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Promoters from *kin1* and *cor6.6*, two *Arabidopsis thaliana* low-temperature- and ABA-inducible genes, direct strong β-glucuronidase expression in guard cells, pollen and young developing seeds

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Abstract

The ability of most higher plants to withstand freezing can be enhanced by cold acclimation, although the freezing tolerance of plant tissues is also affected by their developmental stage. In addition, low temperature has pleiotropic effects on many plant developmental processes such as vernalization. The interaction between plant development and low temperature implies that some genes are regulated by both environmental factors and developmental cues. Although a number of cold-inducible genes from plants have been identified, information concerning their regulation during plant development is limited. In order to understand their developmental regulation and obtain possible clues as to function, the promoters of *kin1* and *cor6.6*, two cold- and abscisic acid (ABA)-regulated genes from *Arabidopsis thaliana*, were fused to the β-glucuronidase (GUS)-coding sequence and the resulting constructs were used to transform tobacco and *A. thaliana*. Transgenic plants with either the *kin1* or *cor6.6* promoter showed strong GUS expression in pollen, developing seeds, trichomes and, most interestingly, in guard cells. During pollen development, maximum GUS activity was found in mature pollen. In contrast, the maximum GUS activity during seed development was during early embryogenesis. These patterns of expression distinguish *kin1* and *cor6.6* from related *lea* genes which are strongly expressed during late embryogenesis. There was no major qualitative difference in patterns of GUS expression between *kin1* and *cor6.6* promoters and the results were similar for transgenic tobacco and *Arabidopsis*. Considering the results described, as well as those in an accompanying paper (Wang *et al.*, 1995, Plant Mol Biol 28: 605–617 (this issue), we suggest that osmotic potential might be a major factor in regulating the expression of *kin1* and *cor6.6* during several developmental processes. The implication of the results for possible function of the gene products is discussed.

Introduction

Temperature is one of the most important environmental factors influencing the physiology, growth and development of plants. Low temperature slows growth and acts as a 'switch' during developmental processes such as vernalization, seed dormancy, bud dormancy (of trees), devel-

opment of underground storage organs and thermoperiodism. Since temperature extremes can be damaging, plants have developed adaptive mechanisms to tolerate substantial temperature variation. For example, through cold acclimation, many plants are able to increase their cold tolerance upon exposure to a low, non-damaging temperature. Although these processes have been studied intensively, the molecular events involved remain poorly understood.

As first steps toward understanding the molecular mechanism of cold acclimation, a number of cold-inducible genes have been identified in recent years, particularly in *Arabidopsis thaliana* [18, 28]. In previous studies the regulation of cold-inducible genes by environmental factors such as low temperature, absciscic acid (ABA) and dehydration has been addressed. Less attention has been paid to their expression during normal (unstressed) plant development. Studies on the developmental regulation of cold-inducible genes are important for several reasons. First, they may help reveal how environmental conditions interract with developmental processes. Second, the functions of most cold-induced genes remain elusive. Studies of developmental expression should provide clues to function since genes are expressed where and when they are required. Third, if some of these genes are to be expressed in heterologous plants for increasing cold tolerance or other purposes, developmental expression should be fully characterized.

Among cold-inducible genes from *Arabidopsis*, there are now at least three cases where two homologous genes are present tandemly in the genome (see references in [32]). One such a pair consists of the genes *kin1* and *cor6.6* [9, 15, 16], which are transcriptionally induced by low temperature as well as ABA and drought. To understand the developmental regulation of cold inducible genes and possible differential expression between the members of the same family, we have cloned a 5.3 kb genomic fragment covering both *kin1*, *cor6.6* and their respective 5′ sequences [31]. The 1.4 kb 5′-regulatory regions of both genes were fused to the β-glucuronidase (GUS)-coding sequence and analysed in transgenic tobacco and *A. thaliana*. Here we characterize patterns of expression during development of unstressed plants. Most of the detailed analyses were performed with transgenic tobacco since its large size and long life-cycle facilitated analysis of tissue- and development-specific expression.

Materials and methods

Constructs and plant transformation

The binary vector constructs containing either *kin1* or *cor6.6* promoter fused to the β-glucuronidase (GUS)-coding sequence and transformation of tobacco and *Arabidopsis* using these constructs were described as in Wang *et al.* ([32], constructs shown in Fig. 3). The transformants were transferred to soil in pots and maintained in a greenhouse. Most analyses were performed with two representative plants from each construct with medium to high levels of GUS activity in leaves. They were: for pHW111, plants 5 and 8; for pHW112, plants 7 and 30; for pHW113, plants 8 and 13; and for pBI121, plants 9 and 26. Plants from the same construct all showed similar patterns of expression.

Quantitation of GUS activity

GUS activity was quantified using fluorometry [13]. Tissues from sources described below were ground in the extraction buffer using disposable plastic tubes and pestles. In addition, 20 mM DTT was included in the extraction buffer for preparing samples from developing seeds to prevent oxidative browning of early-developing seeds (5–10 days after flowering, DAF). The supernatant after two centrifugations was used for GUS assay following the procedure described [13] and for determining protein concentration [5] (using a protein assay kit from BioRad, Mississauga, Ont.). GUS activity was expressed as pmol MU (4-methylumbelliferone) per milligram protein per minute.

Leaf extracts were prepared from leaves of 15–20 cm length unless stated otherwise. Two discs were punched out using the lids of 1.5 ml Eppen-

dorf tubes at neighbouring positions at the center of the leaf and ground with sand in 400 μl extraction buffer. As plants were flowering, mature flowers were excised with a scalpel and were separated into following parts: sepal, petal, anther, stamen filament, stigma and pistil, and ovary. Extracts were prepared from these parts as for leaves. Extracts from anthers were prepared by thorough grinding without sand. Stem and root samples were prepared after seeds had been collected from these plants. Thus plants were several months older. For stem samples, all the major shoots were removed first and the regenerating secondary shoots were used. Extracts were prepared from stem sections about 7–12 mm in diameter. Root 1 samples came from roots excised from the plants and soil carefully removed. For root 2 samples, most roots were removed first and, two weeks later, the fresh regenerating roots were used for preparing the extracts.

For determining the GUS activity in the epidermis, the extract was prepared from strips from the lower epidermis of a leaf. At the same time an extract was also prepared from discs adjacent to the site of epidermal sampling on the same leaf. For analysing expression during seed development in tobacco, flowers were tagged at anthesis. The fruits between 5–30 DAF were removed and seeds detached for preparing extracts. For preparing extracts from seedlings, the cotyledons or first leaves from 16–20 seedlings were randomly collected.

Histochemical localization of GUS activity

GUS activity was histochemically stained following the method as described [13] using 5-bromo-4-chloro-3-indolyl glucuronidase (X-gluc) as the substrate. Fresh tissues, or tissues fixed with 0.2% formaldehyde in 100 mM phosphate buffer (pH 7.0) for 20–25 min, were placed in the staining solution consisting of 1 mg/ml X-gluc, 100 mM phosphate pH 7.0, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 10 mM Na_2EDTA and 0.02% Triton X-100, and processed in 24-well tissue culture plates. Tissues were infiltrated under partial vacuum (50–64 cm Hg) for 10 min, followed by slow vacuum release and subsequent incubation at 37 °C. For comparative analysis the conditions (e.g. incubation time) were kept the same for different treatments. After the staining, tissues were sometimes cleared with 70% ethanol.

