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# Transient Gene Expression in Intact and Organized Rice Tissues

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**Regulated gene expression of chimeric genes has been studied extensively in electroporated protoplasts. The applicability of these assays is limited, however, because protoplasts are not always physiologically identical to the cells from which they are derived. We have developed a procedure to electroporate DNA into intact and organized leaf structures of rice. Optimization of the new gene delivery system mainly involved eliminating explant-released nucleases, prolonging the DNA/explant incubation time, and expanding the pulse time. Using a β-glucuronidase gene under the control of constitutive promoters, we demonstrated that all cell types within a leaf base were susceptible to electroporation-mediated DNA uptake. Although the technique was initially developed for leaf bases of young etiolated rice seedlings, we proved that it was equally applicable both to other monocotyledons, including wheat, maize, and barley, and to other explants, such as etiolated and green sheath and lamina tissues from rice. Transient gene expression assays with electroporated leaf bases showed that the promoter from a pea light-harvesting chlorophyll *a*/*b*-binding protein gene displayed both light- and chloroplast-dependent expression in rice, and that the promoter from the *Arabidopsis S*-adenosylmethionine synthetase gene was, as in transgenic *Arabidopsis* and tobacco, preferentially expressed in cells surrounding the vascular bundles.**

## INTRODUCTION

Despite recent reports of the production of transgenic maize (Rhodes et al., 1988) and rice (Zhang and Wu, 1988; Shimamoto et al., 1989), transformation of monocotyledonous plants is neither as routine nor as reproducible as that of dicotyledonous plants (Potrykus, 1989). As a consequence, analysis of regulatory sequences or of the effects of modifying biochemical pathways through introduction of new genes into Gramineae has been very limited.

Transient gene expression (TGE) assays provide an alternative system to analyze genetically modified plant genes. This approach has used electroporation (Fromm et al., 1985) or polyethylene glycol treatments (Shillito et al., 1985) to introduce genes transiently into plant protoplasts so that their expression can be evaluated. TGE assays offer two advantages over analysis of stable transformants. The major advantage is undoubtedly that gene activity can be measured within hours or days after DNA introduction. By comparison, it takes several weeks or months before stably transformed lines are available for detailed study. Furthermore, because the vast majority of transferred DNA remains extrachromosomal during the time course of a TGE assay (Werr and Lörz, 1986), the analysis is not confounded by influences exerted by chromosomal sequences adjacent to the sites of integrated genes. In transgenic plants these so-called position effects can influence both the level and the specificity of newly introduced genes (Jones et al., 1985; Poulsen et al., 1986).

Whereas inducible gene expression has been analyzed with TGE assays in protoplasts (e.g., Callis et al., 1988; Huttly and Baulcombe, 1989), the question remains whether regulated expression in cells without walls and dissociated from their original organs and tissues is always comparable with the expression in the corresponding intact tissues. Protoplast isolation treatments disrupt the developmental control systems dependent on cell/cell contact or on the position of a cell in an organized structure. Moreover, once protoplasts begin to resynthesize cell walls and divide in culture, they converge to the same phenotype regardless of their origin. This leads to loss of the differentiated state and quite probably of the control processes unique to those tissues. All of these problems are compounded in monocotyledons because the isolation of high-quality protoplasts from differentiated tissue has proven difficult. As a consequence, the standard material for TGE assays in monocotyledons is derived from cell suspension cultures, precluding examination of differential gene expression in different organs.

Several studies demonstrate the limitations of TGE assays in protoplasts. For example, mesophyll protoplasts isolated from illuminated tobacco leaves do not synthesize

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the small subunit of ribulose-1,5-bisphosphate carboxylase (Fleck et al., 1979; Vernet et al., 1982). Similarly, leaf protoplasts from barley leaves do not synthesize abscisic acid in response to osmotic stress, although the intact leaves do (Loveys and Robinson, 1987). In addition, varying terminator sequences of a chimeric gene varied gene expression as much as 60-fold in transgenic tobacco leaves but almost not at all in electroporated tobacco leaf protoplasts (Ingelbrecht et al., 1989).

In the hope of circumventing the limitations of TGE assays with protoplasts and to be able to study regulated gene expression in monocotyledons, we modified existing electroporation conditions in such a way that it became possible to deliver DNA into organized and intact plant tissue. This novel protocol not only retained all the advantages of a TGE assay, but allowed us to investigate, for the first time, the expression of both a light-regulated and a tissue-specific chimeric gene in monocotyledons. The approach was developed for rice, but could easily be adapted for maize, wheat, and barley.

## RESULTS

### Optimization of DNA Introduction into Intact Cells

Based on previous experiments (Dekeyser et al., 1989), we used a construct (*p2′-nptII*) with the promoter from the mannopine synthase *2′* gene of the *Agrobacterium* TR-DNA (Velten et al., 1984) fused to the neomycin phosphotransferase II (*nptII*) gene (Beck et al., 1982) to assess whether transient gene expression could be detected in intact and organized tissue.

Leaf base explants from 7-day-old, etiolated rice seedlings, as shown in Figure 1, were chosen to test the permeability of plant cells because (1) the explants contain several types of differentiated cells making it possible to study tissue-specific expression if the DNA were introduced uniformly, (2) the tissue contains meristematic cells capable of developing into embryogenic callus tissue (Wernicke et al., 1981; Abdullah et al., 1986) so that proliferative capacity after treatment could be monitored, (3) the tissue is soft, indicating a less rigid cell wall, and (4) the explants are easily obtained.

