

RESEARCH ARTICLE

Regulation of the Osmotin Gene Promoter

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By introducing a chimeric gene fusion of the osmotin promoter and β -glucuronidase into tobacco by *Agrobacterium*-mediated transformation, we have demonstrated a very specific pattern of temporal and spatial regulation of the osmotin promoter during normal plant development and after adaptation to NaCl. We have found that the osmotin promoter has a very high natural level of activity in mature pollen grains during anther dehiscence and in pericarp tissue at the final, desiccating stages of fruit development. GUS activity was rapidly lost after pollen germination. The osmotin promoter thus appears to be unique among active pollen promoters described to date in that it is active only in dehydrated pollen. The osmotin promoter was also active in corolla tissue at the onset of senescence. Adaptation of plants to NaCl highly stimulated osmotin promoter activity in epidermal and cortex parenchyma cells in the root elongation zone; in epidermis and xylem parenchyma cells in stem internodes; and in epidermis, mesophyll, and xylem parenchyma cells in developed leaves. The spatial and temporal expression pattern of the osmotin gene appears consistent with both osmotic and pathogen defense functions of the gene.

INTRODUCTION

Since we first reported its isolation (Singh et al., 1985), osmotin (OSM) and OSM-like proteins or the genes encoding these proteins have been extensively studied in several laboratories (Singh et al., 1987a, 1989a, 1989b; LaRosa et al., 1989; Meeks-Wagner et al., 1989; Grosset et al., 1990; Neale et al., 1990; Roberts and Selitrennikoff, 1990; Stintzi et al., 1991; Woloshuk et al., 1991). OSM is a group of cationic proteins that exist in at least two forms, one with a pI of 7.8 and the other with a pI of greater than 8.2; they differ slightly in molecular weight (Singh et al., 1985, 1987a). We showed that OSM is synthesized as a preprotein with a molecular weight of 26,380 and that the molecular weight of its mature form is 23,984 (Singh et al., 1989b). OSM exhibits a very high level of sequence homology with other proteins, including the sweet protein thaumatin, potato pathogenesis-related (PR) protein C, tobacco PR-S, and the maize α -amylase/trypsin inhibitor (Richardson et al., 1987; Singh et al., 1987a).

On the basis of similarities in amino acid sequence and in expression pattern, OSM has been classified as a member of the PR-5-type proteins of tobacco (Bol, 1988; Brederode et al., 1991; Linthorst, 1991). Further studies have shown that the synthesis and accumulation of OSM mRNA are developmentally regulated and controlled by at least six hormonal or

environmental signals, including abscisic acid (ABA), ethylene, tobacco mosaic virus infection, salinity, desiccation, and wounding in both cultured cells and whole plants of tobacco (LaRosa et al., 1985, 1987; Singh et al., 1987b). However, substantial accumulation of the OSM protein itself occurs only in response to osmotic stress and ethylene in tobacco cultivar W38, which shows no hypersensitive response (LaRosa et al., 1992).

Much of the cellular OSM protein was found by immunocytochemistry to be localized in electron-dense (tanninlike) inclusions in the vacuoles. A very low amount of OSM was detected in the ground cytoplasm, but there was no preferential localization of this protein within the cytoplasm. Occasionally, OSM-positive colloidal gold particles were found attached to the tonoplast, but the organelles, plasma membrane, and cell wall were devoid of any gold particles (Singh et al., 1987a). OSM has also been detected in the extracellular matrix of plant cells, although it is not known whether the extracellular OSM is the same as that found in the vacuole or whether it is perhaps an acidic form as are other extracellular PR proteins (Casas et al., 1992).

Transcriptional activation of the OSM promoter was investigated in transformed tobacco plants carrying copies of a fusion of the cloned OSM promoter to the β -glucuronidase (GUS) reporter gene (Nelson et al., 1992). In young seedlings,

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the OSM promoter was found to be transcriptionally activated by exogenous ABA and ethylene. However, the sensitivity of the OSM promoter to ABA decreased with age in both roots and shoots of young seedlings, whereas ethylene-induced promoter activity remained the same over this developmental time period. It was also shown that NaCl shock activated the OSM promoter and that this effect seemed to be mediated through ABA. On the other hand, wounding, which also activated the OSM promoter, seemed to exert its effect through ethylene (Nelson et al., 1992).

To elucidate the function of the OSM gene, several studies have focused on the organ and tissue localization of OSM mRNA and the encoded protein. A high rate of synthesis and accumulation of OSM was found in roots and in "outer stem tissue," which is composed mainly of the epidermis (Singh et al., 1987b). Only low levels of OSM were found in the flower bud, stigma, style, and ovary of mature flowers, and in the cortex and pith of stem. The OSM gene product was undetectable in seeds and barely detectable in leaf tissues (LaRosa et al., 1989, 1992; Singh et al., 1989a, 1989b). Also, developmental regulation of the synthesis and accumulation of OSM transcripts has been demonstrated (Meeks-Wagner et al., 1989; Neale et al., 1990). In tobacco cultivar Samsun NN, FB7-2 transcripts encoding the OSM protein occur in apices of prefloral and floral plants, in stem internode segments, and in leaves of plants possessing an immature inflorescence, with higher levels in older leaves and internodes (Meeks-Wagner et al., 1989; Neale et al., 1990). OSM mRNA also accumulates to high levels in root tissues and 2-week-old seedlings and to moderate levels in floral organs at various stages during development (Neale et al., 1990).

In this study, we report the results obtained from histochemical analyses of GUS expression in specific cell types of transgenic tobacco plants containing an OSM promoter::GUS chimeric gene, which is illustrated in Figure 1. We also report the pattern of OSM promoter activity in these plants after adaptation to a moderately saline (171 mM NaCl) environment. Our data demonstrate that the OSM promoter had the highest level of activity in mature desiccated pollen grains and in placenta and pericarp tissues of the mature fruit. The OSM promoter activity was stimulated during adaptation to NaCl in specific cells and tissues of roots and in xylem parenchyma cells of both stem and leaves.