Most tissues were taken from pot-growing plants and used directly in the staining. In addition, for analysing GUS activity during pollen development, anthers were excised, placed in a tube containing a solution of 13% sucrose in 100 mM phosphate buffer and the pollen squeezed out of anthers with a plastic pestle. The pollen suspension was transferred into a conical tube and centrifuged for 2–3 min at 70–90 × *g*. Before staining, some samples were fixed with 1–1.5 ml of fixative consisting of 0.2% formaldehyde and 0.1% Triton X-100 in 0.1 M phosphate buffer for 25 min, followed by two washes with 13% sucrose in phosphate buffer. For *in vitro* germination experiments, pollen grains were collected from flowers at anthesis and germinated at 24 °C in the dark on glass slides coated with a germination medium consisting of 3 mM H_3BO_3, 1.7 mM $Ca(NO_3)_2$, 10% sucrose and 0.7% agar pH 5.8 [4]. For cryosectioning, tissues were frozen in TissueTekII (Sigma) and were cut into 20 μm sections. Stained sections were mounted in 50% glycerol. Seedlings were germinated on solidified medium in Petri plates. Some roots were also generated from excised tobacco shoot stalks submerged in Hoagland's solution.

Microscopy

Micrographs were taken with either a Wild Heerbrugg dissecting microscope or a Leitz Orthoplan microscope equipped with interference contrast optics.

Results

General survey of GUS activity

Transgenic tobacco plants were obtained from the following constructs with promoter-GUS

fusions: pHW111 containing the *kin1* promoter; pHW112 containing the *cor6.6* promoter; pHW113 containing the *cor6.6* promoter with a 45 bp deletion of the untranslated leader sequence; pBI121 (positive control) containing a cauliflower mosaic virus (CaMV) 35S promoter-GUS fusion and pBI101.2 (negative control) a promoterless GUS construct. The temporal and spatial expression patterns during plant development were examined by histochemical localization and quantitative analysis of GUS activity in different organs (parts) in the absence of external stresses. Results of a general comparison are shown in Fig. 1 (developing seeds or fruits were not included). It was evident that most organs (or floral parts) of pHW (construct-containing) plants showed GUS activity although the level varied significantly. The overall pattern of expression for all pHW plants was clearly different from that of pBI121 plants directed by the CaMV 35S promoter, indicating that the specificity of GUS activity in pHW plants was due to *kin1* or *cor6.6* promoter. More specifically, the *kin1* and *cor6.6* promoter-GUS fusions showed strong expression in anther as well as in developing seeds (shown later) but lacked expression in ovary, relative to the 35S promoter-GUS fusion. In addition, all organs (tissues) from pBI101.2 plants exhibited

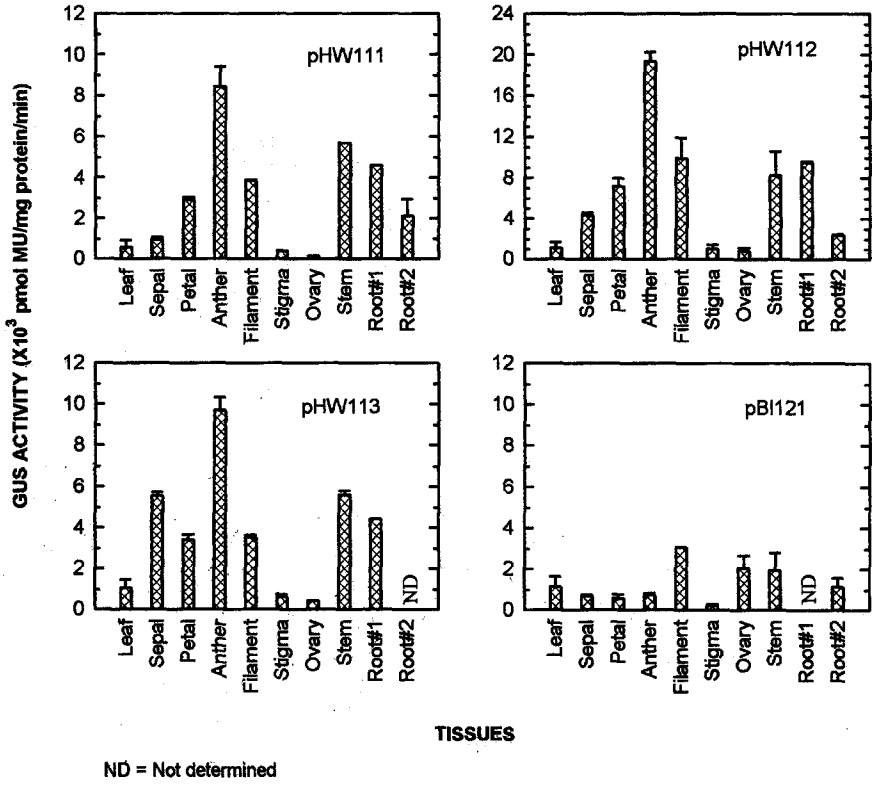


Fig. 1. General survey of GUS activity in different organs and floral parts of R_0 transgenic tobacco plants. Data are from representative plants based on similar magnitudes of GUS expression in leaves. The average (from 3 or more leaves) GUS activity was (in pmol MU per mg protein per minute): 560 for pHW111 and about 1100 for other plants. Extracts were prepared from various sources of individual transgenic plants and used for GUS and protein assays. In most cases two or more samples were obtained (the standard deviation given on top of the bar). Leaf samples were prepared 6–8 weeks after plants were transferred to soil. Floral parts were from flowers before anthesis. Stem and root samples were prepared from older plants maintained in greenhouse by pruning. Stem samples were prepared from secondary shoots after the plants were pruned. Root 1 samples were prepared from plants directly and root 2 samples from regenerating roots two weeks after most of the previous roots had been removed. ND = not determined.

very low GUS activity, less than 30 pmol per mg protein per minute (data not shown). Further analyses were undertaken to determine cell-specific expression within these organs (parts) and the changes occurring during development.

Expression in vegetative tissues

Analysis of GUS activity during leaf development showed that it generally increased with the leaf age. Results from one representative plant (containing pHW112) are presented in Fig. 2. Furthermore it was observed that older plants generally had higher GUS activity (data not shown). These observations indicate that the expression in leaves increased both with the leaf age and plant (shoot) age.