In initial experiments, 25 leaf bases were electroporated using conditions similar to those appropriate for protoplasts (Dekeyser et al., 1989). After 2 days of culture, the electroporated leaf bases showed low levels of neomycin phosphotransferase II (NPTII) activity (data not shown). Leaf bases electroporated with a promoterless *nptII* construct never showed any NPTII activity. To improve the efficiency of DNA introduction, we analyzed (1) factors influencing the DNA/explant interaction, (2) physical parameters related to the electroporation technique, and (3) chemical parameters that might protect the DNA or enhance the viability of the treated tissue.

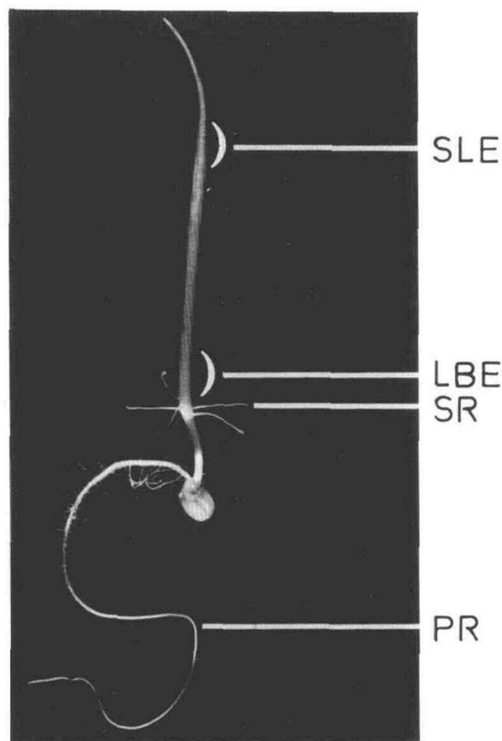


**Figure 1.** Gross Anatomy of a 7-Day-Old Etiolated Rice Seedling.

LBE, position of leaf base explant; SLE, position of sheath/lamina explant; PR, primary root; SR, secondary root.

Figure 2A illustrates that preincubating the isolated leaf bases in electroporation (EPR) medium before adding the plasmid DNA was essential for efficient introduction of DNA. When the preincubation time was prolonged from 15 min to 180 min, a 25-fold increase in NPTII activity was obtained. This result is at least partially related to the leakage of nucleases out of the leaf bases, a phenomenon also observed in excised barley leaves (Galston et al., 1979). This was demonstrated by refreshing the electroporation buffer after 15 min, 30 min, 60 min, 120 min, and 180 min, incubating each fraction with 2 $\mu$g of supercoiled plasmid DNA for 1 hr, and assessing the integrity of the plasmid DNA by agarose gel electrophoresis. Whereas the plasmid DNA incubated with the 180-min fraction remained intact, there was progressively more degradation of the samples in medium removed at the earlier time points (data not shown). Plasmid DNA degradation was even more pronounced when the experiment was done with root explants.

As shown in Figure 2B, another fourfold increase in NPTII activity was obtained by expanding the coincubation time of the plasmid DNA with the leaf bases from 15 min to 60 min.

The number or size of the pores in electroporated cells

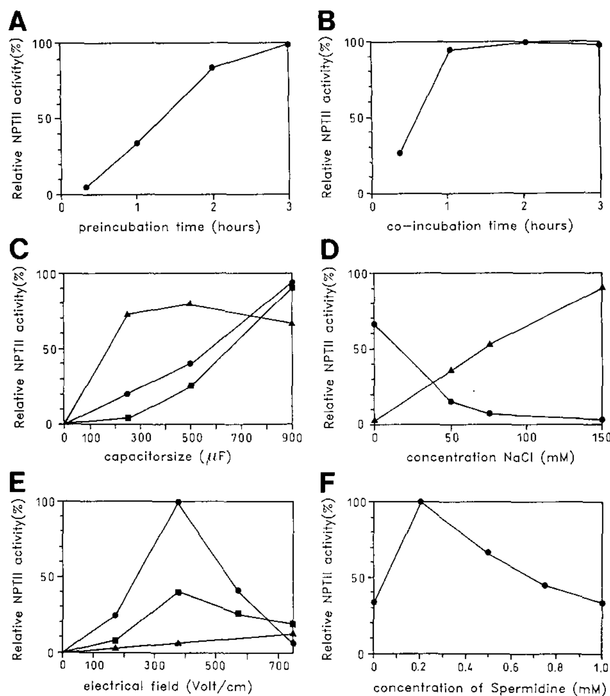


**Figure 2.** Relationship between the Electroporation Parameters and the Level of NPTII Activity in Electroporated Leaf Bases.

Each point represents the amount of radioactive phosphorylated kanamycin detected with that condition relative to the maximum obtained within that experiment and averaged over at least two experiments. If not noted otherwise, the leaf bases were electroporated with the standard protocol.

**(A)** Effect of incubation of leaf bases in electroporation buffer before electroporation.

**(B)** Influence of the time leaf bases are incubated with the plasmid DNA.

**(C)** Effect of the capacitor size and the number of pulses (■, one pulse; ●, three pulses; ▲, five pulses).

**(D)** Effect of the concentration of NaCl in the electroporation buffer during the electrical discharge (▲, with a 900-μF capacitor; ●, with a 200-μF capacitor).

