Plant Cell Physiol. 38(12): 1326–1332 (1997)

The Expression of an Aquaporin Promoter from *Mesembryanthemum crystallinum* in Tobacco

Shigehiro Yamada [1, 4], Donald E. Nelson [1], Eleazar Ley [1], Sheila Marquez [1] and Hans J. Bohnert [1, 2, 3]

[1] *Department of Biochemistry, The University of Arizona, Biosciences West, Tucson, AZ 85721-0088, U.S.A.*
[2] *Department of Plant Sciences and The University of Arizona, Biosciences West, Tucson, AZ 85721-0088, U.S.A.*
[3] *Department of Molecular and Cellular Biology, The University of Arizona, Biosciences West, Tucson, AZ 85721-0088, U.S.A.*

The promoter region of the *MipB* gene encoding an aquaporin from *Mesembryanthemum crystallinum* was isolated and used in a transcriptional fusion to control *uidA* expression in tobacco. The sequence of the promoter was determined for 2 kb upstream of the translation initiation site. Three start sites were utilized with approximately equal frequency, located 176, 170, and 161 bases, respectively, upstream of the translation initiation site. As judged by analysis of GUS expression, promoter *MipB* retains its specificity in transgenic tobacco. In germinating seedlings, all cells showed GUS expression of different intensities with the strongest signals in root meristems. In older seedlings, GUS staining was observed in rapidly expanding cells—root and apical meristem, and lateral root primordia. In mature plants, strong GUS activity was located to glandular trichomes, subepidermal cells of the stem and petioles, to cells surrounding vascular tissues as well as in xylem parenchyma cells. In immature floral organs, GUS expression was strong in sepals, petals, stamen, and pistil. The intensity declined as they matured. In general, this promoter was active in rapidly expanding cells and cells with high water flux capacity, especially in the xylem parenchyma.

Key words: Aquaporin — *Mesembryanthemum crystallinum* — *Nicotiana tabacum* — Promoter analysis — *uidA* expression.

Several members of the MIP (major intrinsic protein) family facilitate the permeation of water through membranes in animals and plants (Fushimi et al. 1993, Chrispeels and Agre 1994, Yamada et al. 1995). These proteins have been termed aquaporins. In plants, aquaporins are located in either the tonoplast (TIP) (Maurel et al. 1993) or the plasma membrane (PIP) (Daniels et al. 1994). PIP and TIP are encoded by a relatively large gene family (Yamada et al. 1995, Park and Saier 1996) which seems to exceed 20 different genes in *Arabidopsis thaliana* (Weig et al. 1997). This complexity may be an indicator of genetic diversification or a sign of functional redundancy in regulatory mechanisms of water conductance. Our interpretation is that multiplicity may be a result of the necessity of precisely controlling water flux in all tissues and cells in response to different developmental and/or environmental cues.

We recently found MIP-homologs in *Mesembryanthemum crystallinum* (common ice plant) as part of a search for transcripts whose expression was affected in roots in response to high salinity (Yamada et al. 1995). Transcripts of seven different genes, termed *Mip*, have been found and characterized (Yamada et al. 1995, Quigley and Ishitani unpublished). Aquaporin function has been documented for two of these ice plant transcripts, *MipA* and *MipB* by *Xenopus* oocyte RNA injection assays and measurements of the influx of water (Yamada et al. 1995). Based on the characterizations and protein parsimony analyses, *MipA* and *MipB* were found to encode PIP-type aquaporins.

We previously reported that *Mip* genes are expressed differentially in tissues and cells, based on RNA gel blot analysis and in situ hybridization (Yamada et al. 1995). Immunological analysis, using peptide antibodies specific for MIPA and MIPB (Kirch and Bohnert unpublished), provided information on the localization of some of these proteins. MIPA and MIPB proteins are present in the epidermis and endodermis of roots, in interstitial parenchyma cells associated with the xylem in the mature root and leaf vascular tissue, and they are expressed more weakly in mesophyll cells. A difference between MIPA and MIPB is the location in the vascular tissue. MIPA is more highly concentrated in phloem and phloem-associated cells, while MIPB is mostly localized in vascular parenchyma and xylem parenchyma cells (Kirch and Bohnert unpublished). Thus, it is apparent that different aquaporins possess different cell specificity in the ice plant.