RESULTS

Patterns of Expression of OSM::GUS Gene Fusion in Vegetative Organs of Unadapted Plants

GUS activity was analyzed in the longitudinal and transverse paraffin sections of roots, in stem internode segments (basal, middle, and apical segments), and in leaves at different stages of development. Low GUS activity was detected in tissues of roots, stems, leaves, and reproductive organs of transformed

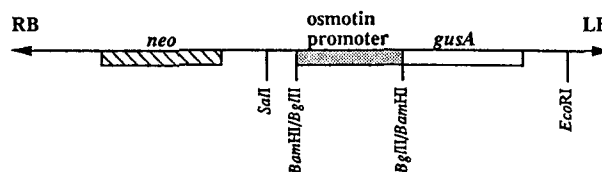


Figure 1. OSM::GUS Fusion Gene Used for Transformation.

RB, right border; LB, left border; *neo*, neomycin phosphotransferase II gene; *gusA*, GUS reporter gene.

unadapted plants. Although osmotin and other basic PR proteins are considered to accumulate in healthy roots, we found that the expression of the OSM::GUS gene was quite low in unstressed hydroponically grown roots. However, high GUS activity was detected in roots of these plants when grown in either soil or vermiculite (data not shown). A higher level of the GUS activity was observed in the epidermis of both roots (in the elongation zone), as shown in Figure 2A, and trichomes and epidermis of basal leaves and stems, as shown in Figures 3D to 3F. No GUS activity was detected in either vascular or parenchymatous tissues of any vegetative plant organs. It must be emphasized that some minor variation in GUS activity (i.e., differences in color intensity but not localization) was observed between analyzed organ samples.

High GUS Activity in Mature Pollen Grains after Anther Dehiscence

GUS activity was analyzed in tissues of different parts of flowers at several developmental stages. Only mature pollen grains and epidermis of petals showed GUS activity, as shown in Figures 4E and 5C, respectively. No GUS activity was observed in any other floral tissue by histochemical GUS assays.

Expression of the OSM::GUS gene in pollen grains occurred only after anther dehiscence (Figures 4B to 4E). Approximately 57% of the pollen grains expressed moderate or high levels of GUS activity. Another 30% showed low GUS activity. This variability in degree of expression could be the result of segregation of the four OSM::GUS genes carried by GUS1B plants. GUS activity decreased rapidly after the initiation of pollen germination *in vitro* (Figures 4G to 4J). After 24 hr of culture on pollen germination medium, almost all (93%) of the pollen grains showed no GUS activity. At no stage of development or germination was moderate or high GUS activity found in pollen grains from nontransformed tobacco plants or plants transformed with a construct consisting of the GUS coding sequence without the OSM promoter (Figures 4A and 4F). Only occasionally were pale blue-stained pollen grains observed in these plants.

When mature pollen grains from transformed OSM::GUS plants were incubated in a GUS assay mixture with 20% methanol, a treatment that according to Kosugi et al. (1990) eliminates the intrinsic GUS activity in tobacco plants, color

intensity was slightly reduced, but the percentage of GUS-positive grains did not change (~90%). GUS activity was not detected in pollen grains from nontransformed plants and plants transformed with GUS coding sequence alone (without promoter) when GUS assay was conducted in the presence of 20% methanol.

Induction of the OSM Promoter during Fruit Development

Before fertilization and fruit formation, the only tissue of the ovary that expressed GUS activity was the vascular tissue in the basal portion of this floral organ, as shown in Figures 5D and 5E. In the ovary of both NaCl-adapted and NaCl-unadapted OSM::GUS-transformed tobacco plants, induction of GUS activity and a gradual increase in GUS intensity were observed at the final stages of fruit development as desiccation of the fruit began, as shown in Figures 6A to 6L. Interestingly, a GUS-positive reaction was found only in the maternal tissues of developing fruit (pericarp and placenta) during desiccation, but not in seeds at any stage of development, including mature, desiccated seeds (Figures 6G to 6L).

Adaptation to NaCl Induces and/or Stimulates Activity of the OSM Promoter

Long-term adaptation of OSM::GUS transgenic plants to moderate concentrations of NaCl (171 mM) modulated the activity of the OSM promoter in temporal and spatial contexts. Paraffin sections were made of roots, stems, and leaves at different stages of development from both types of transgenic plants as well as nontransformed tobacco plants that were adapted for 30, 60, and 90 days to NaCl and used to localize GUS activity. Histochemical analysis of GUS activity was also performed at different stages of flower development, before and after pollination (stages 1, 3, 5, 7, 8, 9, 11, and 12 according to the designation proposed by Koltunow et al. [1990]), and at different stages of fruit formation. GUS activity was carefully monitored in pollen grains during their development and in vitro germination.

Adaptation to NaCl in Roots

OSM::GUS transgenic plants adapted to grow in NaCl for 30 days showed increased GUS activity in root elongation zones (epidermis and root cortex parenchyma) but not in root apical meristems (Figure 2B). After prolonged (60 days) adaptation of plants to NaCl, GUS activity was also observed in the endodermis, cortex, and tissues of the vascular cylinder within the root elongation zone (Figures 2C to 2K). A GUS-positive reaction was never found either in root meristems (Figures 2G and 2K) or in any tissue of the differentiated (basal) zone of the root (Figure 2D).

Stem Adaptation to NaCl

Adaptation to NaCl also increased OSM promoter activity in stem internodes at all stages of plant development. The GUS-positive reaction was restricted to the epidermis and the xylem parenchyma of the stem, but was also occasionally observed in the cortex parenchyma, as shown in Figures 7A to 7C. The pattern of expression or GUS level in these tissues did not change significantly after the initial induction by NaCl.

Leaf Adaptation to NaCl

Although increased activity of the OSM promoter was found in the trichomes, epidermis, mesophyll, and xylem parenchyma of fully developed (expanded) leaves after adaptation to NaCl, the strongest GUS-positive reaction was found in leaf tips (Figures 3A, 3G, and 3J). In young leaves, lower GUS activity was observed in the trichomes. No GUS activity was observed in either shoot apical meristems or leaf primordia. There was a consistent gradient of low to high OSM promoter expression in the youngest to the oldest leaves, and this was confirmed with GUS fluorometric assays using 4-methylumbelliferyl β -D-glucuronide substrate (data not shown).

Adaptation to NaCl in Reproductive Structures

Adaptation of transgenic OSM::GUS plants to NaCl significantly affects OSM promoter activity in flowers. Moderate GUS activity after salt adaptation was found in vascular bundles of the basal part of the ovary, the epidermal layers and the mesophyll of the top (limb) region of petals (Figures 5A to 5C), and in the epidermis and mesophyll of the sepal tips.