When the GUS activity was localized after incubating leaves with the substrate X-gluc, the guard cells of the stomatal complex were intensely stained and, to some degree, the trichomes also, while there was only weak staining in the neighbouring epidermal cells and no staining in other

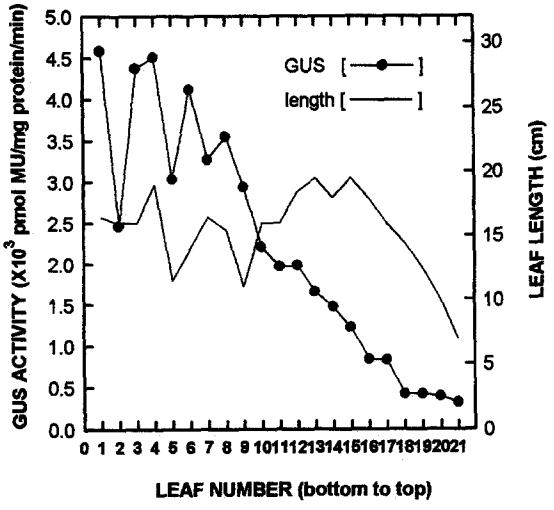


Fig. 2. Variation in GUS activity with leaf size and position. Samples were prepared from different leaves of the same tobacco transformant and GUS activity was measured (presented as pmol MU formed per mg protein per minute). The data shown here is from a representative pHW112 primary transgenic and are average values from duplicate neighbouring leaf discs. Leaves are numbered from the base to the tip.

epidermal cells (not adjacent to the guard cells) (Fig. 3a–g). The strong expression in guard cells was observed in both cotyledons (Fig. 3a–b) and leaves (Fig. 3c). Use of peeled epidermis revealed more clearly the difference in GUS activity among various epidermal cells (Fig. 3d–f). For trichomes, curiously, the intensity of coloration varied considerably from one to another on the same leaf (Fig. 3d). Fixation in 0.2% formaldehyde prior to staining did not alter the pattern, although the intensity of staining decreased with prolonged fixation (data not shown). Therefore, the strong GUS activity in the guard cells was not due to induction of expression during the incubation for staining. The relative level of GUS activity among various cell types of unstressed leaves based on staining density was: guard cells > trichome > other leaf cells.

Transformed plants of pHW111, pHW112 and pHW113 showed the same pattern, indicating that both *kin1* and *cor6.6* are highly expressed in guard cells and that such expression was probably conferred by a *cis*-acting element(s) in the promoter. Although the dramatic difference in the intensity of staining between guard cells and other leaf cells was unlikely to be due to differences in the penetration of substrate, a quantitative assay was nevertheless performed to confirm the results from histochemical localization and to obtain results normalized for protein content. Since it is technically difficult to isolate guard cells without altering the GUS expression (especially when it is directed by a stress-inducible promoter), the epidermis was peeled and the GUS specific activity was compared to activity of the leaf from a similar location on a leaf blade. As shown in Fig. 4, GUS specific activity in epidermis was much higher (7–9 times) than the average activity of punched leaf disc for pHW plants. Since the leaf sample also contained the epidermis (and guard cells), the activity ratio is an underestimate of the difference in GUS activity between epidermis and mesophyll cells. As a comparison, GUS activity in the epidermis of the plants harbouring the CaMV 35S promoter was the same or only marginally higher than the average leaf activity (Fig. 4).

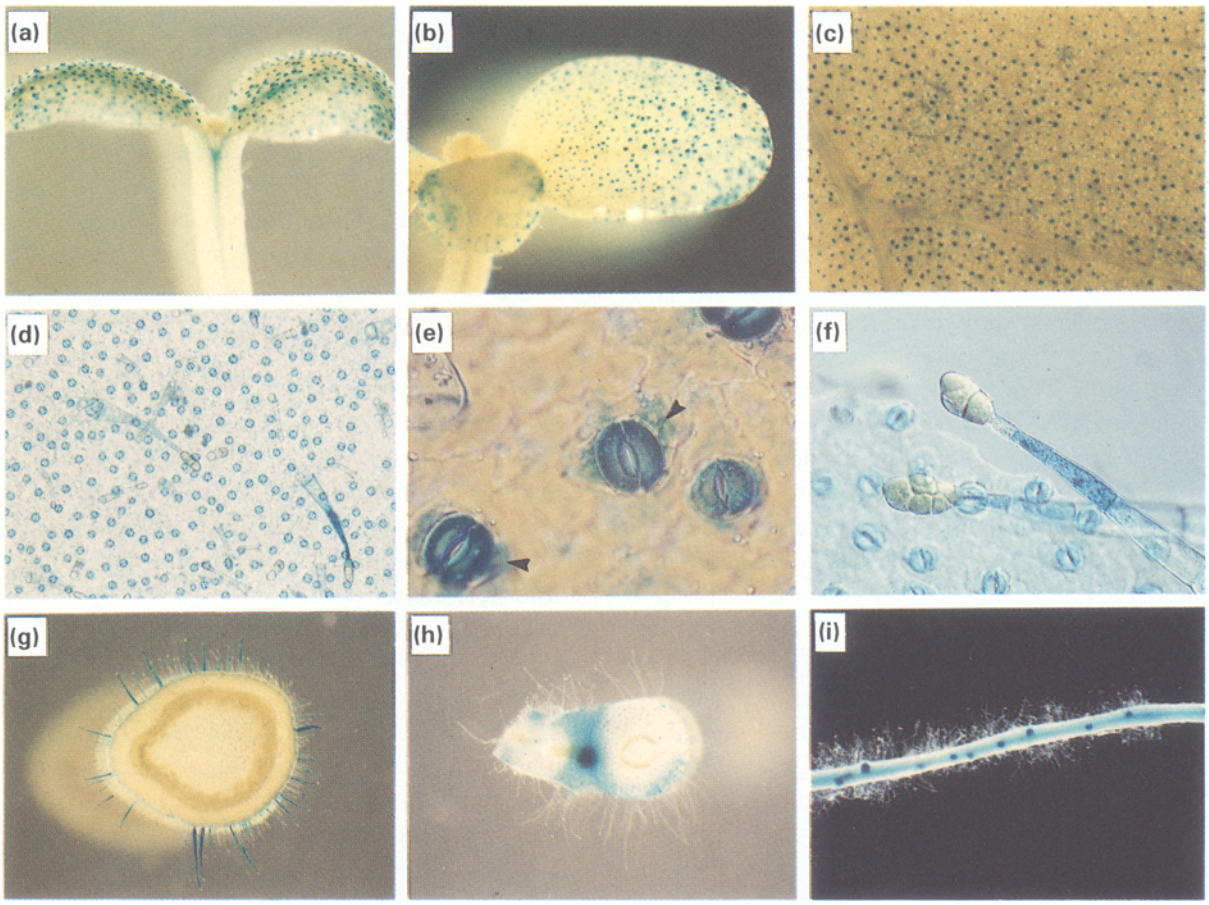


Fig. 3. Histochemical localization of GUS expression directed by either *kin1* (a, b) or *cor6.6* (c–i) promoter in vegetative tissues of transgenic tobacco plants. (a, b) Seedlings growing in Petri plates at two weeks (a) and three weeks (b) showing strong expression in cotyledon guard cells. × 14. (c) Leaf showing strong expression in guard cells. Note that the small veins remained unstained. × 28. (d) Peeled epidermal strip (viewed at low magnification) with guard cells and some trichomes showing strong GUS activity. × 34. (e) Stained guard cells on an epidermal peel. Note some weak staining in neighbouring epidermal cells (arrowheads). × 285. (f) Stained trichomes on an epidermal peel. Note the stained guard cells in the background. × 114. (g) A stem section showing GUS activity in epidermis and trichomes. × 5.3. (h) A stem section showing strong GUS activity in the lateral bud. × 6.7. (i) A root generated from hydroponically cultured shoot showing strong GUS activity in lateral root primordia. × 3.4.