To investigate tissue specificity of the ice plant *Mip* genes in more detail, we conducted GUS-fusion experiments with promoter regions of *Mip* genes. We wished to determine the expression characteristics of this promoter in *Nicotiana tabacum* (var. SR1) as the plant host of *Mip*-promoter::*uidA*(GUS) constructs. A similar experiment using a heterologous system was reported with a *MIP*-related gene from pea (Jones and Mullet 1995). The promoter of

Abbreviations: GUS, β-glucuronidase; MIP, major intrinsic protein; TIP, tonoplast intrinsic protein; PIP, plasma membrane intrinsic protein.

[4] Present address: Japan Tobacco Inc., Plant Breeding and Genetics Research Laboratory, 700 Higashibara, Toyoda, Iwata, Shizuoka, 438 Japan.

Trg31, expressed in transgenic tobacco, reflected the endogenous expression characteristics observed in pea (Guerrero et al. 1990). Similarly, the characteristics of a root-specific promoter have been analyzed in transgenic tobacco (Yamamoto et al. 1991). By studying organ-, tissue- and cell-specificity of the ice plant *MipB* promoter in tobacco we wished to obtain an assessment about the potential of this sequence as a gene control element for transgene expression targeted to specific cells. Prevalent strong expression in meristematic, rapidly expanding cells and in cells of the vascular tissues, in particular in the xylem parenchyma, have been observed.

Materials and Methods

Promoter isolation—A genomic library of the ice plant was constructed using Lambda Dash-II (Stratagene) and screened with *MipB* cDNA. DNA sequencing of a subcloned fragment that contained 5′ upstream sequences of the *MipB* gene was carried out with an automatic DNA sequencer (ABI, model 373).

Gene construction and plant transformation—A 3.2 kb genomic DNA fragment containing the *MipB* promoter was obtained by *Eco*RV digestion from the genomic clone and used for GUS-fusion constructs. The *Eco*RV-generated DNA fragment was inserted into the *Sma*I-site of the vector pBI101 (ClonTech Inc.). This construct, pPMB (Fig. 1), was transferred into *N. tabacum* (var. SR1) by leaf-disk transformation as described (Horsch et al. 1985). Selection, regeneration, growth, and selfing of transgenic plants were carried out following established procedures (e.g., Tarczynski et al. 1992).

Transcription start site determination—Primer extension was carried out according to Ausubel et al. (1988). An oligonucleotide (5′-ATCCTCCTCTTTCCCTTCC-3′) complementary to the sequence near the translation start site was end-labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase. The labeled primer was annealed with 50 μg of total ice plant RNA and extended with Superscript II reverse transcriptase (Gibco-BRL). For a size-ladder, pPMB was sequenced with the same oligonucleotide. Lanes containing unextended olginucleotide were included. Sequencing products and extension products were analyzed by denaturing acrylamide gel electrophoresis.

Expression of GUS in transgenic plants—Plant materials were stained for GUS-activity by the method of Suter-Crazzolara et al. (1995). After staining, seedlings and plant tissues were fixed overnight, briefly dehydrated in 70% ethanol, and hand sectioned, if necessary. Samples were photographed with an Olympus 35AD-4 camera mounted on an Olympus S2H-ILLB microscope. For the analysis of sections, GUS-staining was followed by fixation, dehydration, and embedding in Historesin (Reichert-Jung, Germany) as recommended (Suter-Crazzolara et al. 1995). Sections, 8 or 10 μm thick, were prepared using a Jung RM2035 microtome. Sections were photographed with an Olympus 35AD-4 camera mounted on an Olymbus BH-2 microscope.

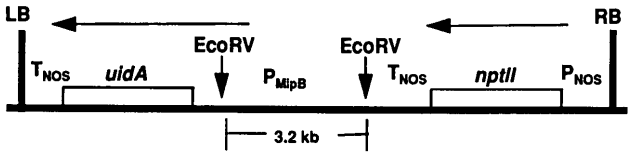

Fig. 1 Schematic presentation of the *MipB* promoter:*uidA*:: NOS terminator construct. A *Eco*RV restriction fragment including the *MipB* promoter from the ice plant (3.2 kb) was inserted into the *Sma*I site of the pBI101 vector located upstream of *uidA* gene. The construct was introduced into *Agrobacterium tumefaciens* LBA 4404 and subsequently into *N. tabacum* (var. SR1). *uidA*, β-glucuronidase; *nptII*, neomycin phosphotransferase; P_{NOS}, promoter of a nopaline synthase gene.

