cyanobacterial strain contained a plasmid that matched pMZ2 in size and restriction sites (data not shown). DNA extracted from the cyanobacterial strain was used to transform *E. coli*. Restriction digests of the plasmid from the *E. coli* transformants gave a pattern that was identical to the original pMZ2 transferred from *E. coli* to *Anabaena* sp. strain PCC 7120, thus providing evidence that the TMV MP cDNA was stably maintained during replication of pMZ2 in *Anabaena* sp. strain PCC 7120 (data not shown).

Detection of TMV MP expression and localization of accumulated MP.

In *E. coli* transcription from the *tac* promoter is efficiently induced by IPTG (de Boer et al. 1983). To determine whether

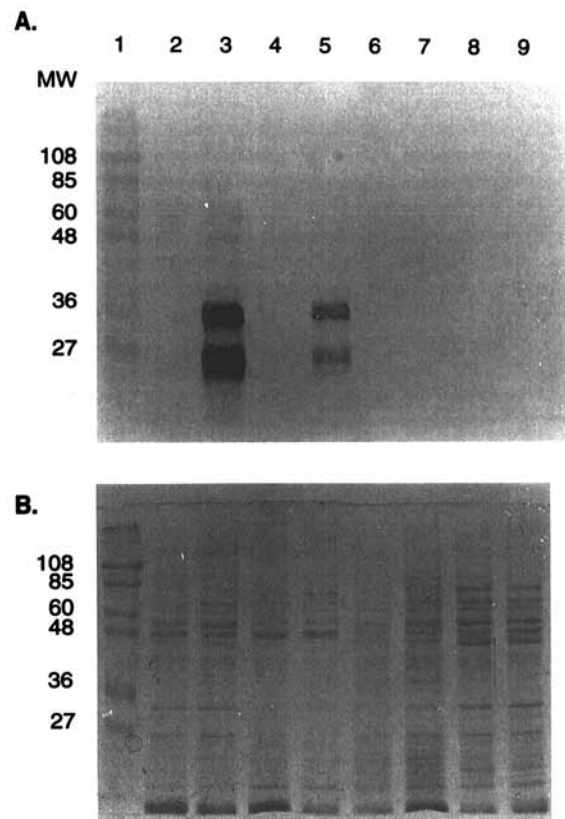


Fig. 1. Expression of tobacco mosaic virus movement protein (TMV MP) in *Anabaena* sp. strain PCC 7120. **A**, Immunoblot analysis of *Anabaena* sp. strain PCC 7120 (pMZ2) (lanes 2–5), *Anabaena* sp. strain PCC 7120 (lanes 6–9), IPTG induced (lanes 3, 5, 7, 9), uninduced (lanes 2, 4, 6, 8), nondifferentiated filaments (lanes 2, 3, 6, 7), and differentiated filaments (lane 4, 5, 8, 9). Lane 1 contains prestained protein molecular mass markers (Sigma) as indicated (kDa). Ten micrograms of protein was applied in each lane. **B**, Coomassie-stained gel as in **A**.

the TMV MP could be expressed in cyanobacteria, 1.0 mM IPTG was added during growth to *Anabaena* sp. strain PCC 7120 containing pMZ2. Immunoblot analysis of total protein extracts from differentiated or nondifferentiated filaments showed a protein of about 32 kDa from IPTG-induced cells of *Anabaena* sp. strain PCC 7120 with pMZ2 (Fig. 1A). In addition, an immunoreactive band with lower apparent molecular mass was observed (lanes 3 and 5). A faint immunoreactive band was detected in noninduced cultures (lane 2), indicating a low level of constitutive expression of MP in *Anabaena* sp. strain PCC 7120 with pMZ2. There was no cross-reactivity of the antiserum to normal cell proteins of wild-type *Anabaena* sp. strain PCC 7120 (lanes 6 to 9). No

immunological reaction was detected in extracts of *Anabaena* sp. strain PCC 7120 cells containing pRL502 (the parent plasmid without the MP gene) with or without treatment with IPTG (data not shown). The increase in the amount of MP after induction with IPTG indicated that the TMV MP gene was expressed under the control of the *tac* promoter in cells grown either with or without a source of fixed nitrogen.

In tobacco plants the MP accumulates in the cell wall fraction of transgenic plant cells that express the MP and show altered plasmodesmata function (Deom et al. 1990). We investigated the subcellular location of MP in *Anabaena* sp. strain PCC 7120 by crude fractionation of vegetative cells. A fraction rich in membranes and cell wall was compared to the soluble fraction of cells obtained from strains with and without pMZ2, either IPTG-induced or noninduced. Immunoblot analysis of MP after SDS/PAGE analysis of the cell fractions is shown in Figure 2. A 32-kDa MP was present in the soluble fraction (Fig. 2A, lane 5) but accumulated predominantly in the crude membrane fraction of IPTG-induced cells (Fig. 2A, lane 1). Immunoreactive bands with apparent molecular masses of less than 30 kDa and more than 32 kDa, which have been reported to accumulate in different tissues of leaves of transgenic tobacco plants (Deom et al. 1990) were also observed in *Anabaena* sp. strain PCC 7120. No cross-reactive

bands were seen in the soluble or crude membrane fractions from wild-type cells; thus, the anomalously migrating immunoreactive proteins are probably components of the TMV MP.

The cell wall, the thylakoid membrane, and the cytoplasmic membrane of IPTG-induced nondifferentiated filaments of cells with pMZ2 were separated on sucrose gradients. The visible absorption spectra of the isolated cytoplasmic and thylakoid membrane preparations from IPTG-induced vegetative cells of this strain was similar to the visible absorption spectra of these subcellular structures isolated from other cyanobacteria (Murata and Omata 1988; Smith et al. 1992) (spectra not shown).