Quantitative analysis showed that GUS specific activities in extracts of shoots and roots of pHW plants were higher than in leaves (Fig. 1). Cross sections of stems revealed expression in trichomes and epidermis (Fig. 3g). In older stems, expression was sometimes observed in vascular tissues and parenchyma cells (data not shown). However, the strongest expression in shoot was observed in lateral bud primordia (Fig. 3h). In roots generated from hydroponically cultured shoots, strong expression was observed in lateral root primordia (Fig. 3i).

Expression in reproductive tissues

The analysis of GUS expression during anther development is shown in Fig. 5. The level of GUS activity in mature anthers was 40–200 times greater than activity in young anthers (flower bud about 5 mm) for all transformants with pHW111, pHW112 and pHW113 while it remained the same in transformants with pBI121 (CaMV 35S promoter) (Fig. 5).

Histochemical staining of detached flower parts showed that GUS activity in sepals (Fig. 6a), pet-

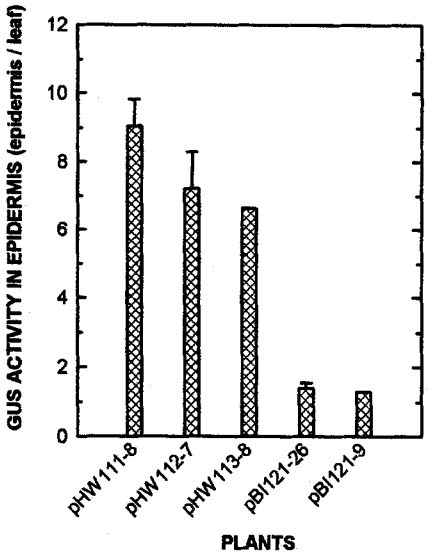


Fig. 4. Quantitation of GUS activity in epidermis of transgenic tobacco. GUS activity (in pmol MU per mg protein per minute) was determined for samples prepared from leaf discs and epidermal peels from the same leaves. The ratio between GUS activity in epidermis and GUS activity of leaf discs were plotted. The number above the bars indicates vector and independent transformant (following the dash). The standard deviation is shown on the top of the bar.

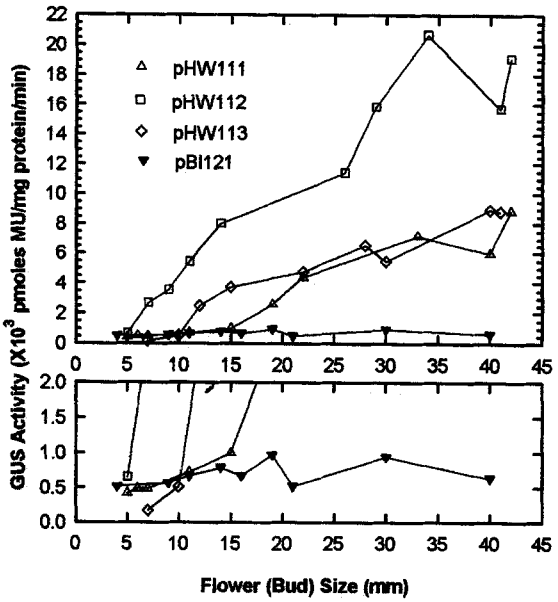


Fig. 5. Quantitation of GUS activity during anther development. Anthers were removed from flower buds of various lengths. The extracts prepared were used for GUS and protein assays. Data from one representative plant of each construct were plotted. a (top). Figure plotted at a larger scale. b (bottom). The lower portion of (a) plotted at a smaller scale.

als (Fig. 6b) and stamen filaments (Fig. 6c) were localized in guard cells and trichomes, as in leaves. There was little activity in the ovary, style and stigma and this corresponded with a reduced number or absence of trichomes and guard cells in these floral parts. Cryo-sections stained under identical conditions indicated that the strongest activity was in guard cells of the sepal (Fig. 6d) and pollen of the anther (Fig. 6e). The anther wall showed little activity, indicating that the GUS activity in mature anther (Fig. 5) was due to the expression in pollen.

Analyses of isolated pollen from flower buds showed progressively increasing GUS staining during pollen maturation (Fig. 6f–h) with maximum expression at anthesis (Fig. 6h–i). The 'cut-off' size for flowers to show visible pollen staining depended on individual plants and incubation times. Obviously, plants with higher levels of GUS expression would show staining of anthers at earlier stages (smaller size) relative to those of plants with lower GUS expression when assayed under the same conditions. In some plants with high GUS expression, mature pollen showed visible staining in 30 min. In addition, germinating pollen demonstrated apparently lower GUS activity than ungerminated pollen from the same plant (compare Fig. 6j to Fig. 6i).

Expression during seed development

Fruits at 5-day intervals after anthesis (DAF, for days after flowering) were hand-dissected and stained for GUS activity. The results (Fig. 7a, c, e, g and i) showed that there was clear expression in fruits at 5–15 DAF with the highest expression observed at 10 DAF (Fig. 7c). The junctions of two carpels showed some activity and low activity was occasionally observed in placenta while longitudinal sections revealed enhanced expression near the receptacle. By far the highest activity was localized in the developing seeds.

Parallel analyses were performed with detached seeds (Fig. 7b, d, f, h and j). Developing seeds of