A high level of OSM::GUS gene expression was found in mature pollen grains of adapted and unadapted plants at anther dehiscence (Figures 4D and 4E). The GUS-positive reaction, however, was visible only in mature, "dry" pollen grains and decreased dramatically during the early stages of in vitro germination (Figures 4H to 4J).

DISCUSSION

We have found that the GUS reporter gene when driven by the OSM promoter exhibits organ-, tissue-, and cell-specific expression patterns. Furthermore, adaptation of OSM::GUS transgenic plants to NaCl resulted in a significant increase in GUS gene expression in specific cells and tissues of roots, stems, and mature leaves. The differential patterns of OSM promoter activity were mostly consistent with less detailed analyses of the distribution of OSM mRNA and protein in plant tissues reported earlier (Singh et al., 1987a, 1987b; LaRosa et al., 1989; Meeks-Wagner et al., 1989; Neale et al., 1990),

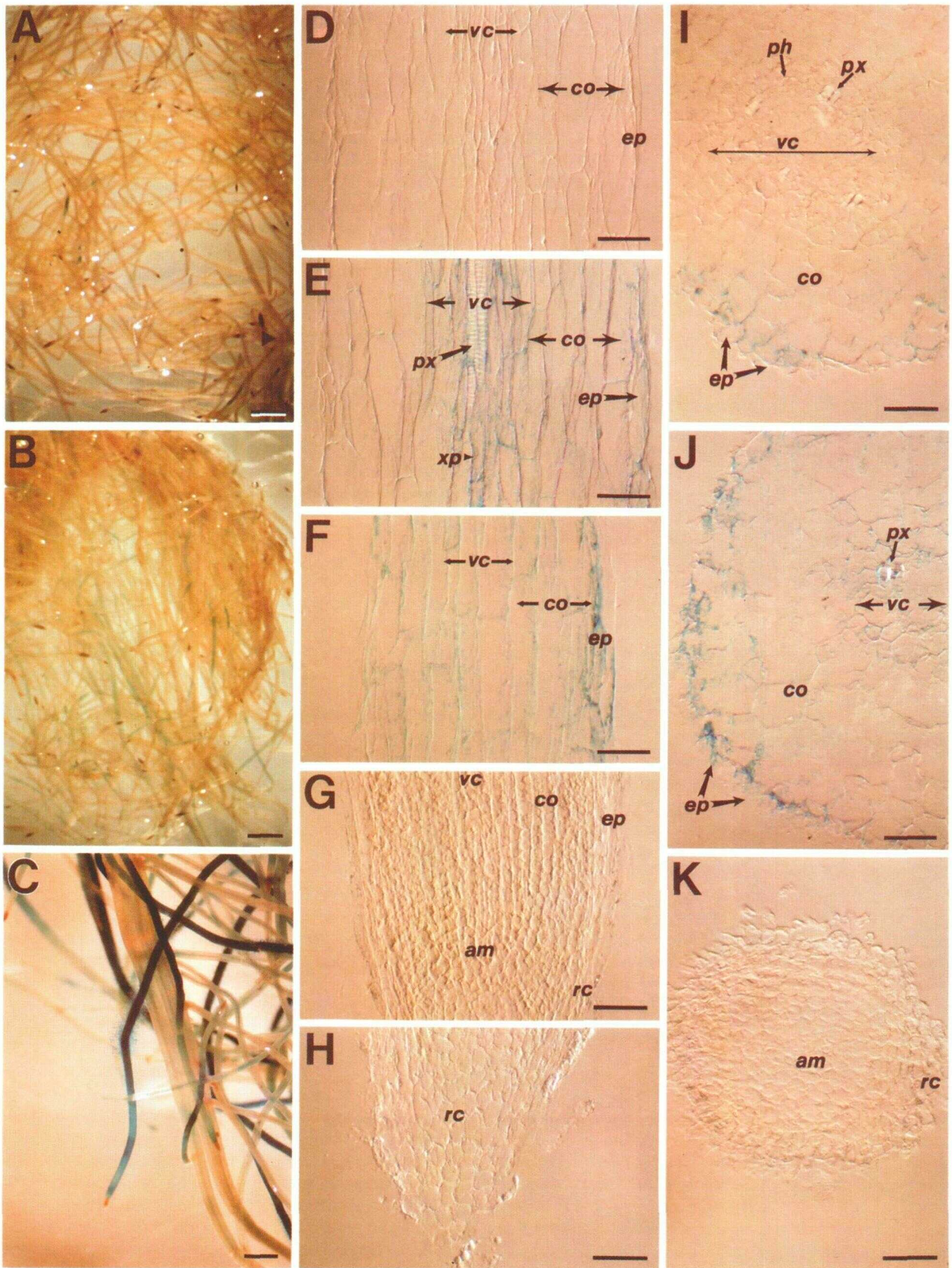


Figure 2. Pattern of OSM Promoter Activity Revealed by GUS Activity in Roots of GUS1B-Transformed Plants.