The proteins of each fraction were separated by SDS/PAGE and blotted to nitrocellulose (Fig. 3). In Figure 3A and B are shown pictures of the same immunoblot, first stained with amido black (Fig. 3B) for the detection of protein, then washed and used for detection of TMV MP. Unfortunately, the amido black-stained protein bands were still visible after washing (compare Fig. 3A with Fig. 3B), although the purple immunoreactive bands (indicated by the arrows) were clearly distinguishable. The protein profile of each membrane frac-

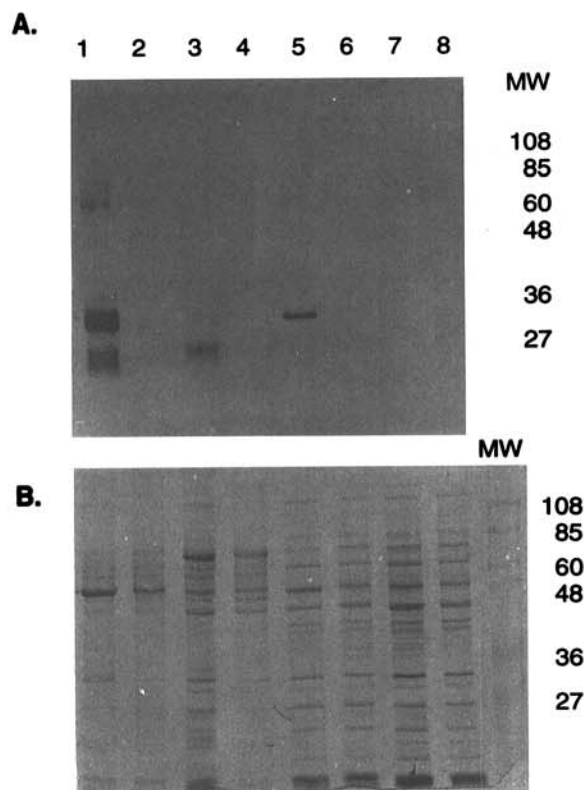


Fig. 2. Tobacco mosaic virus movement protein (TMV MP) in soluble versus membrane fractions. **A**, Immunoblot analysis of crude cell membrane fractions (P2) (lanes 1–4) and soluble fractions (S2) (lanes 5–8) from *Anabaena* sp. strain PCC 7120 (pMZ2) induced with IPTG (lanes 1 and 5) or not induced (lanes 3 and 6), and from wild-type *Anabaena* sp. strain PCC 7120, IPTG-induced (lanes 2 and 7) or not induced (lane 4 and 8). MW indicates prestained protein molecular mass markers (kDa). Ten micrograms of protein was applied in each lane. **B**, Coomassie-stained gel as in A.

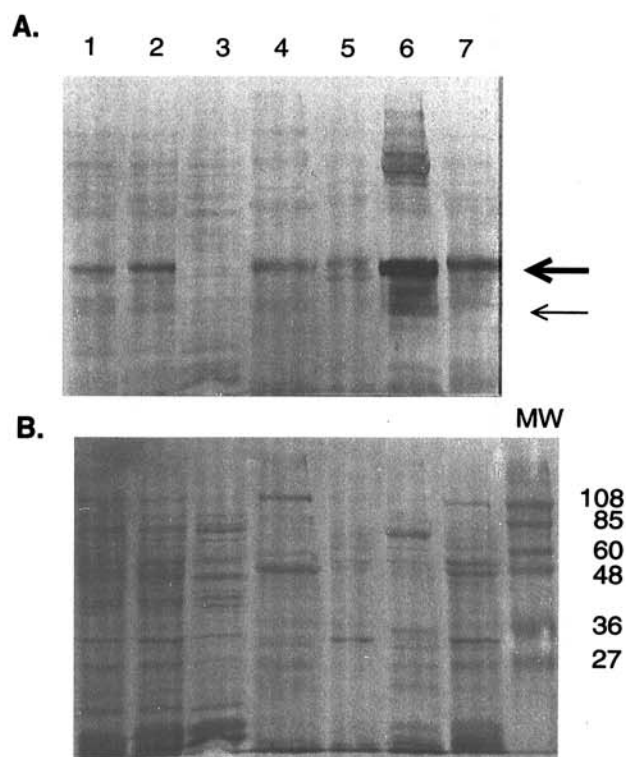


Fig. 3. Subcellular localization of movement protein (MP) in IPTG-induced cells of *Anabaena* sp. strain PCC 7120 (pMZ2). **A**, Immunoblot analysis of MP in total cell protein (lane 1), S1 (crude cell-free lysate) (lane 2), S2 (soluble) (lane 3), CM (cytoplasmic membrane) (lane 4), TM (thylakoid membrane) (lane 5), CW (cell wall) (lane 6), P2 (crude membrane fraction) (lane 7). MW indicates prestained protein molecular mass markers (kDa). Ten micrograms of protein was applied in each lane. Arrows indicate strong (heavy arrow) and weaker (light arrow) immunoreactive bands on the blot that had been previously stained with amido black (see Fig. 4B). The original blot showed purple immunoreactive bands that were easily distinguished from the blue-black amido black stained protein bands. **B**, Amido black-stained nitrocellulose filter before immunoblot analysis.