**(E)** Effect of electrical field strength delivered from different-size capacitors (▲, 200 μF; ■, 500 μF; ●, 900 μF).

**(F)** Influence of the addition of spermidine to all solutions.

is correlated with the duration and the amplitude of the electrical pulse provided. Figure 2C illustrates that with a constant pulse amplitude (375 V/cm) and EPR medium containing 150 mM NaCl, maximal NPTII activity was reached with a 900-μF capacitor. This corresponds to a pulse lasting approximately 300 msec. Pulse times higher than 300 msec, which were obtained by retaining the 900-μF capacitor and decreasing the concentration of NaCl in the EPR medium, decreased the level of NPTII activity (Figure 2D). Figure 2D also shows that using a 200-μF capacitor and EPR medium without NaCl yielded levels of NPTII activity comparable with those obtained with a 900-μF capacitor and 150 mM NaCl in the EPR buffer. The pulse time for both conditions was very similar. Increasing the number of pulses only elevated the level of NPTII activity when the pulse duration was short (Figure 2C). At higher pulse durations, this benefit was lost, quite probably because of the increased damage to the cells.

The effect of amplitude on transient expression is summarized in Figure 2E. If the duration of the pulses was extended (900 μF, 150 mM NaCl, and to a lesser extent 500 μF, 150 mM NaCl), the NPTII activity peaked at an electrical field of 375 V/cm. Under optimal DNA introduction conditions (900 μF, 150 mM NaCl, and 375 V/cm), 33% of the electroporated leaf bases remained capable of initiating callus tissue on LS2.5 medium. This is approximately 50% of the callus initiation observed with unelectroporated leaf base fragments.

Experiments employing DNA concentrations as low as 5 μg/mL produced readily detectable amounts of NPTII activity. Enzyme levels increased linearly as DNA concentrations were raised to 100 μg/mL (data not shown). Both supercoiled and linearized plasmid DNA provided similar levels of NPTII activity. Plasmids used in this paper ranged from 3.5 kb to 15 kb, but the correlation between plasmid size and cell penetration has not been systematically studied.

A time course of gene expression after electroporation showed that NPTII activity after DNA introduction rose to a maximum at 4 days and could be easily detected as early as 1 day and as late as 8 days after electroporation (data not shown).

In an attempt to further improve the efficiency of DNA transfer, we experimented with chemicals that might protect DNA against degradation by nucleases or enhance the viability of the treated tissue. Figure 2F shows that enriching incubation and electroporation solutions with 0.2 mM spermidine, which delays lysis of oat protoplasts and inhibits the activity of nucleases (Galston et al., 1979), improved the NPTII signal threefold. Two compounds that have been used to enhance transformation efficiencies in animal cells, protamine (Wienhues et al., 1987) and chloroquine diphosphate (Luthman and Magnusson, 1983), did not improve the gene transfer efficiency in electroporated leaf bases. All requirements for optimal expression based on these studies are chronologically outlined in Methods.

### Transient Expression in Leaf Bases from Other Cereals

To prove that the delivery of DNA to intact cells has broad applications, we subjected leaf bases from other monocotyledonous crop plants to the same electroporation procedure. Figure 3 shows that high enzyme activity was obtained in maize and wheat using either the *p2′-nptII* or a similar construct driven by the cauliflower mosaic virus 35S promoter. Higher expression might be obtained in

**Figure 3.** General Applicability of Electroporation for Introducing DNA into Gramineae.

Shown is an autoradiogram of an NPTII assay on leaf bases from various cereals electroporated according to the standard protocol with equimolar amounts of either *p2'-nptII* or *p35S-nptII* constructs.

barley or any of the other species by modifying the electroporation conditions, but the precise optimization procedure has not been pursued in these studies.

## Transient Expression in Other Leaf Tissues

To determine whether the electroporation procedure was more generally applicable, we tested four types of explants: leaf bases (LBD) and sheath/lamina tissue from first and second leaves (SLD) from etiolated seedlings (Figure 1), and leaf bases (LBL) and sheath/lamina tissue (SLL) from etiolated seedlings transferred to the light for 8 hr.

Using the optimized conditions for leaf bases from etiolated seedlings, we electroporated the *p2'-nptII* and *p35S-nptII* constructs into the different explants, quantified the NPTII activity according to Reiss et al. (1984), and normalized the results from every independent experiment to the NPTII activity produced in LBD explants electroporated with the *p2'-nptII* gene. As illustrated in Table 1, the variation in relative NPTII activity in three independent experiments is low for LBD, LBL, and SLD explants. The variability determined in extracts from SLL explants is at least partially due to the low absolute NPTII levels, which make accurate measurements difficult. Based on these experiments, the average relative level of NPTII activity in LBD, LBL, SLD, and SLL tissue amounts to 100%, 93%, 18%, and 4%, respectively, when electroporated with the *p2'-nptII* construct, and to 98%, 95%, 17%, and 5%, respectively, when electroporated with the *p35S-nptII* gene. These results are shown in Figure 4. A third promoter derived from a *Nicotiana plumbaginifolia* extensin gene fused with the same *nptII* reporter sequence (M. De Loose, unpublished results) directs 20 times less activity in LBD explants (Table 1). These results show that the 2' and 35S promoters are similarly expressed in rice leaf tissue although it should be noted that in protoplasts the 2' promoter was consistently found to be 3 times stronger than the 35S promoter (Dekeyser et al., 1989).