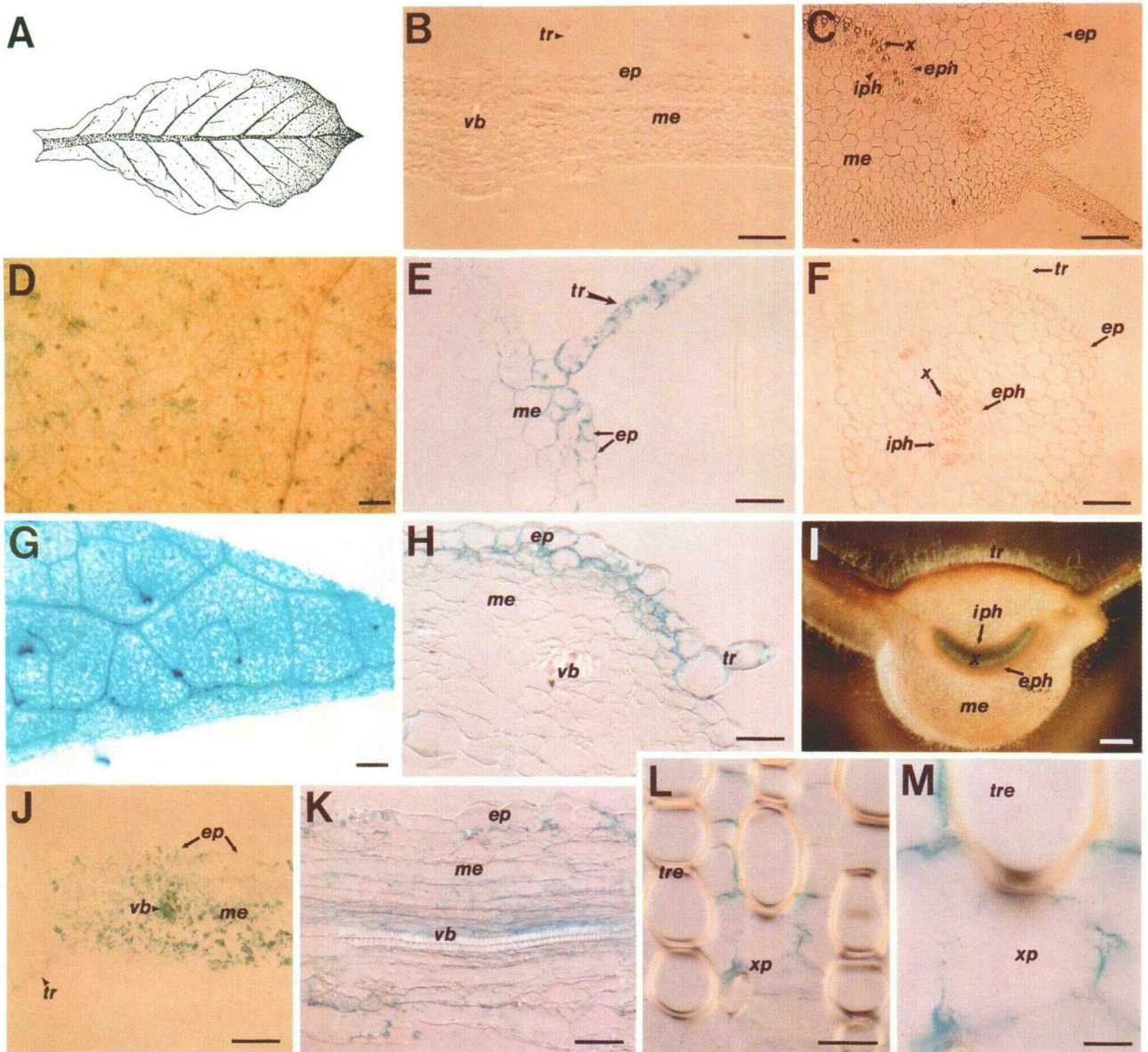


Figure 3. Pattern of OSM Promoter Activity Revealed by GUS Activity in Leaves of Tobacco Transformants.

(A) General pattern of GUS activity in fully expanded leaves of GUS1B-transformed plants (stippled area represents region of GUS activity). (B) and (C) Transverse sections of leaves from plants transformed with the GUS coding sequence alone. Bars = 100 μ m. (D) Portion of leaf of GUS1B-transformed plant unadapted to NaCl. Bar = 0.7 mm. (E) and (F) Transverse sections of leaf blade and petiole from GUS1B-transformed plant unadapted to NaCl. Bars = 50 μ m. (G) Portion of leaf tip of fully expanded leaf from GUS1B-transformed plant adapted to NaCl. Bar = 1 mm. (H) to (M) Transverse sections of fully expanded leaves from GUS1B plants adapted to NaCl. (H) shows leaf blade; bar = 50 μ m. (I) shows petiole; bar = 1 mm. (J) shows leaf tip; bar = 100 μ m. (K) shows leaf blade; bar = 50 μ m. (L) and (M) show portions of vascular bundle; bar in (L) = 30 μ m and bar in (M) = 10 μ m. ep, epidermis; eph, external phloem; iph, internal phloem; me, mesophyll; tr, trichome; tre, tracheary element; vb, vascular bundle; x, xylem; xp, xylem parenchyma.

Figure 2. (continued).

(A) Roots from plants unadapted to NaCl. Bar = 1.25 mm. (B) Roots from plants adapted to 171 mM NaCl for 30 days. Bar = 1.25 mm. (C) Roots from plants adapted to 171 mM NaCl for 60 days. Bar = 0.7 mm. (D) to (H) Longitudinal sections through different root zones from plants adapted to NaCl. Bars = 50 μ m. (I) to (K) Transverse sections through different zones of roots from plants adapted to NaCl. Bars = 50 μ m. (D), (E), (I), and (J), differentiated zone; (F), elongation zone; (G) and (K), meristematic zone; (H), root cap. am, apical meristem; co, root cortex; ep, epidermis; ph, phloem; px, protoxylem; rc, root cap; vc, vascular cylinder; xp, xylem parenchyma.