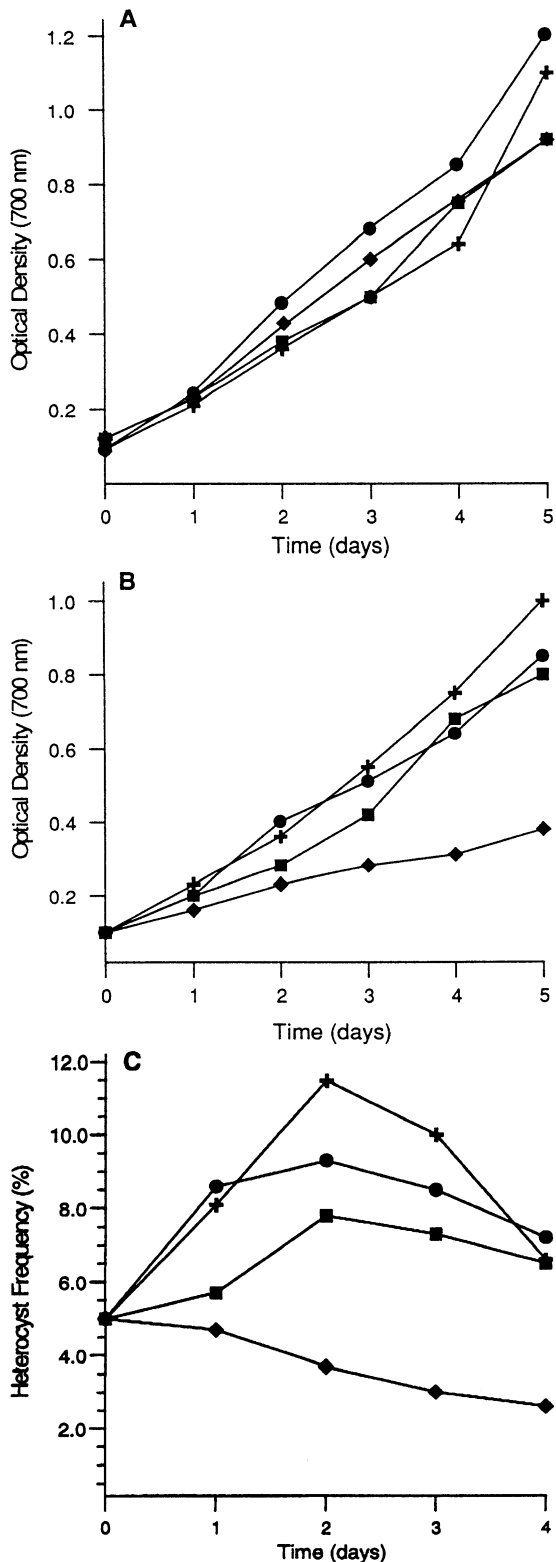


Fig. 4. Growth and heterocyst differentiation of *Anabaena* sp. strain PCC 7120 cells expressing MP. **A**, Cells were grown in media containing nitrate to repress heterocyst differentiation and induced with 1 mM IPTG at zero time. **B**, Cells were grown in media lacking a source of fixed nitrogen to mid log phase, diluted, and induced with IPTG at zero time. **C**, Heterocyst frequency for cultures shown in **B**. Cross = *Anabaena* sp. strain PCC 7120, without IPTG. Bullet = *Anabaena* sp. strain PCC 7120, with IPTG. Square = *Anabaena* sp. strain PCC 7120 (pMZ2), without IPTG. Diamond = *Anabaena* sp. strain PCC 7120 (pMZ2), with IPTG.

tion (lanes 4 to 6) indicated a manifold purification as compared to the crude membrane fraction (lane 7) or the soluble fraction (lane 3). The TMV MP was detected in the CM (lane 4), the TM (lane 5), and the CW (lane 6) fractions. The MP antibody primarily detected a protein migrating at approximately 32 kDa (Fig. 3A, heavy arrow). Other minor immunoreactive bands were detected in the CW and other membrane fractions. The most intense minor band is indicated by the light arrow (Fig. 3A). Based on the intensity of immunoreactive bands in lanes containing approximately equal amounts of protein, the TMV MP was consistently found to be most abundant in the cell wall fraction.

The effect of MP gene expression on growth and heterocyst differentiation of *Anabaena* sp. strain PCC 7120.

The effect of expression of the TMV MP gene on growth and heterocyst differentiation was determined for IPTG-induced cultures grown with or without nitrate. In the absence of IPTG, *Anabaena* sp. strain PCC 7120 (pMZ2) grew well with (Fig. 4A) or without (Fig. 4B) nitrate. In the presence of IPTG and nitrate, *Anabaena* sp. strain PCC 7120 (pMZ2) grew as well as wild-type cells (Fig. 4A). In contrast, IPTG-induced cells with pMZ2 grew poorly in the absence of a source of fixed nitrogen (Fig. 4B). After several days with IPTG, cells grown without nitrate became starved for fixed nitrogen, degraded the biliproteins, and turned yellow. Wild-type cells grew well with or without nitrate and were not affected by IPTG (Fig. 4A and B). Cells with a plasmid carrying a mutated MP gene (pMZ11) (with an insertion mutation at amino acid 23 in the MP gene) grew at the same rate as cells without a plasmid in media without nitrate, with or without IPTG (Fig. 5). Cells carrying the parent plasmid without the TMV MP gene (pRL502) grew at the same rate as cells without a plasmid in media with or without nitrate, with or without IPTG (data not shown).