## Light-Regulated Expression

To analyze whether the electroporated leaf bases were sufficiently unperturbed to allow photoregulated gene expression, a construct consisting of the promoter and the transit peptide sequence from the light-inducible pea *lhcpAB80* gene fused to the *nptII* gene (Van den Broeck et al., 1988) was electroporated into the four different explants (Figure 4). Whereas no enzyme activity was detected in etiolated explants, NPTII activity in the green SLL tissue reached levels averaging 3 times to 5 times higher than those obtained with the *p2'-nptII* and *p35S-nptII* genes (Table 1). This demonstrates that the expression from the pea promoter sequence is light-regulated in leaf tissue from rice. Light, however, is not the only factor governing the expression in this tissue: we were unable to detect NPTII activity in leaf bases from light-grown seedlings. To determine whether this tissue-dependent difference mirrors the expression of the endogenous light-harvesting chlorophyll proteins (LHCPs), all tissues were examined cytologically.

Using the electron microscope, LBL tissues were found to possess amyloplasts but no chloroplasts, as shown in

**Table 1.** Variability of NPTII Activity in Intact Leaf Tissue Electroporated with Chimeric Genes

| | LBD | LBL | SLD | SLL | Experiment |
|---|---|---|---|---|---|
| *p2'-nptII* | 100 | 97 | 18 | 2 | 1 |
| | 100 | 100 | 15 | 5 | 2 |
| | 100 | 82 | 21 | 4 | 3 |
| *p35S-nptII* | 105 | 102 | 17 | 7 | 1 |
| | 92 | 93 | 17 | 3 | 2 |
| | 97 | 89 | 18 | 4 | 3 |
| *pext-nptII* | 5 | ND[a] | ND | ND | 1 |
| | 6 | ND | ND | ND | 2 |
| *plhcp-nptII* | UD[b] | UD | UD | 8 | 1 |
| | UD | UD | UD | 10 | 2 |
| | UD | UD | UD | 27 | 3 |

Four different leaf explants from 7-day-old rice seedlings have been electroporated with four chimeric genes in three independent experiments. The NPTII activity present in the electroporated tissues was analyzed (Reiss et al., 1984) and normalized to the NPTII activity in leaf bases from dark-grown seedlings (LBD) electroporated with the *p2'-nptII* construct. See Figure 4 for description of other explants.

[a] ND, not determined.

[b] UD, not detected.

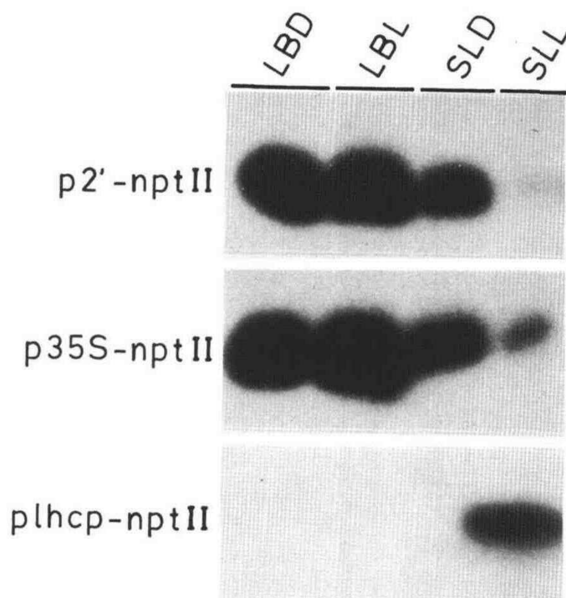


**Figure 4.** Developmental Control over Transient Gene Expression.

Autoradiogram of an NPTII assay performed on leaf bases (LB) or mixed leaf sheath and lamina tissue (SL) isolated from dark-grown (D) or light-grown (L) seedlings and electroporated with equimolar amounts of photo-insensitive (*p2'-nptII* and *p35S-nptII*) or photo-regulated (*plhcp-nptII*) reporter genes.

Figure 5F. In contrast, the SLL mesophyll tissue contained chloroplasts with well-developed thylakoid membranes and grana (Figures 5B and 5D). The cells from the etiolated LBD and SLD explants contained amyloplasts and etioplasts, respectively (Figures 5A, 5C, and 5E). The development of mature photosynthetic organelles was found to coincide with the synthesis of the endogenous rice LHCP. With antibodies raised against the pea LHCP, which have been shown to cross-react with *Arabidopsis*, tobacco, and alfalfa LHCPs (G. Engler, personal communication), we could readily detect LHCP associated with the thylakoid membranes of chloroplasts in mesophyll cells from the sheath/lamina tissue of light-grown seedlings (Figures 5B and 5D). No cross-reacting protein could be detected in parenchyma cells of any of the other explants.

We conclude that the success or failure to detect NPTII activity from the *plhcp-nptII* constructs correlates with the expression of the endogenous rice *lhcp* gene(s). In addition, photoregulation of both the endogenous and foreign genes depends in part on the developmental stage of the plastids, as was previously noticed in tobacco (Simpson et al., 1986).

The mobility of the LHCP-NPTII protein in the enzymatic assay was greater than expected for the fusion protein and, in fact, migrated where the protein would be if the *lhcp* transit peptide had been removed. This might indicate that the NPTII protein is targeted to the chloroplasts, but this has not been rigorously demonstrated here. However, the ribulose-1,5-bisphosphate carboxylase small subunit transit peptide from pea targets the NPTII protein to chloroplasts of barley mesophyll protoplasts (Teeri et al., 1989), so the ability of monocotyledonous cells to utilize dicot chloroplasts transit sequences may be a general phenomenon.