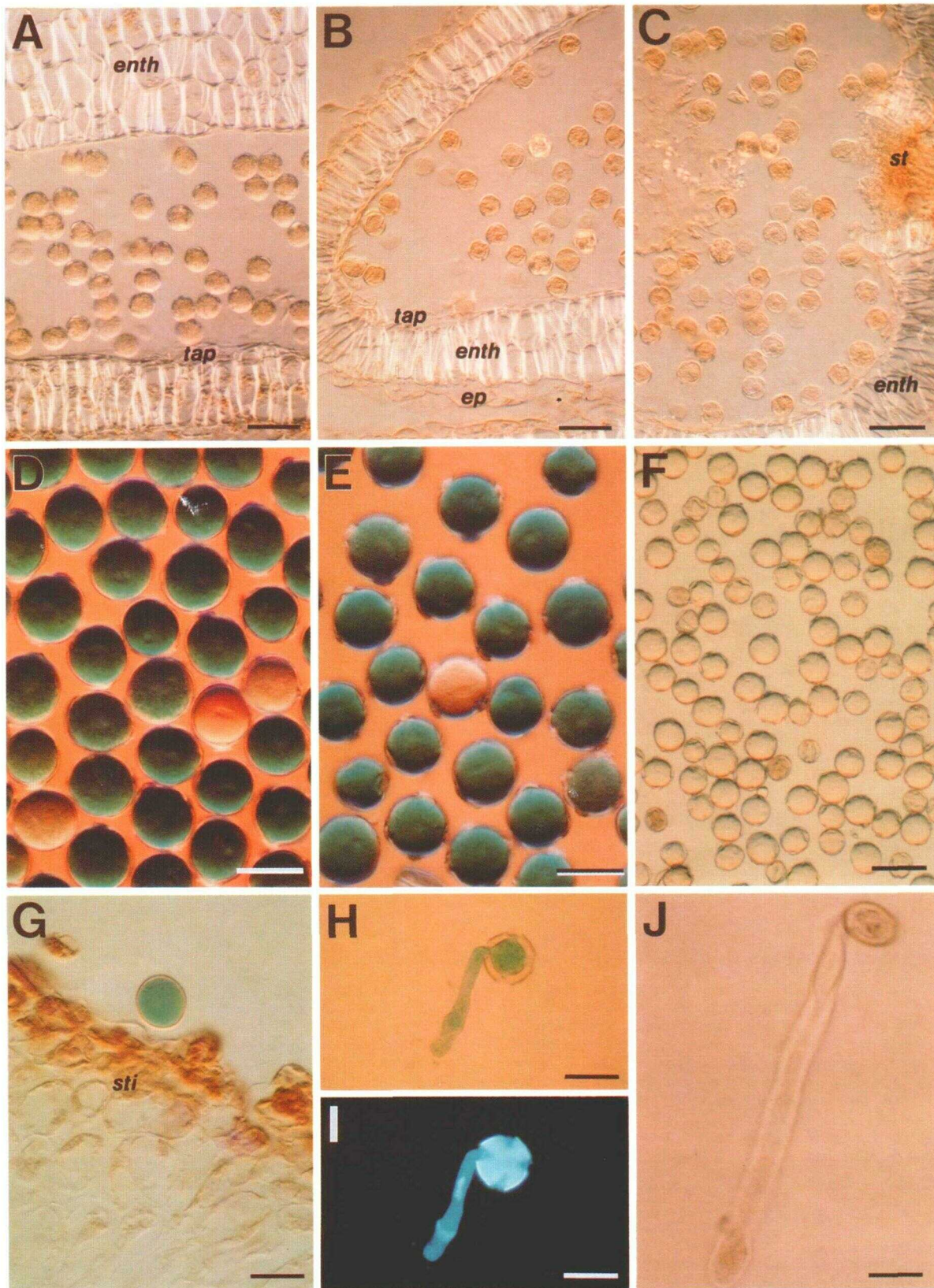


Figure 4. Patterns of OSM Promoter Activity Revealed by GUS Assay in Anthers and Pollen Grains in GUS Transformants and GUS1B-Transformed Plants.

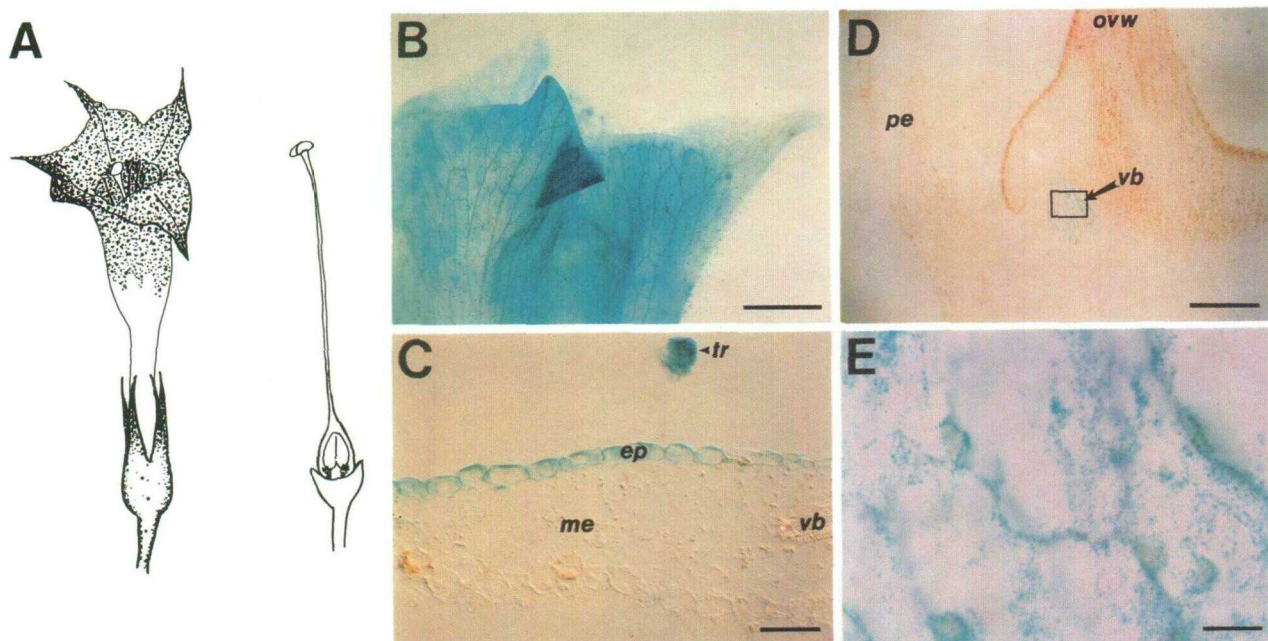


Figure 5. Pattern of OSM Promoter Activity in Flowers of GUS1B-Transformed Plants.

(A) General pattern of OSM promoter activity represented by stippled regions.

(B) Corolla from GUS1B plant adapted to NaCl. Bar = 0.5 mm.

(C) Transverse section of petal from GUS1B-transformed plant adapted to NaCl. Bar = 50 μ m.

(D) Longitudinal section through basal portion of flower from NaCl-adapted GUS1B-transformed plant. Bar = 300 μ m.

(E) Portion of vascular bundle (framed in Figure 4D) from basal portion of ovary from GUS1B-transformed plant adapted to NaCl. Bar = 10 μ m. ep, epidermis; me, mesophyll; ovw, ovary wall; pe, petal; tr, trichome; vb, vascular bundle.

confirming the validity of the GUS assay as an indicator of transcriptional activity of the OSM gene.

OSM::GUS Gene Is Highly Expressed during Natural Desiccation of Both Pollen Grains and Specific Fruit Tissues

In general, histochemical data confirmed that there is relatively low-level activity of the OSM promoter in plants not adapted to osmotic stress. The major exceptions are pollen grains and the pericarp of the fruit. Both pollen and tobacco fruits desiccate during the latter stages of development, and the pattern

of OSM expression is consistent with the osmotic regulation of this promoter in other tissues. Seeds that also naturally desiccate, however, do not exhibit any expression of the OSM promoter, indicating an important developmental control over the osmotic responsiveness of this promoter. It will be interesting to determine if there are seed-expressed versions of the OSM gene in tobacco as was reported for OSM-like proteins of other species (Vigers et al., 1991).