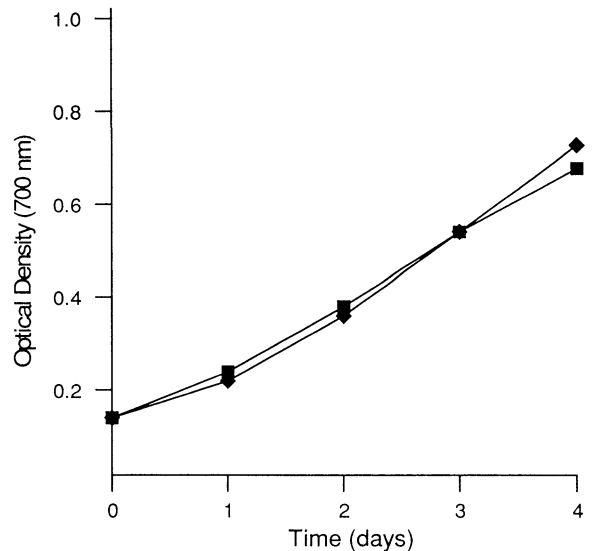


Fig. 5. Growth of *Anabaena* sp. strain PCC 7120 cells containing plasmid pMZ11 (with an insertion in MP cDNA). Cultures were grown with nitrate, with or without IPTG, then washed free of nitrate and resuspended in media lacking nitrate, with or without IPTG. Square = *Anabaena* sp. strain PCC 7120 (pMZ11), without IPTG. Diamond = *Anabaena* sp. strain PCC 7120 (pMZ11), with IPTG.

The frequency of heterocysts was compared in the IPTG-induced and noninduced cells with pMZ2 or without the plasmid (Fig. 4C). IPTG-treated cells with pMZ2, which grew poorly, had the lowest frequency of heterocysts. The heterocyst frequency decreased from 5% to about 2% after induction of MP. Cells with pMZ2 that were not IPTG-induced had a lower heterocyst frequency than wild-type cells (induced or not). All other cultures showed a normal initial increase in heterocyst frequency (8 to 12%), followed by a decrease to about 7%. The heterocyst frequency was similar in filaments of wild-type cells grown with or without IPTG; thus the poor growth and reduced heterocyst frequency in filaments expressing the MP was not due to IPTG addition per se. In addition, IPTG-induced cells containing pMZ11 (with an inser-

tion mutation in the MP gene) grew normally and differentiated 5 to 10% heterocysts in a medium lacking fixed nitrogen. Cells containing pRL502 (lacking the MP gene, but containing *luxAB* under the control of the *tac* promoter) also grew normally and differentiated heterocysts in a medium lacking fixed nitrogen (data not shown).

The effect of TMV MP on *de novo* heterocyst differentiation was determined after nitrogen stepdown. Cells of *Anabaena* sp. strain PCC 7120 (pMZ2) were grown with nitrate in the presence or absence of IPTG for 48 hr prior to removal of nitrate from the medium. In the presence of nitrate there appeared to be no adverse effect of IPTG on the growth of this strain (see Fig. 3A). However, IPTG-treated cultures formed very few heterocysts after nitrogen stepdown and the cultures did not grow (Fig. 6A and B). Cultures that were not treated with IPTG differentiated heterocysts, but the frequency was lower than in the wild-type cells. In contrast, cells without pMZ2 produced heterocysts within 24 hr of nitrogen stepdown, exhibiting similar frequencies with or without IPTG treatment. Cells with pRL502 or pMZ11 also differentiated heterocysts normally in the presence of IPTG and the cultures grew well (data not shown).

DISCUSSION

The TMV MP gene, under the control of an inducible *tac* promoter, was stably maintained and expressed in *Anabaena* sp. strain PCC 7120 on a replicating plasmid, pMZ2. An increase in MP after IPTG induction provided evidence that the MP gene was expressed in *Anabaena* sp. strain PCC 7120 under the control of the *tac* promoter. Oliver et al. (1986) reported the *in vitro* synthesis of a 32-kDa peptide and a 28-kDa peptide from TMV MP cDNA. It was suggested that the lower molecular mass component was formed by initiation of MP synthesis at an internal AUG, in frame with the MP gene. The 32-kDa immunoreactive form of the protein was the only form detected in the soluble fraction of induced cyanobacterial cultures; thus it probably represents the form in which MP was synthesized in *Anabaena* sp. strain PCC 7120. Membrane fractions contained the lower molecular mass components of MP, possibly due to proteolysis. In *Anabaena* sp. strain PCC 7120 the higher molecular mass MP components may be multimers of TMV MP or may represent stable interactions of MP with specific cellular components. Such an explanation was proposed by Deom et al. (1990) for the 52-kDa component observed in transgenic tobacco plants expressing the TMV MP.

Physiological studies indicated that all strains used in these experiments grew well when induced with IPTG in the presence of a source of fixed nitrogen. Therefore, MP does not appear to inhibit normal growth of vegetative cells. Growth in an environment lacking combined nitrogen requires the differentiation of heterocysts from vegetative cells to supply fixed nitrogen to the filament and requires the transfer of metabolites between vegetative cells and heterocysts. Cultures of differentiated filaments fixing N_2 grew well with low-level expression of MP, but grew poorly and produced few heterocysts after addition of IPTG, which induced high-level expression of MP. This effect was not attributable to the presence of a replicating plasmid carrying a foreign gene that was highly expressed, since *luxAB* expression from the same pro-