Results

Promoter isolation—A clone of genomic DNA was obtained that contained the entire *MipB* coding region including 3.2 kb upstream of the 5′ end of the cDNA. After subcloning, deletion analysis and mapping, a DNA sequence, 2,196 nucleotides upstream of the *Eco*RV-site in the 5′ UTR of the ice plant *MipB*, was found to contain the 5′ end of the cDNA and upstream regions. The DNA sequence was determined and has been deposited into the EMBL DNA database (accession No. U32551). Analysis of the promoter fragment (CGC program: "FindPattern") indicated the presence of several sequences which could serve as TATA elements, but no deletion analysis was done. In addition, the presence of ABRE-elements (putatively responding to ABA signaling) and *myb*-like elements (which may be recognized by helix-turn-helix-binding factors) is indicated. However, these elements are located more than 300 nucleotides upstream of the transcription start sites (see below) and their involvement in the regulation of the *MipB* promoter is hypothetical.

Gene construction and transformation—pPMB resulted in a transcriptional fusion transcript 16 nucleotides upstream of the translation initiation codon of *uidA*. It contained 143 nucleotides of the 5′ leader of the longest *MipB* transcript. Fifteen independent transgenic lines were obtained and the inheritance of the *MipB* promoter::GUS construct and the kanamycin resistance were analyzed after self-pollination of primary transformants (Table 1). T1 seeds for several lines segregated in a ratio of 3 : 1 with respect to both kanamycin resistance and GUS activity. Further experiments used T2 and T3 generations of five lines indicating single insertions, pMB1, 3, 5, 10 and 14, respectively (Table 1).

Transcription start determination—The transcription start site of *MipB* in the ice plant was determined by primer extension with an oligonucleotide complementary to the region close to the translation start site. Three transcription start sites were identified, although the site closest to the translation start was sometimes less pronounced (Fig. 2). The transcription start sites are located 176, 170, and 161 nucleotides upstream of the translation start. The 5′ UTR region does not contain any potential open reading frames longer than 15 nucleotides.

Expression of GUS in transgenic plants—MipB promoter activity in transgenic tobacco was analyzed by GUS

Table 1 Inheritance of P_{MipB}-GUS and K_m^R in transformants of tobacco

Line	No. K_m^R (%)	No. GUS^+ (%)
pMB1	42/52 (77)	13/15 (87)
pMB2	15/15 (100)	11/16 (69)
pMB3	51/67 (76)	13/15 (87)
pMB4	21/23 (91)	12/13 (92)
pMB5	37/48 (77)	7/ 9 (78)
pMB6	52/52 (100)	13/14 (93)
pMB7	16/22 (73)	0/46 (0)
pMB8	35/40 (88)	12/15 (80)
pMB9	55/56 (98)	15/17 (88)
pMB10	34/48 (71)	17/20 (85)
pMB11[a]	57/58 (98)	48/53 (91)
pMB12	80/80 (100)	10/10 (100)
pMB13	55/56 (98)	17/17 (100)
pMB14	33/45 (73)	32/45 (72)
pMB15	48/50 (96)	0/25 (0)

Wild type tobacco (*Nicotiana tabacum*, var. SR1) did not survive on 50 $\mu g\ ml^{-1}$ kanamycin and did not show any blue staining. Plants putatively containing single locus inserts (pMB1, 3, 5, 10 and 14) were used for further analyses. Several pMB-lines contain multiple inserts on different chromosomes. Two lines were K_m^R, but did not show GUS activity.

[a] pMB11 are male sterile. After a backcross with SR1 as the pollen donor, several inserts were indicated.