## Generalized Permeability of Electroporated Cells to DNA Transfer

None of the 150 rice leaf bases electroporated in the absence of DNA and incubated with 5-bromo-4-chloro-3-indolyl-$\beta$-D-glucuronic acid (X-gluc) showed $\beta$-glucuronidase (GUS) activity in any tissue, as shown, for example, in Figure 6A. On this basis, leaf bases were electroporated with the $\beta$-glucuronidase gene (Jefferson et al., 1987) under control of either the cauliflower mosaic virus (CaMV) 35S promoter (*p35S-uidA*) or the 2' promoter (*p2'-uidA*), stained with X-gluc 4 days after treatment, and analyzed to determine which cells were susceptible to DNA introduction. At the macroscopic level, these electroporated leaf bases displayed, like electroporated protoplasts (Gallie et al., 1989), variation in expression of the $\beta$-glucuronidase gene. Five percent to 20% of the leaf bases electroporated with either of the two chimeric constructs produced GUS at detectable levels as judged by the presence of blue-stained cells. Of the positively reacting leaf bases, 5% to 20% stained uniformly blue, the others only showed sectors of blue-stained cells. We believe that the differential expression levels in the leaf bases might be at least partially related to the position of the tissue in the electroporation chamber and to the fact that each fragment possesses a nonuniform resistance. Consequently, different leaf bases may receive slightly different electrical pulses.

Histological examination of several leaf bases demonstrated that both complete and partially stained leaf bases showed a similar cellular expression pattern. In leaf bases electroporated with the *p2'-uidA* construct, high $\beta$-glucuronidase activity was detected in inner and outer epidermis, parenchyma, and vascular bundle cells (Figure 6B). This illustrates that the 2' promoter is similarly expressed in different cell types from the rice leaf base and that all cells, ranging from approximately 10 $\mu$m to 100 $\mu$m in diameter, can be permeabilized by the electrical discharge. It also proves that one electrical pulse can introduce DNA simultaneously into more than six layers of cells. The *p35S-uidA* gene was similarly expressed (Figures 6C and 6D), further indicating that the DNA is randomly introduced, not preferentially delivered, to a specific cell type. Because there is also no clear correlation between the cut surfaces