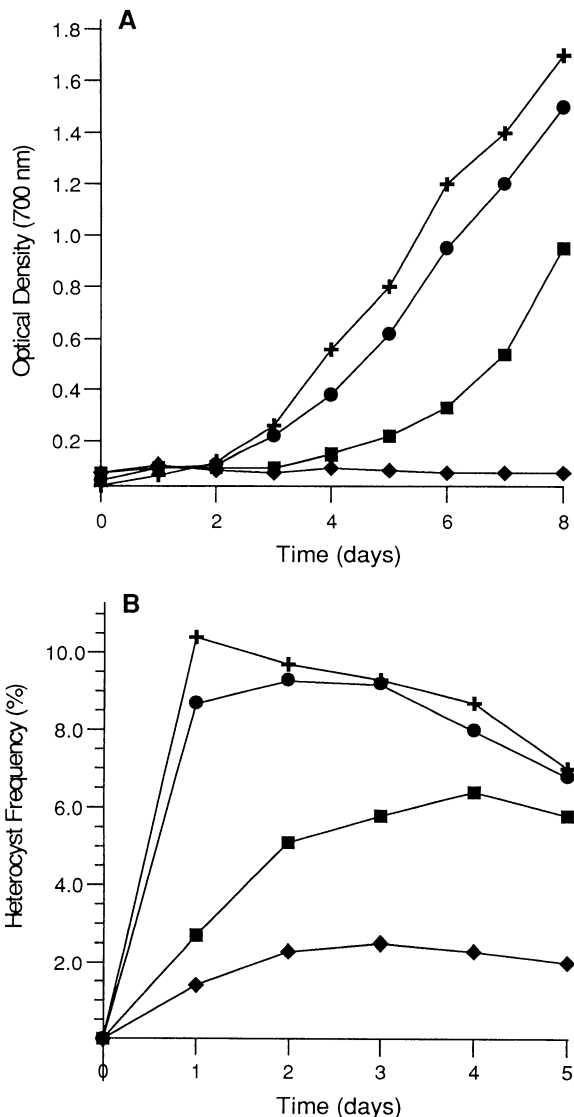


Fig. 6. The effect of movement protein on *de novo* differentiation of heterocysts. Cultures were grown with nitrate, with or without IPTG, then washed free of nitrate and resuspended in media lacking nitrate, with or without IPTG. **A**, Growth. **B**, Heterocyst frequency. Cross = *Anabaena* sp. strain PCC 7120, without IPTG. Bullet = *Anabaena* sp. strain PCC 7120, with IPTG. Square = *Anabaena* sp. strain PCC 7120 (pMZ2), without IPTG. Diamond = *Anabaena* sp. strain PCC 7120 (pMZ2), with IPTG.

motor had no such effect on heterocyst differentiation. Similarly, the effect disappeared when the MP gene was inactivated by an insertion mutation. The inability of undifferentiated filaments expressing MP protein at high level to produce heterocysts upon nitrogen stepdown supports the idea that MP interferes with heterocyst differentiation. In the absence of heterocysts, the cultures died. The inability of cultures expressing high levels of MP to differentiate heterocysts may be attributable to MP-induced alterations in cell-to-cell signaling or in cell-to-cell transport of metabolites.

Cell fractionation studies of transgenic plants and TMV-infected plant tissue show MP accumulating in the cytoplasmic membrane and cell wall (Deom et al. 1990). In cyanobacteria the cytoplasmic membrane and cell wall form the junctions that separate cells in a filament and, thus, would be potential sites for cell-to-cell communication. Analysis of subcellular fractions obtained from vegetative cells showed that MP accumulated in all membrane fractions, but particularly in the cell wall fraction. Cyanobacteria have a typical gram-negative cell wall comprising a peptidoglycan layer outside the cytoplasmic membrane and an outer membrane. The binding of MP to the outer membrane portion of the cell wall is indicated by its presence in the SDS-solubilized portion of the cell wall fraction of lysozyme treated cells. Thus, the presence of MP in the outer membrane requires either that there be connections between the inner and outer membranes or that MP is translocated to the outer membrane. Structural data show the cell wall closely associated with microplasmodesmata located in the area of contact between cells (Lang and Fay 1971). The binding of MP to structures such as the cell wall and cytoplasmic membrane is consistent with the idea that MP in *Anabaena* sp. strain PCC 7120 may interfere with cell-to-cell communication since the transfer of substances into and out of the cell is controlled by the cytoplasmic membrane and possibly the cell wall membrane.

The physiological studies show that MP adversely affected growth of *Anabaena* sp. strain PCC 7120 under conditions requiring interaction between cells. After nitrogen stepdown, which induces heterocyst differentiation, intercellular communication is important for heterocyst pattern development (Wolk 1991). During diazotrophic growth, fixed carbon must be transported from vegetative cells to heterocysts and fixed nitrogen must be transported from heterocysts to vegetative cells in the filament (reviewed in Wolk 1982). The inefficiency of heterocyst formation when MP is made suggests that MP interferes with the normal response to physiological signals for heterocyst differentiation. Our subcellular localization data are consistent with the hypothesis that MP interacts with structures associated with intercellular transport of nutrients.

Interestingly, the ability of TMV MP to modify the molecular size exclusion limit in the study of Deom et al. (1990) was dependent on the developmental stage of the leaf. Plasmodesmata of young leaf mesophyll cells expressing MP constitutively were able to restrict the movement of the larger fluorescent probes that moved freely in MP-expressing mesophyll cells of mature tobacco leaves. The authors proposed that a critical level of MP may be required before plasmodesmata are modified by MP binding. In *Anabaena* sp. strain PCC 7120 the level of MP was controlled by IPTG. Only at a high level of expression was MP associated with poor growth and

low heterocyst frequency (Figs. 4B, 4C, 6A, 6B).

The current belief that cell-to-cell communication channels are important for normal development was based on experiments involving gap junctions. The injection of antibodies against a 27-kDa gap-junction protein selectively blocks cell-to-cell dye movement and causes developmental defects in embryos (Warner et al. 1984). Similarly, high-level expression of MP represses cell differentiation in *Anabaena* sp. strain PCC 7120.

METHODS AND MATERIALS

Organisms and growth conditions.

Anabaena sp. strain PCC 7120 strain was grown in an eightfold dilution of the medium of Allen and Arnon (1955) (AA/8) or in AA/8 media supplemented with 2.5 mM NaNO₃ and 2.5 mM KNO₃ (AA/8 and nitrate). Cyanobacterial cultures were maintained on AA or on BG-11 (Allen 1968) media solidified with 1.5% Difco Bacto agar (Thiel et al. 1989). Strains containing a plasmid were grown on agar media supplemented with either 25 µg ml⁻¹ neomycin (Nm), or 2.5 µg ml⁻¹ each streptomycin (Sm) and spectinomycin (Sp); or in liquid media with Nm at 5 µg ml⁻¹ or with 0.5 µg ml⁻¹ each Sm and Sp. All strains were routinely grown as 50-ml cultures in 125-ml Erlenmeyer flasks at 30°C on a reciprocal shaker under cool-white fluorescent lights (approximately 50 µeinsteins m⁻² s⁻¹).