OSM promoter activity was found to be localized in mature, desiccated pollen grains during anther dehiscence and was not found either in immature pollen or in sporophytic tissues of the anthers. Anther- and pollen-specific gene expression has been extensively examined. cDNA clones corresponding

Figure 4. (continued).

(A) and (B) Longitudinal sections of GUS assayed anther (before dehiscence) from GUS transformant adapted to NaCl (A) and GUS1B-transformed plant unadapted to NaCl (B). Bars = 50 μ m.

(C) Transverse section of GUS assayed anther (before dehiscence) from GUS1B plant adapted to salt. Bar = 50 μ m.

(D) and (E) mature pollen grains from GUS1B-transformed plants unadapted (D) and adapted (E) to NaCl. Bars = 30 μ m.

(F) Mature pollen grains from GUS transformant adapted to NaCl. Bar = 50 μ m.

(G) Longitudinal section of pistil from GUS1B-transformed plant adapted to NaCl. Bar = 30 μ m.

(H) and (I) GUS1B pollen grain after 4 hr of in vitro germination. (H), after GUS assay; (I), after staining with 4,6-diamidino-2-phenylindole. Bars = 30 μ m.

(J) GUS1B pollen grain after 12 hr of in vitro germination. Bar = 30 μ m.

enth, endothecium; ep, epidermis; st, stomium; sti, stigma; tap, tapetum.

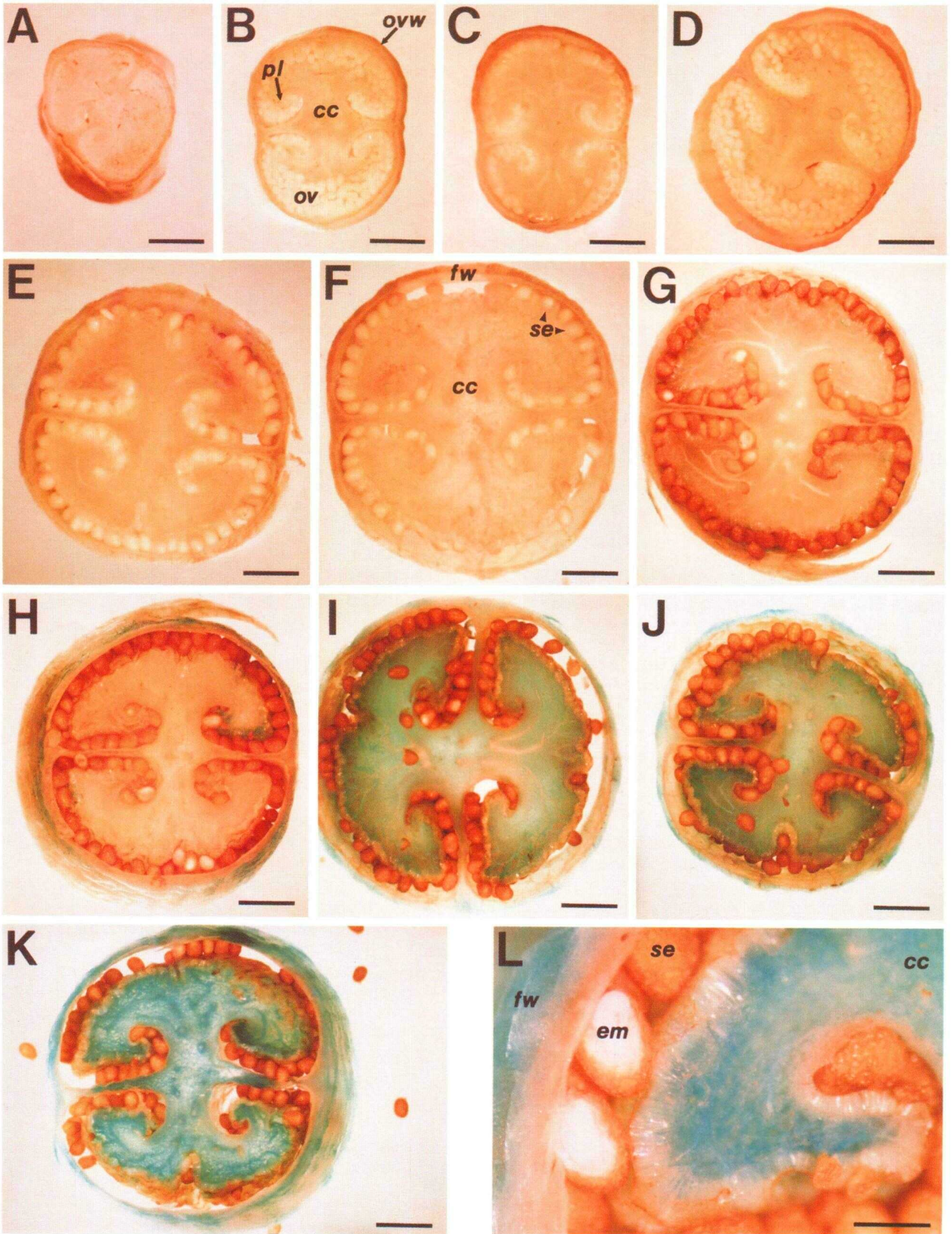


Figure 6. Pattern of OSM Promoter Activity at Different Stages of Ovary and Fruit Development in GUS1B-Transformed Plant.

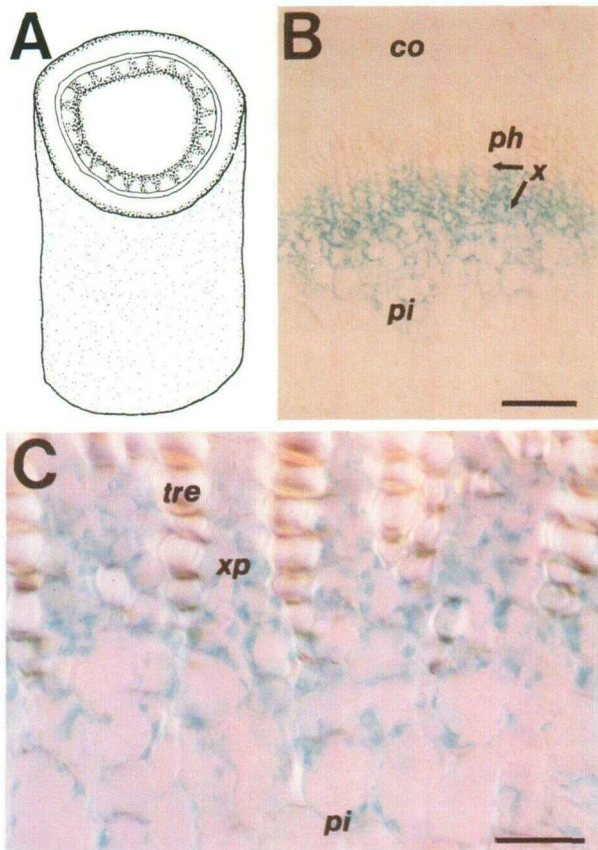


Figure 7. Pattern of OSM Promoter Activity in Stem of GUS1B-Transformed Plants Adapted to NaCl.