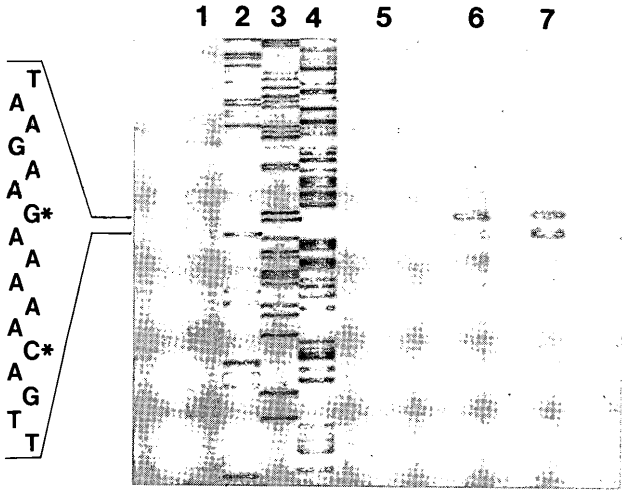

Fig. 2 Determination of the start site of transcription of *MipB* gene in *M. crystallinum*. An oligonucleotide complementary to the sequence near the translation start site was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled primer was annealed to 50 mg of total RNA isolated from *M. crystallinum* and extended with Superscript II reverse transcriptase (Gibco-BRL). The extension products and sequencing products of pPMB were analyzed by denaturing acrylamide gel electrophoresis. The sequence ladder (lane 1 to 4: ACGT), a lane with the primer, without extension (free), and a primer extension lane (root) are shown.

staining. In germinating seedlings, strong expression of the reporter gene was observed in the root tip (Fig. 3A). In older seedlings, GUS staining extended through the vascular system of the root with strongest staining in the endodermis and xylem parenchyma (Fig. 3B, and not shown). These characters were uniformly displayed by all independently generated transgenic lines, five of which were studied in detail. Strong expression equal to that in the primary root tip was observed in developing lateral root primordia from the earliest stages of primordia formation (Fig. 3C). In seedlings and young plants, the apical meristem also stained strongly (Fig. 3D). Vascular tissues of both the cotyledons (Fig. 3D) and the true leaves, and glandular trichomes of true leaves (see leaf in Fig. 3C) showed modest expression of the reporter gene. Glandular trichomes are missing from the cotyledons (Fig. 3D).

At higher magnification, staining could be located in specific cells, cells of the interstitial parenchyma associated with the xylem (Fig. 3E) and cells surrounding the phloem and secondary phloem elements (data not shown). Staining declined with increasing distance from the vascular bundle. In addition, a sub-epidermal cell layer was stained in the stem and petiole (Fig. 3F).

Flowers of the transgenic tobacco also showed expression of GUS (Fig. 4). In flower buds, stages 3 to 8 according to Goldberg (1988), prior to opening of the corolla most tissues and cells showed GUS activity in the gradient which followed expansion of cells in the individual flower organs (Fig. 4A). The tissues of the flower tended to lose GUS activity when they had matured. For example, in immature pistil, most cells showed GUS activity (Fig. 4A), while at maturity only the tip of the pistil showed GUS activity (Fig. 4B). During seed formation, GUS activity progressed such that, initially, all cells of the seeds were stained (Fig. 5A), with strongest staining found in the endosperm, integuments and the cells lining the seed cavity. As the seeds matured, activity within seeds declined, however the placental tissues and cells lining the seed cavity retained GUS activity (Fig. 5B).

Discussion

In a previous study (Yamada et al. 1995), we had demonstrated cell-specific expression of *Mip*-transcripts from the ice plant. By in situ hybridization under stringent conditions, we observed signals for *MipB* only in root tips. When antibodies raised against peptides specific for individual MIP were used (Kirch and Bohnert unpublished), there was more general expression of MIPB in the ice plant than indicated by the in situ hybridization of transcripts, although at vastly different intensities. Highest expression was seen in the root tip and in lateral root primordia, moderate expression in the apical meristem and lower