E. coli HB101 containing various plasmids was grown in Luria broth or on Luria agar at 30°C or 37°C, supplemented when appropriate with ampicillin (50 µg ml⁻¹), kanamycin (50 µg ml⁻¹) chloramphenicol (25 µg ml⁻¹), Sp (50 µg ml⁻¹), or tetracycline (12 µg ml⁻¹).

Subcloning the TMV MP gene and transfer to *Anabaena* sp. strain PCC 7120.

The cDNA for the MP gene of TMV (Oliver et al. 1986; Deom et al. 1987) was provided by Deom as an insert in the plasmid pT7-7. The MP cDNA was excised from the vector pT7-7:MP with *Bam*HI and *Xba*I, producing a 1.0-kb fragment that was then inserted between the *Bam*HI and *Xba*I sites of pUC118 to create pMZ1. The MP gene was excised from pMZ1 with *Bam*HI and *Sal*I and cloned between those same sites in plasmid pRL502 (kindly provided by Jeff El-hai). pRL502 is a cyanobacterial shuttle vector that replicates in *Anabaena* sp. strain PCC 7120. It is based on pRL25 (Wolk et al. 1988) and contains *luxAB* (Engebrecht et al. 1983) fused to the *tac* promoter. The *luxAB* genes were removed from pRL502 by digestions with *Bam*HI and *Sal*I and replaced with the MP gene (under the control of the *tac* promoter) creating plasmid pMZ2. pRL502 also contains a *lacI*^q gene encoding the *lac* repressor and a kanamycin/neomycin resistance gene (*npt* from Tn5). Plasmid pMZ11 was constructed to contain an insertion mutation inactivating the MP gene. It was constructed by inserting a Sm/Sp resistance gene (*aadA*) with *Bam*HI ends into the *Bgl*III site of the MP gene interrupting the MP at leucine (the 24th amino acid) in plasmid pMZ1. The interrupted MP gene was then subcloned into pRL502, under the control of the *tac* promoter, as described above for the construction of pMZ2.

Plasmid pMZ2, pRL502, or pMZ11 was transformed into *E. coli* HB101 containing the plasmid pRL528 that provides

the *trans*-acting *mob* gene function required for conjugation. pRL528 also encodes methylases that protect plasmids from restriction by two cyanobacterial restriction enzymes, *Ava*I and *Ava*II (Elhai and Wolk 1988). Plasmid pMZ2, pRL502, or pMZ11 was transferred by conjugation to *Anabaena* sp. strain PCC 7120 from *E. coli* HB101 using the conjugal plasmid pRL443 (a derivative of RP4 that is Km^r) (Elhai and Wolk 1988). The triparental mating was carried out essentially as described by Elhai and Wolk (1988) except that cells on the mating filter were transferred after 24 h to 50 ml of AA/8 and nitrate liquid medium and incubated with shaking in the light for 24 h before the cells were collected by centrifugation and plated on BG-11 agar containing Nm at 25 µg ml⁻¹ or Sm/Sp at 2.5 µg ml⁻¹ each. Antibiotic-resistant exconjugant colonies were clearly visible within 1 week. DNA extracted from axenic exconjugant colonies was analyzed for the presence of transferred plasmid. Plasmid pMZ2 was identified by the presence of the 1.0-kb MP gene fragment after *Bam*HI and *Sa*II enzyme digestion, and, after Southern transfer, by hybridization to the TMV MP-cDNA. The presence of pRL502 and pMZ11 in *Anabaena* sp. strain PCC 7120 was verified by agarose gel electrophoresis and by Southern analysis using a portion of the vector, pRL502, as a probe. In addition, *Anabaena* sp. strain PCC 7120 colonies carrying pRL502 (with *luxAB*) were identified visually in a darkroom by their light production in the presence of *n*-decanaldehyde (placed in the lid of the petri dish) (Schmetterer et al. 1986). Wild-type colonies of *Anabaena* sp. strain PCC 7120 produced no light.

Detection of TMV movement protein in *Anabaena* sp. strain PCC 7120.

Expression of the MP gene under the control of the *tac* promoter was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to cultures at 1.0 mM final concentration. Total cell protein was extracted from cell pellets from 5–15 ml of culture by boiling for 3 min in protein gel loading buffer (Sambrook et al. 1989). Protein concentration was assayed by BCA assay (bicinchoninic acid) (Pierce) with bovine serum albumin (BSA) as the protein standard. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) (4% stacking gel, 12% separating gel) and electroblotted onto nitrocellulose (Schleicher & Schuell; Keene, NH, BA85). The antigen (MP) was detected by immunoreaction to rabbit antibody raised against a synthetic peptide corresponding to 19 amino acids near the carboxy-terminus of the TMV MP (Atkins et al. 1991; Berna et al. 1991). The rabbit antiserum was detected by reacting with alkaline phosphatase-conjugated goat antirabbit IgG as secondary antibody.

Subcellular fractionation.

Cultures of *Anabaena* sp. strain PCC 7120 grown in AA/8 and nitrate were induced with IPTG for 48 hr, harvested, washed, and treated with lysozyme and EDTA according to Murata and Omata (1988). The cells were washed twice by centrifugation in 20 mM TES-NaOH (pH 7.0), 600 mM sucrose to remove EDTA, and mechanically lysed in a slurry of buffered glass beads by alternately vortexing and chilling on ice for 1 min. After DNase I treatment (15 min at 37°C), a cell free lysate (S1) was obtained by centrifugation at 5,000 ×

g for 10 min. In experiments comparing subcellular fractions of IPTG-induced to noninduced cultures, S1 was obtained as described above, but lysozyme and DNase I treatment were omitted.