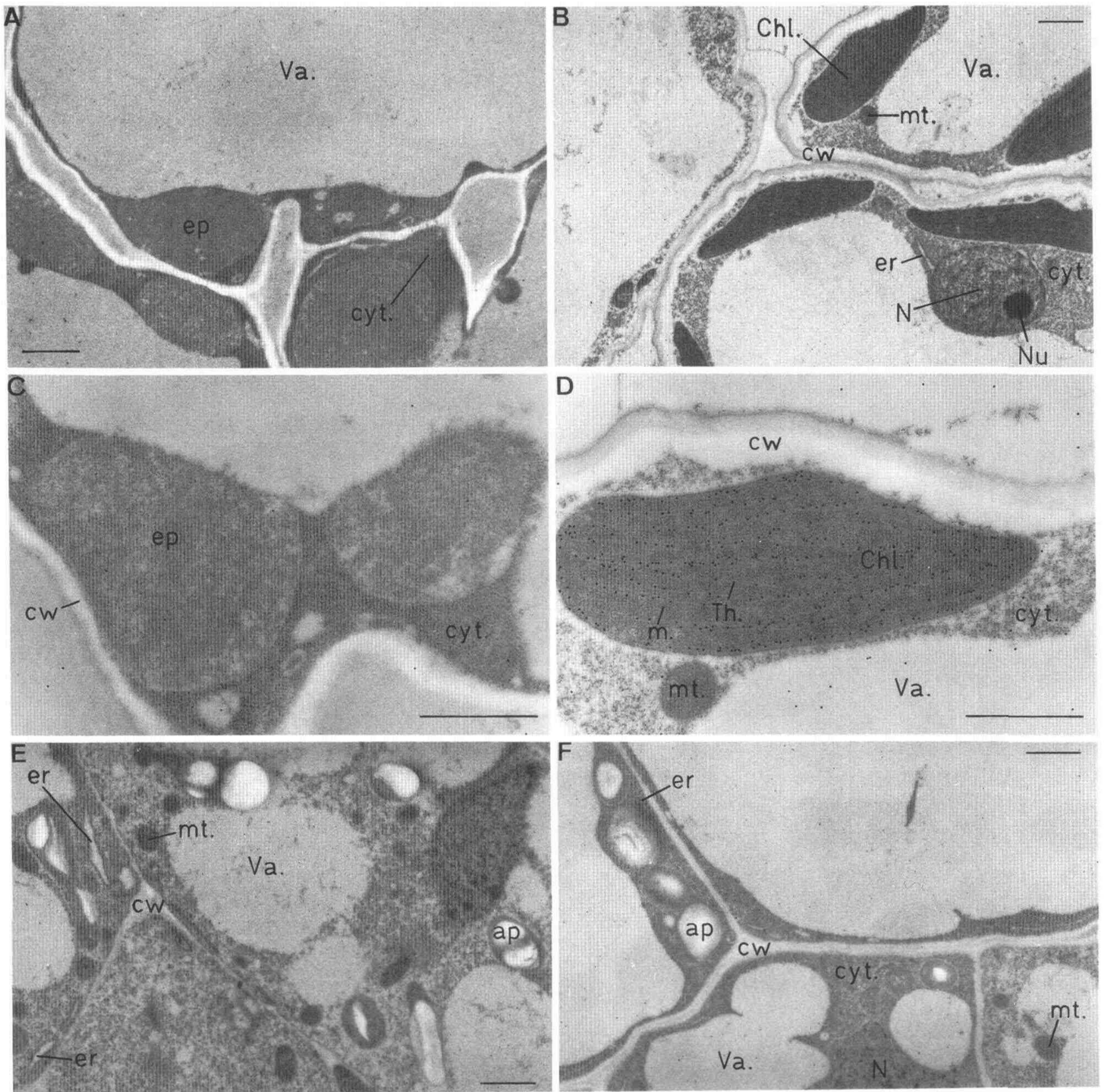


**Figure 5.** Degree of Photomaturation in 7-Day-Old Rice Explants.

**(A)** and **(C)** Chloroplast development in mesophyll cells in leaf sheath and leaf lamina from dark-grown rice seedlings.

**(B)** and **(D)** Chloroplast development in mesophyll cells in leaf sheath and leaf lamina from light-grown rice seedlings.

**(E)** Structure of parenchyma cells in dark-grown leaf bases.

**(F)** Structure of parenchyma cells in light-grown leaf bases.

All sections have been cross-reacted with antibodies against the pea LHCP and treated subsequently with 15-nm, protein-A gold to demonstrate that the LHC protein has been synthesized in light-grown sheath and lamina tissues. The abbreviations used are: ap, amyloplast; Chl, chloroplast, cw, cell wall; cyt, cytoplasm; ep, etioplast; er, endoplasmic reticulum; m, matrix, mt, mitochondria; N, nucleus; Nu, nucleolus; th, thylakoid membrane; Va, vacuole. The black bar in Figures A to F is 1 $\mu$m.

and the sites of staining, the plasmid DNA is not entering the explants exclusively where cell walls have been physically damaged.

### Tissue-Specific Expression

In *Arabidopsis* and tobacco, a construct (*psam-uidA*) containing the promoter from the *Arabidopsis S*-adenosylmethionine synthetase (*sam*) gene fused to the bacterial *uidA* coding region is preferentially expressed in vascular tissue (Peleman et al., 1989a, 1989b). Therefore, this reporter system was chosen to assess whether tissue-specific gene expression can be demonstrated after electroporation. RNA gel blot analysis, using the coding region of a rice *sam* gene as a probe, verified that the endogenous *sam* gene family was expressed in 4-day-old to 8-day-old seedlings (data not shown).

At the macroscopic level, the staining pattern generated by *psam-uidA*-electroporated leaf bases was different from the pattern in *p2′-uidA*- and *p35S-uidA*-electroporated explants. From 250 treated leaf bases, seven randomly selected blue-stained leaf bases were sectioned and analyzed histologically. All of them showed a similar expression pattern, as illustrated in Figures 6E to 6I. The vast majority of the GUS activity was restricted to cells surrounding the vascular bundles. Occasionally, we observed some randomly stained epidermis cells, but none of the leaf bases showed the nearly uniform staining seen with 35S or 2′ promoters.

## DISCUSSION

Electroporation is a very efficient technique to introduce DNA into many types of protoplasts. Previous reports have indicated that intact plant cells can be permeabilized by electroporation. For instance, sugar beet suspension cultures electroporated with a chloramphenicol acetyltransferase (*cat*) gene showed CAT activities 2 times above background, but higher levels were only obtained after treatment with pectolytic enzymes (Lindsey and Jones, 1987). This type of enzymatic treatment could be as disruptive for promoter regulation as complete cell wall removal. This approach contrasts with the one reported in this paper in which DNA is delivered efficiently into untreated plant cells using electroporation. It is not clear whether all plants have susceptible tissues, but our approach has succeeded for rice, maize, wheat, and barley. Under optimized conditions, the NPTII activity directed by the *p2′-nptII* and *p35S-nptII* reporter genes amounts to more than 100 times the background activity.

Plasmid DNA might be entering cells in any of several ways. A priori (1) the DNA might enter only damaged cells, or (2) the electrical discharge may be able to disrupt both cell walls and cell membranes creating pores in both barriers, or (3) the DNA might penetrate passively through pores of the cell wall during the incubation period with leaf material so that the electrical shock is needed only to permeabilize the membrane. Whereas the first hypothesis is unlikely because we could demonstrate that the expression of the introduced reporter genes is not restricted to wound sites, we cannot discriminate between the second and the third hypotheses at this time.

Three modifications of the rice protoplast electroporation conditions were necessary to obtain optimal nucleic acid transfer into intact leaf cells. First, excised tissue had to be preincubated for 3 hr in electroporation buffer before transfer to fresh medium containing the plasmid DNA to remove nucleases excreted by damaged cells. Second, the coincubation of the plasmid DNA with the plant material had to be prolonged. This probably reflects the slow diffusion of DNA into the intercellular spaces and/or the slow penetration of DNA through the cell wall pores. Third, efficient DNA introduction required a pulse duration up to 300 msec, which is 5 times to 10 times higher than the pulses used to electroporate protoplasts.