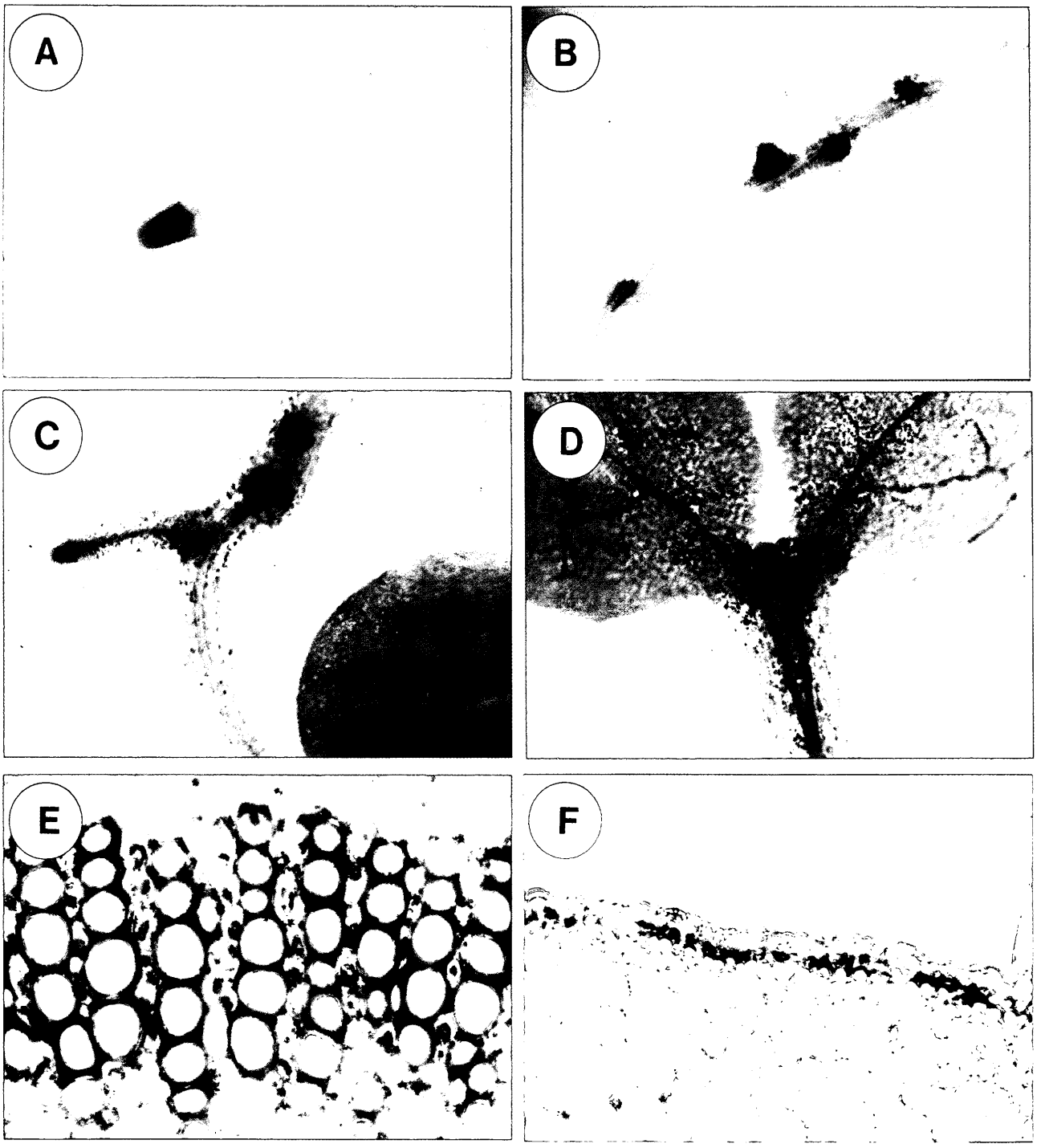

Fig. 3 GUS expression in germination seedlings and young plants. Plant materials were stained for GUS-activity. After staining, seedlings were fixed overnight, briefly dehydrated in 70% ethanol, and hand sectioned, if necessary. (A) Root tip of a six days-old seedlings. (B) Staining in the vascular system of the primary root. (C) Lateral root primordia in the root/hypocotyl transition zone. Also included is a true leaf with GUS-staining of glandular trichomes. (D) Seedling apical meristem with hypocotyl and the cotyledons. (E) Enlarged stem vascular bundle with staining of xylem parenchyma cells. (F) Sub-epidermal layer in the stem. In leaves, this layer expands into palisade cells which showed weak GUS activity.