S1 was fractionated by flotation centrifugation on a discontinuous sucrose density gradient to isolate the cytoplasmic membrane (CM), thylakoid membrane (TM), and cell wall (CW) fractions (Murata and Omata 1988; Omata and Murata 1983; Peschek et al. 1988). This yielded the CM fraction (yellow band) at 30% sucrose, the TM fraction (green band) at 39 to 50% sucrose, and a CW pellet at 50% sucrose. The CM and TM fractions were collected separately and diluted threefold with buffer (10 mM TES-NaOH, pH 7.0, 10 mM NaCl). The CW pellet was resuspended in buffer and all fractions were concentrated by centrifugation (130,000 × *g* for 1.6 hr in a Ty 65 rotor). The pellets were resuspended in buffer and subjected to two additional cycles of centrifugation on separate discontinuous sucrose density gradients producing manifold purified CM, TM, and CW fractions. The CM and TM fractions were analyzed spectrophotometrically for identification of their characteristic membrane pigments (Omata and Murata 1983). S1 was also subjected to high-speed centrifugation (1.6 h at 130,000 × *g* in a Ty 65 rotor), giving the soluble supernatant fraction (S2) and a pellet (P2) representing the crude membrane fraction (containing the cell wall with cytoplasmic and thylakoid membranes). All subcellular fractions were stored at -70°C in 10% glycerol 10 mM Tris-HCl, pH 7.0, prior to protein determination, SDS/PAGE analysis and immunoblotting. Protease inhibitors (1.0 mM phenyl methyl sulfonyl fluoride, 10 mM benzamide HCl, 10 µg ml⁻¹ phenanthroline, 10 µg ml⁻¹ aprotinin, 10 µg ml⁻¹ leupeptin, and 10 µg ml⁻¹ pepstatin A) were present throughout subcellular fractionation procedures (Barrett 1980).

Physiological studies.

To determine the effect of MP expression on growth and heterocyst differentiation, cells were diluted from log phase liquid culture to OD₇₀₀ of 0.025 to 0.1 and induced by the addition of 1.0 mM IPTG. For nitrogen stepdown experiments, cultures were shifted after 48 h of growth with IPTG in AA/8 and nitrate to nitrate-free AA/8 containing IPTG. Control cultures were shifted to the same medium lacking IPTG. All strains with a plasmid were grown with the appropriate antibiotic. Growth was measured as OD₇₀₀. Filaments were examined by light microscopy (600×) for determination of heterocyst frequency.

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LITERATURE CITED

- Allen, M. B., and Arnon, D. I. 1955. Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. *Plant Physiol.* 30:366-372.
- Allen, M. M. 1968. Simple conditions for the growth of unicellular blue-green algae on plates. *J. Phycol.* 4:1-4.
- Atkins, D., Hull, R., Wells, B., Roberts, K., Moore, P., and 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.