(A) General pattern of GUS activity is shown by stippled regions. (B) and (C) Transverse sections of the stem. Bar in (B) = 100 μ m; bar in (C) = 30 μ m. co, cortex; pi, pith; ph, phloem; tre, tracheary elements; x, xylem; xp, xylem parenchyma.

to mRNAs expressed in all sporophytic cell layers of the anthers, except the tapetum, have been isolated from tobacco (Goldberg, 1988; Drews and Goldberg, 1989), and tapetal-specific cDNA clones have been characterized in tomato (Smith et al., 1987) and tobacco (Goldberg, 1988). Also, pollen-specific cDNA clones have been isolated from corn and spiderwort (Mascarenhas, 1988; Hanson et al., 1989). McCormick et al. (1987) have described the cloning and characterization of anther-specific cDNA clones from tomato. Five of these, designated as LAT (late anther tomato), pLAT51, pLAT52, pLAT56,

pLAT53, and pLAT59, showed a progressive increase in steady state levels of corresponding mRNAs during anther and pollen maturation, and the mRNAs were present at very high levels in isolated pollen. In addition, an LAT59 promoter construct was found to be expressed in roots and seeds, whereas LAT52 was expressed in seeds (Twell et al., 1990, 1991). The spatial and temporal patterns of the relatively enhanced OSM promoter activity in both roots and mature pollen seem to be somewhat similar to the expression patterns exhibited by pLAT56 and pLAT59. However, the OSM promoter activity in developing pollen grains is induced only during desiccation.

OSM Promoter Remains Silent or Is Rapidly Suppressed Early during Pollen Germination

Microspore mitosis seems to be an important deterministic switch in pollen development because after mitosis there is a rapid activation of many genes followed by massive synthesis of new mRNAs. Some of these transcripts are presumably involved in pollen maturation, whereas others appear to be stored and translated upon pollen germination and/or pollen tube growth (Mascarenhas, 1988). The OSM gene appears to belong to the former group of genes, being expressed only in mature, desiccated pollen grains. Even though GUS is considered to be a highly stable enzyme, a dramatic decrease in GUS activity during the first 6 hr of pollen grain germination and pollen tube growth was observed. This result suggests that the GUS enzyme is rapidly degraded in germinating pollen and that there is greatly reduced OSM promoter activity at this time. This also is in apparent contrast to the expression of LAT mRNAs that persist during pollen germination *in vitro*, concentrating toward the tips of the growing tubes (Ursin et al., 1989). We are not aware of any other "pollen-specific" promoters that exhibit this specific pattern of expression.

OSM Promoter Activity in Pollen Grains May Be Related to the Dehydration/Rehydration Processes

It is well known that pollen grains undergo desiccation to a level as low as 10% water content during late stages of maturation (Heslop-Harrison, 1987). The abundance of GUS in mature pollen grains of OSM::GUS transgenic plants and the dramatic decrease in expression of the gene driven by the OSM promoter during hydration of the pollen and subsequent germination suggest that the activity of the OSM promoter is related to changes in water potential or perhaps ABA content of the pollen, because both have been shown to increase the

Figure 6. (continued).

(A) to (D) Transverse sections of ovaries. Bars = 2 mm. (E) to (L) Transverse sections through fruits. Bars in (E) to (K) = 2 mm; bar in (L) = 0.5 mm. cc, central column; em, embryo; fw, fruit wall; ov, ovules; oww, ovary wall; pl, placenta; se, seeds.

levels of OSM mRNA in other tissues (LaRosa et al., 1992). It is not known, however, if ABA levels increase during pollen desiccation. Although ABA levels are known to increase during seed desiccation, this apparent increase of ABA does not activate the OSM promoter in seeds.

OSM May Be Involved in Reorganization of Membranes Affecting Permeability

Recently, Roberts and Selitrennikoff (1990) and Vigers et al. (1991) reported the isolation of an antifungal protein from corn (zeamatin) whose N-terminal amino acid sequence was considerably similar to that of OSM. According to these authors, zeamatin appears to act by permeabilizing the plasma membranes of fungi. This hypothesis was also suggested by Woloshuk et al. (1991); they postulated that because of the hydrophobic nature of the AP24 protein (identified as OSM), it may interact with the plasma membrane. Such interactions could disrupt the membrane of the fungi causing lysis and inhibition of hyphal growth. Electron microscopy studies of the dehydration and hydration of gramineaceous pollen led Heslop-Harrison (1979) to suggest that the membranes of pollen cells are likely to be ineffective as an osmotic barrier because their organization and/or permeability changes during dehydration. During pollen grain germination, upon initial hydration, the integrity of the pollen cell membrane is restored, presumably leading to the recovery of its semipermeability properties and capacity for controlling the movement of solutes. Restoration of membrane integrity is an essential prerequisite for pollen germination, and the inability of the membrane to regain its normal structure is one of the causes of the loss of pollen viability (Simon, 1974; Shivanna and Heslop-Harrison, 1981; Heslop-Harrison, 1987).