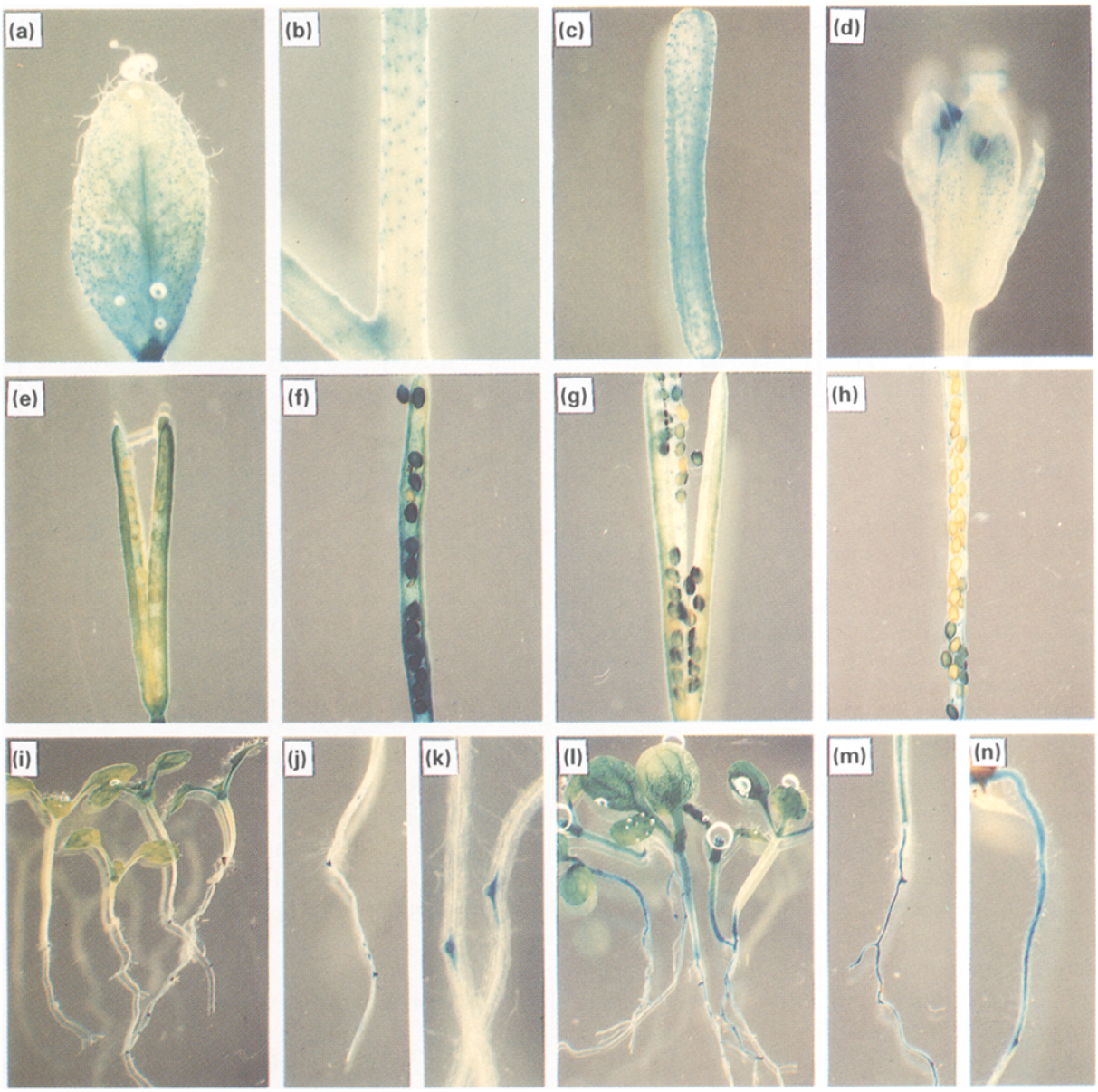
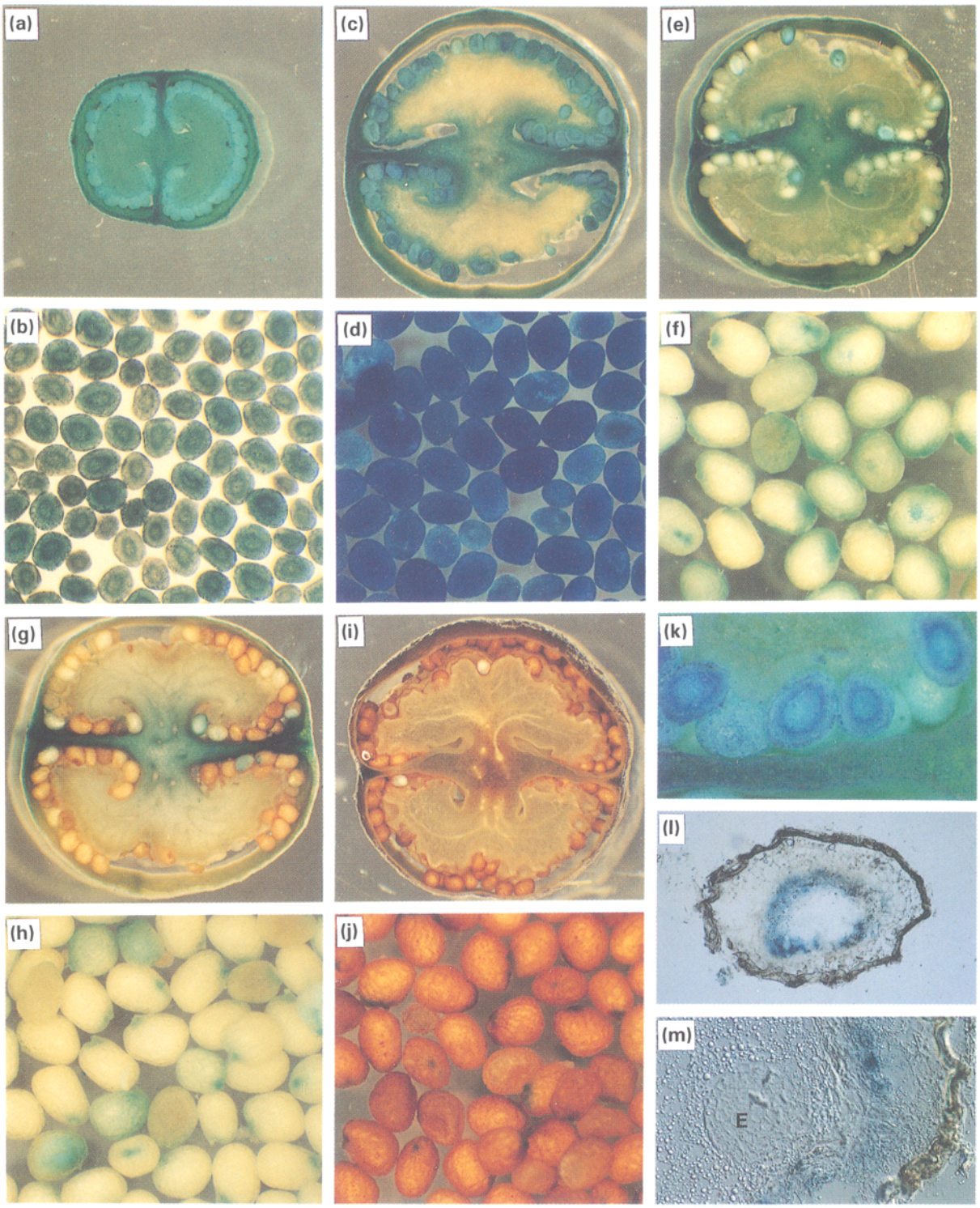


Fig. 6. GUS analysis in reproductive tissues of transgenic tobacco plants using floral parts (a–c), cryo-sections (d–e), isolated pollen (f–i) and germinating pollen (j). (d–e) were sectioned from the same flower of a pHW112 plant and stained for the same lenght of time. Pollen in (f–h) were isolated from flower buds of a pHW111 plant and had 16 h incubation for GUS staining. Pollen in (i–j) were from a pHW112 plant and had 5 h incubation for GUS activity. (a) Sepal viewed from the outer face. Note staining in guard cells and trichomes. × 14. (b) Petal viewed from the inner face. × 14. (c) Stamen filament attached to the inner side of petal. Note numerous stained trichomes. × 14. (d) Cryo-section of sepal (S) and petal (P) showing strong staining of guard cells (arrow heads) and faint staining in the epidermis. × 38. (e) Cryo-section of anther (about two days prior to anthesis) showing strong GUS activity in pollen but not in the anther wall (W). × 152. (f) Developing pollen from a flower bud of 17 mm. × 152. (g) Developing pollen from a flower bud of 27 mm. × 152. (h) Mature pollen from a flower bud of 40 mm. × 152. (i) Mature pollen from a flower bud of 43 mm (pHW112). × 152. (j) Germinating pollen after 12 h of *in vitro* germination. Note an ungerminated pollen at the center with strong GUS staining and much weaker staining for the germinating pollen (tubes). × 152.