We initially monitored the expression of two commonly used chimeric genes, the *p2′-nptII* and the *p35S-nptII*. In rice explants, the highest expression of either promoter was obtained in leaf bases (LB) from both dark- (D) and light-grown (L) seedlings. In the etiolated sheath and lamina tissue from the first and second foliar leaves (SLD), the expression of both constructs was reduced fivefold, and in green sheath and lamina tissue (SLL), the NPTII activity reached only 5% of the activity in leaf bases. Because light can stimulate changes in the cell wall (Kim et al., 1989), it seems likely that the explant-dependent differences are at least partially due to differences in the efficiency of entry of DNA. On the other hand, because tissue-specific expression is maintained in these explants, we cannot exclude the possibility that some of the difference is caused by explant-specific cellular signals.

In contrast to the transcript 2′ and CaMV 35S promoters, the light-inducible pea *lhcpAB80* promoter is highly expressed in light-exposed, chloroplast-containing SLL tissue from rice and is not expressed at detectable levels elsewhere. The expression seems to correlate with the expression of the endogenous rice *lhcp* genes that were monitored immunologically. Whereas it has been reported that a monocotyledonous *lhcp* promoter is photoregulated in transgenic tobacco (Lamppa et al., 1985), we provide evidence that a dicotyledonous promoter is appropriately regulated in a monocotyledonous tissue. This further supports the idea that the components of the phototransduction pathway and the promoter sequences necessary for light-regulated expression have been highly conserved among distantly related species. Nevertheless, differences exist that need further study because a wheat ribulose-1,5-bisphosphate carboxylase small subunit promoter was not expressed in transgenic tobacco plants (Keith and

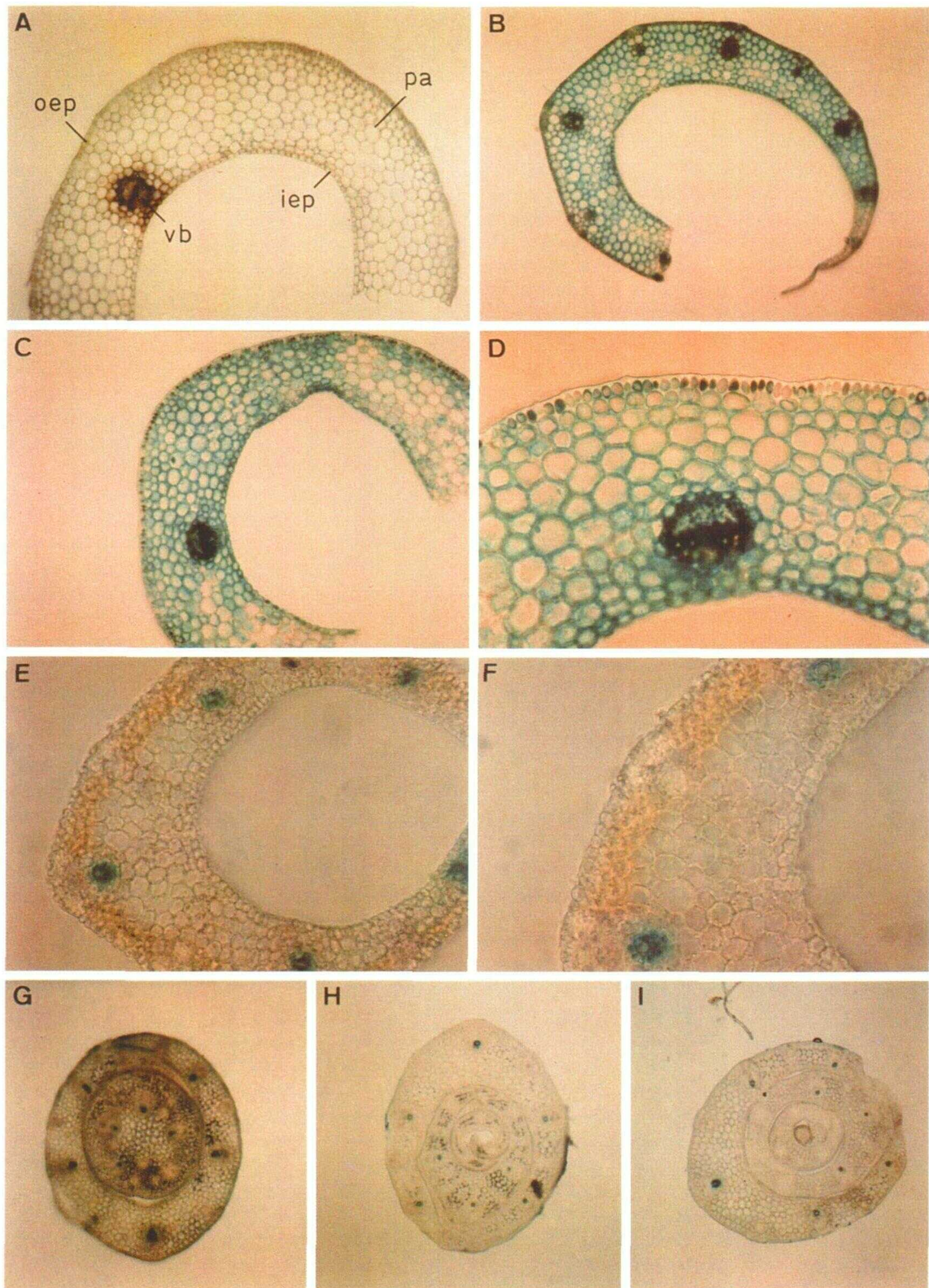


**Figure 6.** Potential for Tissue-Specific Gene Expression in Electroporated Leaf Bases.

**(A)** Leaf base explants electroporated without DNA. The abbreviations used are: iep, inner epidermis; oep, outer epidermis; pa, parenchyma; vb, vascular bundle. Magnification ×100.