We have observed (Singh et al., 1987a; Nelson et al., 1988) that the OSM protein associates with the tonoplast and plasma membrane of tobacco cells but not chloroplast or mitochondrial membranes. Also, Yen et al. (1991) have reported the identification of an OSM-like protein that is associated with the plasma membrane from the common ice plant, a halophyte. It is therefore possible that OSM participates in the dehydration protection of membranes of desiccated pollen cells and that this protection involves the reorganization of membranes in the desiccated state, causing them to become potentially leaky if their normal structure is not restored during rehydration. If this is indeed the case, it could explain why the protein product of a gene that is dramatically regulated by desiccation or osmotic stress is so apparently active in the induction of leakiness of fungal membranes (Vigers et al., 1991). The OSM gene may have evolved as part of a mechanism causing permeabilization that protects pollen membranes during dehydration. Later, this gene may have been modified to allow sequestration of the insoluble protein in vacuoles so that the OSM protein released from the vacuole by pathogen attack would specifically permeabilize the vulnerable hydrated

plasma membranes of pathogens. It will be important to determine the intracellular localization of the OSM protein in these different tissues to test this hypothesis.

The Pattern of OSM Promoter Activity in NaCl-Adapted Plants Is Consistent with Both an Osmotic Adaptation Mechanism and a Pathogen Defense Mechanism

Both the temporal and spatial patterns of OSM promoter activity observed here have considerable similarity to the map of Na⁺ and K⁺ transport and distribution in whole plants constructed by Wolf et al. (1991). These similarities are related to low K⁺/high Na⁺ and include the following observations:

- (1) There is an increase in GUS promoter activity, particularly in root, stem, and part of leaf tissues during and after adaptation to high salt.
- (2) Relatively high expression of GUS activity was found in roots in general and compared with other plant organs and specifically in the root elongation zone but not in the root tip meristem.
- (3) Higher GUS activity was observed in older compared with younger leaves and stem internodes.
- (4) No GUS activity was detected in apical meristems and leaf primordia.
- (5) High GUS activity could be seen in xylem parenchyma of both stem and leaves.

This similarity between Na⁺ and K⁺ distribution within whole plants and the pattern of OSM promoter activity tends to support the hypothesis that the expression of the OSM gene is an adaptive response of plants to osmotic stress. Yet, several aspects of the developmental pattern of OSM promoter activity also suggest that the OSM has a natural role in defense against pathogens, especially those pathogens that may induce symptoms of osmotic stress such as bacterial or fungal wilt pathogens. For instance, these pathogens first tend to attack root tissues near or at the zone of cell elongation. They also can spread to and disrupt the vascular tissues. Both sites exhibit relatively active OSM::GUS expression.

The OSM gene may be osmotically responsive so that the defense mechanism of plants will recognize the induced osmotic stress symptoms of several pathogens such as bacterial and fungal wilt pathogens. This recognition of the pathogen by its induction of stress symptoms rather than by a specific chemical signal released by the pathogen would make it virtually impossible for the pathogen to evolve a counter-defense mechanism to avoid specific recognition by the host. Such a defensive response can be envisaged as the plant's "second line" of defense, which is more slowly activated at a later stage of pathogen attack but would not rely on the early but easily "mutable" specific elicitors produced by specific pathogens.

METHODS

Plant Transformation

A DNA fragment containing 1.8 kb of sequence in the 5' direction from the transcription start of the osmotin (OSM) gene (Nelson et al., 1992) was inserted in the BamHI site of pBI101.1, which is at the 5' end of the gene encoding β -glucuronidase (GUS) (Jefferson et al., 1987). This construct was transferred to *Agrobacterium tumefaciens* and introduced into *Nicotiana tabacum* cv W38 by *A. tumefaciens*-mediated tobacco leaf disc transformation. Plants were regenerated directly from the leaf discs. Several independent primary transformants were obtained, and three were examined histologically. Data obtained from only one of them, designated GUS1B, which contained four copies of the OSM gene construct and expressed the highest level of GUS activity (Nelson et al., 1992), are reported here. However, the pattern of expression in the other two plants was consistent with that observed with GUS1B. Tobacco plants transformed with a construct consisting of only the GUS coding sequence (GUS transformants) were used as control plants.

Growth and Adaptation of Tobacco Plants to NaCl

Vegetatively propagated transgenic tobacco plants carrying the OSM::GUS chimeric gene or the GUS gene without the OSM promoter were grown hydroponically in quarter-strength MS salts (Murashige and Skoog, 1962) and adapted stepwise to a concentration of 170 mM NaCl by increasing the NaCl concentration by 42.5 mM every 3 days. Plants were grown in the presence of 170 mM NaCl for 4, 8, and 12 weeks before tissue samples were taken for GUS analyses. Hydroponically cultured plants of the same age grown without NaCl were used as unadapted controls.

Histochemical Assay for GUS Enzyme Activity

The GUS enzyme assay was used to measure the relative activity of the OSM promoter in OSM::GUS gene fusion transformants. Histochemical staining for GUS activity was performed according to the procedure of Jefferson et al. (1987), with the modifications proposed by Koltunow et al. (1990). Samples of plant tissues and organs were fixed for 15 min under vacuum in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1% formaldehyde, 0.1% Triton X-100, and 0.1% β -mercaptoethanol. Samples were then rinsed several times with 0.1 M phosphate buffer containing 0.1% β -mercaptoethanol, followed by a rinse with 0.05 M phosphate buffer, pH 7.0. For the GUS reaction assay, a buffered solution (0.05 M sodium phosphate buffer, pH 7.0) of 1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid cyclohexylammonium salt (Biosynth AG, Staat, Switzerland) containing 0.1% β -mercaptoethanol and 0.1% Triton X-100 was used. After the GUS reaction, organ or tissue samples were fixed for 4 hr in a 2% glutaraldehyde solution in 0.05 M phosphate buffer, pH 7.2, or 10% formaldehyde, 20% ethanol, 5% acetic acid. Chlorophyll was extracted from tissues during dehydration in an ethanol series prior to embedding in Tissue Prep 2 embedding wax (Fisher Scientific, Fair Lawn, NJ). Paraffin sections (10- to 50- μ m-thick) were cut and deparaffinized. The slides were viewed and photographed using a microscope (Optiphot model; Nikon, Tokyo) set for bright-field illumination or Nomarski differential interference contrast.

For pollen grains, along with the procedure described above, the GUS assay proposed by Kosugi et al. (1990) was used to eliminate intrinsic GUS activity by the addition of methanol at 20% volume to the GUS assay mixture.

In Vitro Germination of Pollen Grains

Pollen was collected from flowers at anthesis and germinated on microscopic slides coated with solidified medium containing 3 mM H_3BO_3 , 1.7 mM $Ca(NO_3)_2$, 10% sucrose, and 0.7% agar at pH 5.8 (Bino et al., 1987). Pollen grains were germinated at 24°C in the dark in a humid chamber prior to GUS assay.

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