Fig. 7. Histochemical localization of GUS activity during fruit and seed development in a pHW112 plant using either fruit sections (a, c, e, g, i, k), detached developing seeds (b, d, f, h, j) or cryo-sections (1 m). (a, b) 5 DAF (days after flowering). (a) × 4.6 and (b) × 14. (c, d) 10 DAF. (c) × 4.6 and (d) × 14. (e, f) 15 DAF. (e) × 4.6 and (f) × 14. (g, h) 20 DAF. (g) × 4.6 and (h) × 14.



(i, j) 25 DAF. (i) × 4.6 and (j) × 14. (k) Seeds at 10 DAF sectioned and stained on fruit. × 19. (l) Section of developing seed at 10 DAF showing strong staining in cells surrounding the embryo sac and the adjacent endosperm. × 152. (m) Section of developing seed at 10 DAF showing globular embryo (E). Note the small size relative to the seed. × 380.

5 DAF showed clear activity, which was manifested in a ring-shape structure (Fig. 7b). By 10 DAF GUS activity was maximal with more prominent staining in the same region as observed in 5 DAF seeds (Fig. 7d). However, it was difficult to identify the high-activity cells in the whole seeds since the staining was intense and may have diffused. At 15 DAF the staining of developing seeds was much reduced and there was some localized activity on the surface of the seed (Fig. 7f). At 20 DAF and later, little GUS staining was observed in the maturing seeds (Fig. 7h and 7j).

The GUS activity in the developing seeds was quantified and the results are shown in Fig. 8. GUS activity peaked around 10 DAF and then decreased, remaining low during seed maturation. Since the absolute activity varied significantly between individual plants, the ratio between the GUS activity at 10 DAF and that at 30 DAF (10/30 DAF ratio) was used to better compare the change of GUS activity during seed maturation among different plants. As shown in Table 1, the 10/30 DAF ratio for pHW112 plants averaged 228. The ratio was lower for pHW111 plants (average of 8.0) and for pHW113 plants (average of 18.3). Thus, it seemed that the relative expression of pHW112 (*cor6.6*) plants appeared stronger than that of pHW111 (*kin1*) plants in 10 DAF seeds. However, the ratios for pHW111 and pHW113 might be less accurate since only two

Table 1. The GUS activity ratio between 10 DAF and 30 DAF tobacco seeds from transformants with various constructs. Flowers were tagged at anthesis. Specific GUS activity was determined from 2–4 samples at each time point for each plant. The calculated result for each plant is the ratio between the averages of GUS activities at 10 and 30 DAF.

Construct	GUS activity ratio = 10/30 DAF seeds (average ± SD)	Number of plants
pHW111	8.0 ± 5.3	2
pHW112	228 ± 133	4
pHW113	18.3 ± 2.5	2
pBI121	1.6 ± 0.9	4

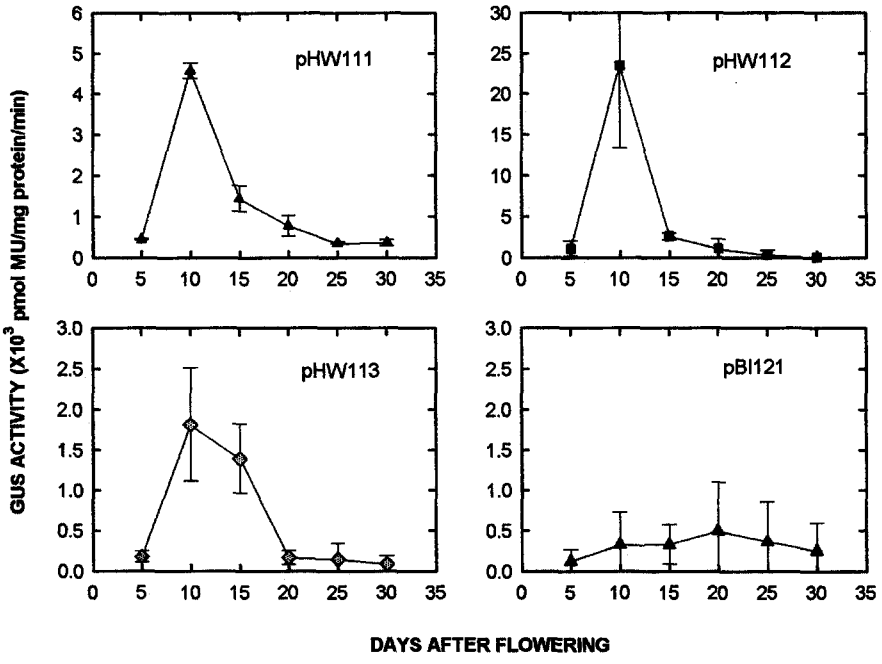


Fig. 8. Quantitation of GUS activity during seed development. Seeds were removed from capsules at 5-day intervals after anthesis. The extracts prepared were used for GUS and protein assays. Data from one representative plant of each construct were plotted: (a) pHW111, (b) pHW112, (c) pHW113 and (d) pHI121.

plants were used from each of the two constructs in these analyses and these plants had lower basal levels of expression than pHW112 plants. Nevertheless, the 10/30 DAF ratios for all three pHW constructs were much higher than the ratio of pBI121 with an average of 1.6.

To exclude the possibility that difficulty in substrate penetration might have hindered the staining of more developed seeds, cryo-sections and dissected embryos were examined. The cryo-sections of detached seeds further demonstrated that the strongest expression in developing seeds of 10 DAF was localized in cells surrounding the embryo sac membrane (inner integument) and