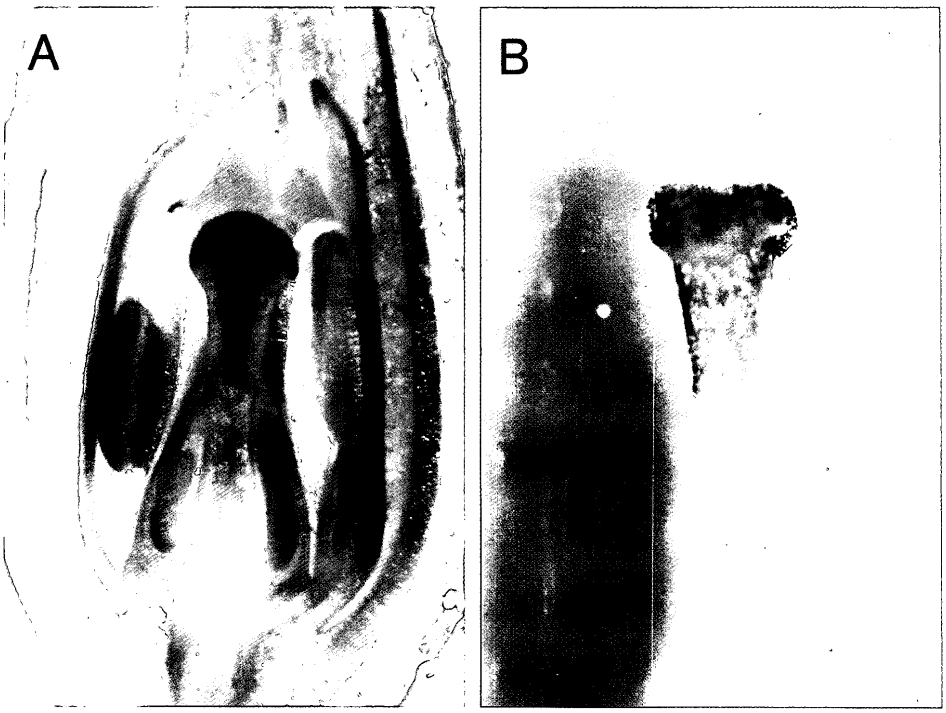

Fig. 4 GUS expression in the floral tissues. Plant materials were stained for GUS-activity. After staining, seedlings were fixed overnight, briefly dehydrated in 70% ethanol, and hand sectioned, if necessary. (A) Staining in immature, expanding flower tissues. (B) Tip of pistil of mature flower.

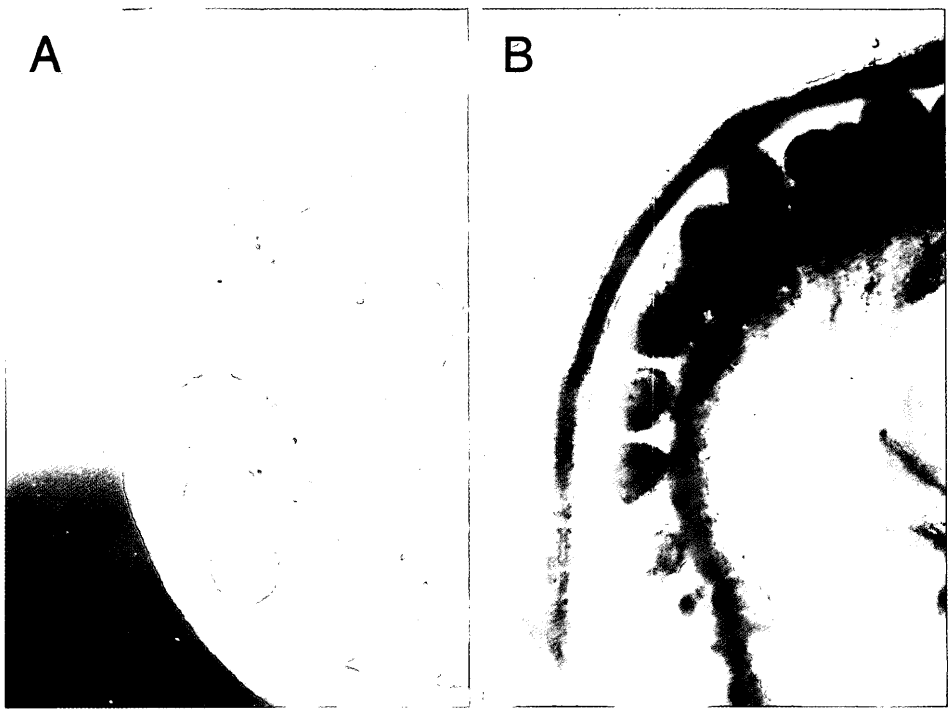

Fig. 5 GUS expression during seed formation. Plant materials were stained for GUS-activity. After staining, seedlings were fixed overnight, briefly dehydrated in 70% ethanol, and hand sectioned. (A) Young seed pod. (B) Seed pod close to maturity.