- Barrett, A. J. 1980. The many forms and functions of cellular proteinases. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* 39:9-14.
- Berna, A., Gafny, R., Wolf, S., Lucas, W. J., Holt, C. A., and Beachy, R. N. 1991. The TMV movement protein: Role of the C-terminal 73 amino acids in subcellular localization and function. *Virology* 182:682-689.
- Bryant, D. A. 1987. The cyanobacterial photosynthetic apparatus: comparison of those of higher plants and photosynthetic bacteria. Pages 423-500 in: *Photosynthetic Picoplankton, Canadian Bulletin of Fisheries and Aquatic Science, Vol. 214*. T. Platt and W. K. W. Li, eds. Department of Fisheries and Oceans, Ottawa.
- Buikema, W. J. and Haselkorn, R. 1991. Isolation and complementation of nitrogen fixation mutants of the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* 173:879-1885.
- Citovsky, V., Knorr, D., Schuster, G., and Zambryski, P. 1990. The P30 movement protein of tobacco mosaic virus is a single-strand nucleic acid binding protein. *Cell* 60:637-647.
- Citovsky, V., Wong, M. L., Shaw, A. L., Prasad, B. V., and Zambryski, P. 1992. Visualization and characterization of tobacco mosaic virus movement protein binding to single-stranded nucleic acids. *Plant Cell* 4:397-411.
- Citovsky, V., and Zambryski, P. 1991. How do plant virus nucleic acids move through intercellular connections? *Bioessays* 13:373-379.
- de Boer, H., Comstock, L., and Vasser, M. 1983. The *tac* promoter: A functional hybrid derived from the *trp* and *lac* promoters. *Proc. Natl. Acad. Sci. USA* 80:21-25.
- Deom, C. M., Lapidot, M., and Beachy, R. N. 1992. Plant virus movement proteins. *Cell* 69:221-224.
- Deom, C. M., Oliver, M., and Beachy, R. N. 1987. The 30-kilodalton gene product of tobacco mosaic virus potentiates virus movement. *Science* 237:387-394.
- Deom, C. M., Schubert, S., Wolf, S., Holt, C., Lucas, W. J., and 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., Haudenschild, J. S., Hull, R. J., Wolf, S., Beachy, R. N., and 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.
- Elhai, J., and Wolk, C. P. 1988. Conjugal transfer of DNA to cyanobacteria. *Methods Enzymol.* 167:747-754.
- Engbrecht, J., Neelson, K., and Silverman, M. 1983. Bacterial bioluminescence: Isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell* 32:773-781.
- Fleming, H., and Haselkorn, R. 1973. Differentiation in *Nostoc muscorum*: Nitrogenase is synthesized in heterocysts. *Proc. Natl. Acad. Sci. USA* 70:2727-2731.
- Gafny, R., Lapidot, M., Berna, A., Holt, C. A., Deom, C. M., and Beachy, R. N. 1992. Effects of terminal deletion mutations on function of the movement protein of tobacco mosaic virus. *Virology* 187:499-507.
- Giddings, T. H., Jr., and Staehelin, L. A. 1978. Plasma membrane architecture of *Anabaena cylindrica*: Occurrence of microplasmodesmata and changes associated with heterocyst development and the cell cycle. *Cytobiol.* 16:235-249.
- Haselkorn, R. 1978. Heterocysts. *Annu. Rev. Plant Physiol.* 29:319-344.
- Lang, N. J., and Fay, P. 1971. The heterocysts of blue-green alga. II. Details of ultrastructure. *Proc. R. Soc. B* 178:193-203.
- Lapidot, M., Gafny, R., Ding, B., Wolf, S., Lucas, W. J., and Beachy, R. N. 1993. A dysfunctional movement protein of tobacco mosaic virus that partially modifies the plasmodesmata and limits virus spread in transgenic plants. *Plant J.* 4:959-970.
- Lucas, W. J., and Wolf, S. 1993. Plasmodesmata: The intercellular organelles of green plants. *Trends Cell Biol.* 3:308-315.
- Murata, N., and Omata, T. 1988. Isolation of cyanobacterial plasma membranes. *Methods Enzymol.* 167:245-251.
- Nishiguchi, M., Motoyoshi, F., and Oshima, N. 1978. Behaviour of a temperature sensitive strain of tobacco mosaic virus in tomato leaves and protoplasts. *J. Gen. Virol.* 39:53-61.
- Ohno, T., Takamatsu, N., Meshi, T., Okada, Y., Nishiguchi, M., and Kiho, Y. 1983. Single amino acid substitution in 30K Protein of TMV defective in virus transport function. *Virology* 131:255-258.
- Oliver, M. J., Deom, C. M., De, B. K., and Beachy, R. N. 1986. *In vitro* transcription and translation of cloned cDNAs encoding the 30-kDa protein gene of TMV. *Virology* 155:277-283.
- Omata, T., and Murata, N. 1983. Isolation and characterization of the cytoplasmic membranes from the blue-green alga (cyanobacterium) *Anacystis nidulans*. *Plant Cell Physiol.* 24:1101-1112.
- Peschek, G. A., Molitor, V., Trnka, M., Wastyn, M., and Erber, W. 1988. Characterization of cytochrome *c* oxidase in isolated and purified plasma and thylakoid membranes from cyanobacteria. *Methods Enzymol.* 167:437-449.
- Peterson, R. B., and Wolk, C. P. 1978. High recovery of nitrogenase activity and of ⁵⁵Fe-labeled nitrogenase in heterocysts isolated from *Anabaena variabilis*. *Proc. Natl. Acad. Sci. USA* 75:6271-6275.
- Robards, A. W., and Lucas, W. J. 1990. Plasmodesmata. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 41:369-419.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schmetterer, G., Wolk, C. P., and Elhai, J. 1986. Expression of luciferases from *Vibrio harveyi* and *Vibrio fischeri* in filamentous cyanobacteria. *J. Bacteriol.* 167:411-414.
- Smith D., Bendall, D. S., and Howe, C. J. 1992. Occurrence of a photosystem II polypeptide in non-photosynthetic membranes of cyanobacteria. *Mol. Microbiol.* 6:1821-1827.
- Thiel, T., Bramble, J., and Rogers, S. 1989. Optimum conditions for growth of cyanobacteria on solid media. *FEMS Microbiol. Lett.* 61:27-32.
- Tomenius, K., Clapham, D., and 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.
- Waigman, E., Lucas, W. J., Citovsky, V., and Zambryski, P. 1994. Direct functional assay for tobacco mosaic virus cell-to-cell movement protein and identification of a domain involved in increasing plasmodesmatal permeability. *Proc. Natl. Acad. Sci. USA* 91:1433-1437.
- Warner, A. E., Guthrie, S. C., and Gilula, N. B. 1984. Antibodies to gap-junctional protein selectively disrupt junctional communication in the early amphibian embryo. *Nature* 311:127-131.
- Wilcox, M., Mitchison, G. J., and Smith, R. J. 1973. Pattern formation in the blue-green alga, *Anabaena*. I. Basic mechanisms. *J. Cell Sci.* 12:707-723.
- Wolf, S., Deom, C. M., Beachy, R. N., and 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., and Lucas, W. J. 1991. Plasmodesmatal function is probed using transgenic tobacco plants that express a virus movement protein. *Plant Cell* 3:593-604.
- Wolk, C. P. 1982. Heterocysts. Pages. 359-386 in: *The Biology of Cyanobacteria*. N. G. Carr and B. A. Whitton, eds. Blackwell Scientific Publishers, London.
- Wolk, C. P. 1991 Genetic analysis of cyanobacterial development. *Curr. Opin. Genet. Dev.* 1:336-341.
- Wolk, C. P., Cai, Y., Cardemil, L., Flores, E., Hohn, B., Murry, M., Schmetterer, G., Schrautemeier, B., and Wilson, R. 1988. Isolation and complementation of mutants of *Anabaena* sp. strain PCC 7120 unable to grow aerobically on dinitrogen. *J. Bacteriol.* 170:1239-1244.
- Zimmer, D., and Hunter, T. 1983. Point mutation in the 30K open reading frame of TMV implicated in temperature-sensitive assembly and local lesion spreading of mutant Ni 2519. *EMBO J.* 2:1893-1900.