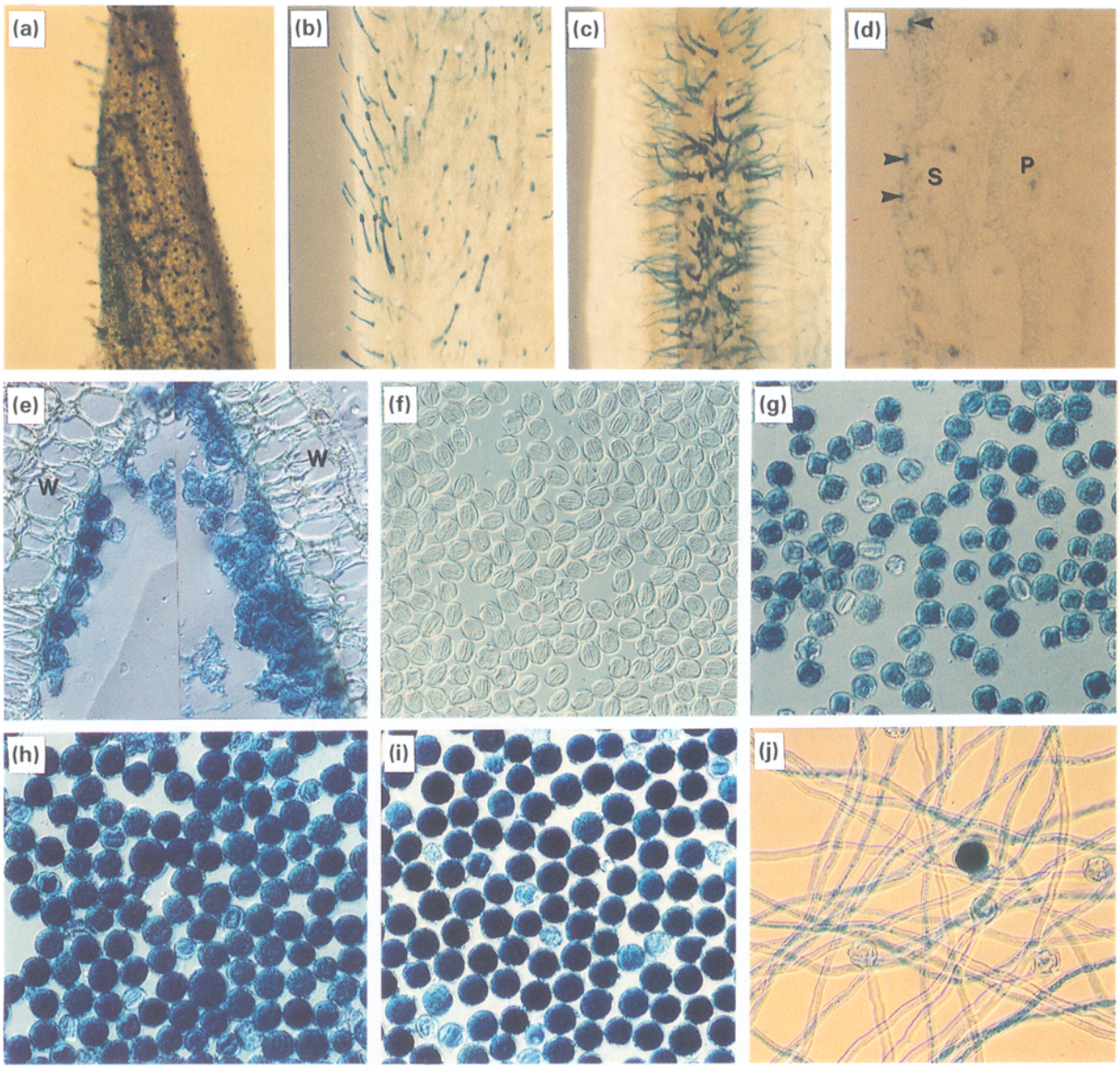


Fig. 9. Histochemical localization of GUS activity in transgenic pHW112 *Arabidopsis* plants. (a) Leaf. × 13. (b) Stem. × 13. (c) Sepal. × 13. (d) Flower. × 13. (e, f, g, h) Seeds in successive developmental stages. × 4.9. (i) Seedlings under normal conditions. × 3.2. (j, k) Roots showing strong expression in lateral root primordia. (j) × 6.6 and (k) × 27. (l) Seedlings treated with ABA for 24 h. × 3.2. (m, n) Roots from ABA-treated seedlings showing strong expression in root tips and vascular tissues. (m) × 6.6 and (n) × 13.

perhaps the adjacent endosperm cells (Fig. 7k–l). A more definite localization however requires further experiments using embedded tissue and microscopy. Embryos from 10 DAF seeds were observed to be at the globular stage and did not show any staining (Fig. 7m). At this stage, dissecting out the embryos was still difficult due to their small size and fragility. Embryos from 15 DAF seeds however could be easily dissected out, identified as being at torpedo stage and were not stained (data not shown).

Expression in transgenic Arabidopsis

Since the above results, although intriguing, were from a heterologous system, transgenic *Arabidopsis* plants were also obtained with pHW112 and analysed similarly. Results showed similar patterns of GUS expression during plant development (Fig. 9). GUS activity was particularly strong in guard cells of leaf, stem and flower tissues (Fig. 9a–d), in pollen (Fig. 9d) and in developing seeds (Fig. 9e–h). Quantitation of GUS activity in developing seeds showed strong expression around early-mid stage of development (Fig. 10).

Similar to tobacco, the basal GUS activity in seedlings was generally low (Fig. 9i) whereas strong expression was observed in the lateral root primordia and the leaf base where lateral buds would emerge (Fig. 9j–k). The expression in guard cells of *Arabidopsis* seedlings, however, was less obvious than the tobacco seedlings. Seedlings were also analysed to assess the effect of several inducing treatments on tissue-specific GUS expression. Seedlings exposed to low temperature treatment (5 °C) were similar to the control seedlings (Fig. 9i). Seedlings subjected to ABA (Fig. 9l–n) or osmotic treatments showed obvious increase in GUS staining, which was more noticeable in the vascular tissues (Fig. 9m–n). Quantitative analysis showed that the relative induction was similar in leaves (Fig. 8 in [32]) and in stem and root tissues (data not shown).

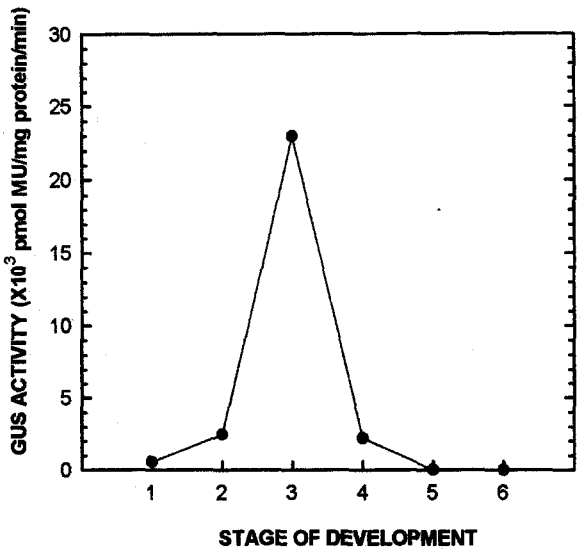


Fig. 10. Quantitation of GUS activity during seed development in trangenic pHW112 *Arabidopsis* plant. The developing siliques on continuously growing shoots were evenly divided into 6 stages from the early stage to mature seeds. The seeds isolated were assayed for GUS activity. Each datum represents the average of two samples from two shoots.

Discussion

Developmental regulation of cold-inducible genes

The expression of most cold inducible genes is also regulated by other environmental conditions (e.g. drought) and exogenous ABA. In addition, they are probably responsive to intrinsic developmental signals. A comprehensive understanding of gene regulation can only be achieved by identifying all the major factors influencing the expression. Thus, we have focused on two homologous *Arabidopsis* genes, *kin1* and *cor6.6*, in an attempt to elucidate some general mechanisms of regulation for cold-inducible genes. Transgenic plants with promoter-GUS fusions were analysed by both histochemical and quantitative assays. The histochemical staining, although able to localize GUS activity, is a qualitative measure of total GUS activity in cells and tissues, whereas the *in vitro* assay provides quantitative data that are normalized for protein content. GUS activity directed by a constitutive CaMV 35S promoter was analysed as a comparison to exclude the ef-

fect of GUS sequence itself on the expression pattern.

Results from the analyses of transgenic plants containing either *kin1* or *cor6.6* promoter revealed that the highest GUS activity was in guard cells of the stomatal complex, trichomes, pollen and developing seeds. The activity in pollen and developing seeds was even higher than that in ABA and osmoticum treated leaves [32]. For instance, the plant pHW112-30 had an average GUS activity (in nmol MU per mg protein per minute) of 4.84 for ABA-treated leaves, around 19 for mature pollen and 23 for seeds at 10 DAF. In addition, expression was also observed in lateral root and bud primordia, in the receptacle, in tissues near the joint of two carpels and in vascular tissues of stressed seedlings. However, histochemical staining indicated that the level of expression in these tissues was lower than that in mature pollen and developing seeds.