amounts were detected in the leaf vasculature. Although we need further work to clarify the difference in signals comparing *MipB* transcripts and MIPB protein, the level to which transcripts accumulate is apparently not proportional to the protein level. It also seems likely that the MIPB (peptide)-antibodies recognized other MIP whose transcripts have not yet been found. This is understandable considering that, for example in *Arabidopsis thaliana*, 23 different MIP-related sequences have been detected up until now (Weig et al. 1997). In addition, the GUS-detection is more sensitive than RNA gel blot analysis or in situ hybridization. Thus, it is possible that the accumulation of *MipB* transcripts could be below the detection limit of the methods we used in the previous study (Yamada et al. 1995). Here, we used the promoter of the ice plant *MipB* gene to probe for its specificity in tobacco. The *MipB* promoter in tobacco shows very similar expression patterns to that in the ice plant found by immunological analysis.

Strongest expression of GUS is observed in the tips of the roots of seedlings, young and mature transgenic tobacco. Less intense GUS expression is observed in other cells and tissues. Strong staining is also found in cell layers surrounding vascular bundles of the leaf, in xylem parenchyma cells and in glandular trichomes which are active in secretion. We observed relatively high GUS activity also in floral meristems and immature floral organs of the transgenic tobacco. Lower expression occurs in a single subepidermal cell layer of the stem and petiole. Even less staining, albeit still above control background levels, is found in mesophyll cells of the leaf. Cells in which the promoter is most active are characterized by one of two criteria: these cells are either rapidly expanding irrespective of their tissue context, or the cells have expanded but are localized at positions where high rates of water flux have been documented or can be expected. The first criterion is fulfilled by all meristematic cells, expanding cells close to meristems, and cells in early developmental stages of flowers. The second criterion is fulfilled by cells surrounding mature vascular systems, xylem parenchyma cells, cells of the sub-epidermis, and the glandular trichomes. These cell types are characterized by high water flux.

The movement of water and its relationship to the presence and activity of aquaporins is an important topic for our understanding of water flux in plants (Chrispeels and Agre 1994). Aquaporins have been shown to mediate water flux. For example, the antisense expression of an aquaporin coding region in *Arabidopsis thaliana* diminished water movement into cells, and strong antisense expression led to a phenotype that is reminiscent of water deficit (Kaldenhoff et al. 1995). Water flux and its control are particularly important under water stress conditions, when the external osmotic potential becomes lowered to the extent that roots would be desiccated if water flux were not regulated. We are interested in aquaporin expression and regulation of activity during salt stress in a halophytic plant. According to transcript analysis, aquaporin mRNAs decline after salt shock transiently (Yamada et al. 1995), while aquaporin mRNA in Arabidopsis thaliana increased (Yamaguchi-Shinozaki et al. 1992). One aspect of our work is the analysis of aquaporin promoters and cell-specific gene expression which has already been done for different aquaporins in several other glycophytic plant models (Yamamoto et al. 1991, Kaldenhoff et al. 1995, Jones and Mullet 1995, Yamada et al. 1995, Johansson et al. 1996, Kaldenhoff et al. 1996). From these analyses—not all experiments have included stress conditions—it is clear that aquaporins exist that are regulated in different ways. The problem is compounded by the number of aquaporin genes that are found in plants. A comprehensive analysis of the cell specificity of all aquaporin genes and their proteins in one species will be necessary to distinguish those genes/proteins that respond to stress by either up-regulation or turnover. From the limited analyses that have been reported, we expect that gene complexity allows plants to control aquaporin protein expression precisely and efficiently under a variety of growth conditions. The *MipB* promoter from the ice plant seems to retain its cell specificity precisely in transgenic tobacco and provides a heterologous promoter element for the transgenic expression of proteins in tobacco. As judged by its cell-specificity and identical expression in independently generated lines, this promoter will prove useful. Transgenes under the control of the *MipB* promoter will be most strongly targeted to meristems and tissues with high water flux capacity, especially to cells associated with the xylem. Heterologous expression studies, which we are presently extending to the expression of other *Mip*-promoters, will be helpful in distinguishing the roles of individual aquaporins, and eventually understanding the controlling mechanisms of water flux in plants.

We thank Patricia Adams and Chris Michalowski for help with photography and sequence analysis. The work has been supported by the U.S. Department of Agriculture (NRI-CGP, Plant Responses to the Environment) and, in part, by the Arizona Agricultural Experimental Station. S.Y. was supported by a Japan Tobacco Inc. graduate fellowship. The contribution of E.L. was part of undergraduate senior thesis in the Dept. of Biochemistry.

References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1988) *Current Protocols in Molecular Biology*. Wiley and Sons, New York.

Chrispeels, M.J. and Agre, P. (1994) Aquaporins: Water channel proteins of plant and animal cells. *Trends Biol. Sci.* 19: 421–425.

Daniels, M.J., Mirkov, T.E. and Chrispeels, M.J. (1994) The plasma membrane of *Arabidopsis thaliana* contains a mercury-insensitive aquaporin that is a homolog of the tonoplast water channel protein TIP. *Plant Physiol.* 106: 1325–1333.

Fushimi, K., Uchida, S., Hara, Y., Hirata, Y., Marumo, F. and Sasaki, S.

(1993) Cloning and expression of apical membrane water channel of rat kidney collecting tubule. *Nature* 361: 549–552.

Goldberg, R.B. (1988) Plants: novel developmental processes. *Science* 240: 1460–1476.

Guerrero, F.D., Jones, J.T. and Mullet, J.E. (1990) Turgor-responsive gene transcription and RNA levels increase rapidly when pea shoots are wilted. Sequence and expression of three inducible genes. *Plant Mol. Biol.* 15: 11–26.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Wallroth, M., Eichholtz, D., Rogers, S.G. and Fraley, R.T. (1985) A simple and general method for transferring genes into plants. *Science* 227: 1229–1231.

Johansson, I., Larsson, C., Ek, B. and Kjellbom, P. (1996) The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^{2+}. *Plant Cell* 8: 1181–1191.

Jones, J.T. and Mullet, J.E. (1995) Developmental expression of a turgor-responsive gene that encodes an intrinsic membrane protein. *Plant Mol. Biol.* 28: 983–996.

Kaldenhoff, R., Koelling, A., Meyers, J., Karmann, U., Ruppel, G. and Richter, G. (1995) The blue light-responsive AthH2 gene of *Arabidopsis thaliana* is primarily expressed in expanding as well as in differentiating cells and encodes a putative channel protein of the plasmalemma. *Plant J.* 7: 87–95.

Kaldenhoff, R., Kolling, A. and Richter, G. (1996) Regulation of the *Arabidopsis thaliana* aquaporin gene AthH2 (PIP1b). *J. Photochem. Photobiol.* 36: 351–354.

Maurel, C., Reizer, J., Schroeder, J.I. and Chrispeels, M.J. (1993) The vacuolar membrane protein g-TIP creates water specific channels in *Xenopus Oocytes*. *EMBO J.* 12: 2241–2247.

Park, J.H. and Saier, M.H., Jr. (1996) Phylogenetic characterization of the MIP family of transmembrane channel proteins. *J. Membr. Biol.* 153: 171–180.

Suter-Crazzolara, C., Klemm, M. and Reiss, B. (1995) *Methods in Plant Cell Biology*. Edited by Galbraith, D., Bourque, D.P. and Bohnert, H.J. Vol. 50, pp. 421–434. Academic Press, San Diego.

Tarczynski, M.C., Jensen, R.G. and Bohnert, H.J. (1992) Expression of a bacterial *mltD* gene in transgenic tobacco leads to production and accumulation of mannitol. *Proc. Natl Acad. Sci. USA* 89: 2600–2604.

Weig, A., Deswarte, C. and Chrispeels, M.J. (1997) Molecular analysis of the major intrinsic protein (MIP) gene family in *Arabidopsis thaliana*. *Plant Physiol.* (in press).

Yamada, S., Katsuhara, M., Kelly, W.B., Michalowski, C.B. and Bohnert, H.J. (1995) A family of transcripts encoding water channel proteins: Tissue-specific expression in the common ice plant. *Plant Cell* 7: 1129–1142.

Yamaguchi-Shinozaki, K., Koizumi, M., Urao, S. and Shinozaki, K. (1992) Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana*: sequence analysis of one cDNA that encodes a putative transmembrane channel protein. *Plant Cell Physiol.* 33: 217–224.

Yamamoto, Y.T., Taylor, C.G., Acedo, G.N., Cheng, C.-L. and Conkling, M.A. (1991) Characterization of cis-acting sequences regulating root-specific gene expression in tobacco. *Plant Cell* 3: 371–382.

(Received July 25, 1997; Accepted September 29, 1997)