Recently, studies of gene regulation using promoter-GUS fusions indicated that some cold inducible genes are also expressed in non-stressed plants. The promoter of *A. thaliana rd29A* (*cor78*) was shown to direct relatively high GUS expression in trichomes of leaves [11, 35]. Weak GUS activity was also observed in vascular tissues of seedlings, the transitional regions between roots and stems, and petioles [35]. Although most of these tissues showed low to moderate levels of GUS activity with chimaeric *kin1/cor6.6* promoter-GUS genes, the striking expression in guard cells, pollen and developing seeds as observed for *kin1/cor6.6* promoters was not present for the *rd29A* promoter. During preparation of this manuscript, Baker *et al.* [2] showed that the promoter from *cor15a* directed strong GUS expression in anther but not in guard cells or seeds.

The same patterns of GUS expression directed by both *kin1* and *cor6.6* promoters indicate that they contain similar sequences conferring the tissue- and development-specific expression. In addition, the expression directed by the *cor6.6* promoter was similar between transgenic tobacco and *Arabidopsis* plants, indicating a conservation of the transcriptional regulation mechanism for these promoters between the two species. One difference was that strong expression in the lateral roots of *Arabidopsis* seedlings was not present in the lateral root primordia of tobacco seedlings. This expression, however, was observed in lateral roots from tobacco shoots cultured without constant aeration.

Expression in guard cells, mature pollen and developing seeds

To our knowledge this is the first report on the preferential expression of cold-inducible genes in guard cells, although expression in epidermis (relative to mesophyll cells) was reported for *lea* and *osmotin* genes [3, 8, 14]. Guard cells have a high osmolarity (relative to other leaf cells) during stomatal opening, due to an influx of K^+ [7, 12] and internal release of counterions [1]. For instance the concentration of K^+ is about 0.4 M in *Vicia faba* guard cells and could be as high as 0.8 M in tobacco [23]. Although the underlying factor responsible for the expression of *kin1* and *cor6.6* in guard cells is yet to be identified, it might be related to the adjustment of cells to high osmolarity, considering also their expression in other tissues (see discussion below). Some weak staining was usually present in epidermal cells neighbouring the guard cells, but not in other non-adjacent epidermal cells, coinciding with the K^+ osmotic gradient [24], although it could also be a non-specific activity diffused from the guard cells. The expression in guard cells apparently was not environmentally induced by water stress since seedlings growing in Petri plates (where humidity was close to 100%) also showed strong expression (Fig. 3a and 3b).

High-level promoter-directed GUS expression in pollen was observed recently for another cold-inducible gene, *cor15a*, although the time course was not examined [2]. Similar results were obtained for at least two other desiccation and osmoticum-inducible genes [14, 21]. The expression is probably due to desiccation and high osmolarity during pollen maturation. It is likely that such expression would be found for other desiccation-inducible genes and perhaps other

cold-inducible genes. In addition, it is noteworthy that the promoter-directed GUS activity of *kin1* and *cor6.6* decreased after pollen germination, as was observed for the *osmotin* gene [14].

Strong gene expression during embryogenesis, especially at late stages of maturation and desiccation, is typical of *lea* genes. They are of particular interest since most of them are also responsive to ABA and desiccation and many cold-inducible genes have sequence homology to *lea* genes. Based on their sequence similarity, *lea* genes were initially classified into three groups [6]. Promoters from some of these genes all conferred a high level of GUS expression during embryo maturation and desiccation in transgenic tobacco, consistent with results of mRNA analysis [19, 26, 34]. Among the cold inducible genes from *Arabidopsis*, the majority have sequence homology to *lea* genes with *cor47* [9], *rab18* [17] and *cab85* [22] belonging to Group 2 and the *cor15* [33] family to Group 3. As for *kin1/cor6.6*, Gilmour *et al.* [9] noted a low homology at the amino acid level between COR6.6 and *Brassica* LEA76 (a Group 3 *lea*) [10]. Expression of *kin1/cor6.6* is also induced by dehydration, osmoticum and ABA as typical of *lea* genes. In addition, they are hydrophilic, like most LEA proteins. Despite these similarities, however, *kin1* and *cor6.6* are unique in that they showed strong transient expression during early seed development outside the embryos, but not in the embryos during late embryogenesis. This is consistent with northern blot results showing no *cor6.6* expression in seeds during later developmental stages [9].

Functional implication for KIN1 and COR6.6

The observations that *kin1* and *cor6.6* are highly expressed in several types of tissues (cells) strongly imply that their products play a different role in these developmental processes than the suggested antifreeze activity [16]. The strong expression in guard cells and pollen and their induction by osmoticum and dehydration in leaves [32] suggest that *kin1* and *cor6.6* expression is related to cellular osmotic potential. This simple rationale is apparently inconsistent with their diminishing expression during desiccation of the seeds. However, GUS staining revealed that the expression was in cells surrounding the embryo sac and perhaps in the endosperm, not in the embryo (Fig. 7d and 7l). Previous studies indicated that osmolarity indeed decreased in this environment during embryo development. Rietsema et al. [25] observed that a lower concentration of sucrose was required for successful culture of more developed *Datura* embryos, with pre-heart embryos requiring about 12% sucrose and mature embryos being able to grow on 0% sucrose. Direct measurement of the endosperm liquid in *Phaseolus vulgaris* from heart stage to late cotyledon stage showed a decrease in osmolarity [27].

Therefore, GUS is predominantly expressed in cells and developmental processes with elevated osmolarity (low osmotic potential), suggesting that KIN1 and COR6.6 may be involved in the adjustment to low osmotic potential. A role in osmo-protection is not inconsistent with their induction by low temperature. It is interesting to observe that the expression patterns of *kin1* and *cor6.6* resemble that of the osmotin gene promoter (expressed in old leaves, epidermis and pollen) [14]. However, the lack of expression during embryo maturation and desiccation indicates that the desiccation signal can be overriden by developmental signals.

It is not clear if the high expression in root and shoot primordia is a manifestation of high osmolarity or of a distinct developmental cue. However, since ABA is involved in the formation of modified lateral roots during drought stress [30], expression in root primordia is not entirely surprising and warrants further investigation.

Cis-*acting elements for development-specific expression*

Since all pHW113 plants bearing a 45 bp deletion of the transcribed leader sequence in the full-length *cor6.6* promoter had the same expression patterns as pHW112 plants, developmental ex-

pression was likely mediated through *cis* elements in the 5′ untranscribed region. Comparisons of the *kin1* and *cor6.6* promoters with other promoters sharing similar expression patterns might be useful. However, there has been no consensus sequence identified as a pollen-specific *cis* element [20]. The *kin1* and *cor6.6* promoters do not contain the 52/56 box and 56/59 box sequence motifs identified in the promoter regions of pollen-specific tomato LAT genes [29]. There has been very little information on guard cell-specific expression. Thus, elements in the two promoters conferring the development-specific expression remain to be elucidated. Since the expression was strong in tissues and cells with high osmolarity, an interesting question is whether expression in some developmental processes (e.g. in pollen and developing seeds) is regulated through the same *cis* element conferring ABA-inducible expression. Current experiments are underway to address this and other questions concerning the sequences conferring environmentally induced expression and developmentally specific expression of the two genes, and to determine possible links between them.

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