Chua, 1986), and neither the pea nor the *Arabidopsis* small subunit promoters were able to provide detectable NPTII activity in electroporated green leaf tissue (data not shown). Our failure to detect *pssu-nptII* expression might be due to evolutionary differences between the different plants or may indicate that further modifications need to be made to the leaf tissue electroporation system.

Indirectly, the light-inducible expression of the *lhcpAB80* promoter implies that plasmid DNA could be electroporated into rice mesophyll cells. More precise information about the susceptibility of the cells to electroporation-mediated gene transfer has been obtained by histological analysis of leaf base explants electroporated with β-glucuronidase reporter genes.

The *p35S-uidA* gene was expressed in cells of the inner and outer epidermis, parenchyma, and vascular bundles of rice. A similar expression pattern of the CaMV 35S promoter has been observed in transgenic tobacco leaf tissue (Benfey et al., 1989), suggesting that the regulatory sequences of this promoter are conserved sufficiently to permit uniform expression in both monocotyledon and dicotyledon leaves. The uniformity of this expression and that shown by *p2'-uidA* also demonstrates that plasmid DNA can be delivered into leaf explants without significant bias. Consequently, reproducible nonuniform cellular expression patterns obtained after chimeric genes have been electroporated into leaf bases most likely reflect differential gene regulation rather than differential DNA introduction. For example, in all sections investigated, the *psam-uidA* construct was preferentially expressed in cells within and surrounding the vascular strands. This agrees with the expression pattern of the *psam-uidA* construct in transgenic *Arabidopsis* and tobacco leaves (Peleman et al., 1989a, 1989b), indicating that the regulatory mechanism for this type of expression is most probably not perturbed by our TGE assay.

The wide range of cells that can express DNA after electroporation and the observation that one electrical pulse can introduce nucleic acids simultaneously into more than six cell layers lead us to conclude that the potential of electroporation-mediated gene transfer might match the potential of the biolistic method (Klein et al., 1987). The particle gun protocol has the advantage that it uses less DNA and that it has been more elaborately investigated (for a review, see Sanford, 1988); our method offers the advantage that it can be performed with a simple, commonly available electroporation apparatus, that it has a high penetration power into adjacent cell layers, and that there is no need to construct DNA-binding tungsten particles.

Based on the expression of the *p2'-uidA* and *p35S-uidA* constructs, it seems likely that plasmid DNA can enter the meristematic leaf base cells located between the vascular bundle and the lower epidermis (Morrish et al., 1987). The frequency of this event, the amount of DNA entering the cell, and the competence of these cells to integrate foreign DNA into their genome will determine the probability of recovering transgenic embryogenic callus and, consequently, transformed rice plants. The suitability of this method to obtain transformed callus tissue from electroporated leaf bases and to transfer DNA into other meristematic explants, such as immature embryos, is under investigation.

## METHODS

### Plant Material

Seeds from rice (*Oryza sativa* cv Taipei 309) kindly supplied by the International Rice Research Institute (Manila, Philippines) were sterilized according to Thompson et al. (1986). To germinate, seeds were transferred to Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 1% sucrose and 0.8% Difco agar, and grown in the dark at 25°C. If greening of seedlings was necessary, 6-day-old plants were transferred to the light for 8 hr. Wheat (*Triticum aestivum* cv Fidel), barley (*Hordeum vulgare* cv Gerbel), and maize (*Zea mays* line 2418) seeds were sterilized for 30 min in 4.5% commercial bleach, rinsed five times in sterile water, and grown like rice seeds. Wheat and barley seeds were obtained from NV Clovis Matton (Adelgem, Belgium) and the maize seeds from line 2418 produced by de Coopérative de Pau (Lescar, France).

### Plasmids

CsCl-purified plasmid DNA was used in the electroporation experiments. The plasmid concentration was determined spectrophotometrically and verified by agarose gel electrophoresis. All chimeric constructs, with the exception of the *p35S-uidA* and *p2'-uidA* genes, which are terminated by the 3' region of gene 7, are terminated by the 3' region of the octopine synthase gene. The *p2'-nptII*, *p35S-nptII*, *plhcp-nptII*, and *pext-nptII* constructs are

**Figure 6.** (continued).

**(B)** Leaf base explants electroporated with plasmids encoding *p2'-uidA*. Magnification ×100.

**(C)** and **(D)** Leaf base explants electroporated with plasmids encoding *p35S-uidA*. Magnification ×100 [**(C)**] and ×250 [**(D)**].

**(E)**, **(F)**, **(G)**, **(H)**, and **(I)** Leaf base explants electroporated with plasmids encoding *psam-uidA*. Magnification ×100 [**(E)** and **(F)**] and ×60 [**(G)**, **(H)**, and **(I)**].

After 4 days leaf bases were stained with X-gluc and sectioned (see Methods).

**(A)** through **(F)** Bright-field micrographs show transverse sections through isolated leaves.

**(G)** through **(I)** Bright-field micrographs show transverse sections through the first three foliage leaves tightly rolled inside each other. Positive staining reactions show that the location of the blue precipitate depends on the construct used.

carried by pLD1 (8.3 kb; Dekeyser et al., 1989), pGemOCS (3.5 kb; Ingelbrecht et al., 1989), pGABTneo2 (8.2 kb; Van den Broeck et al., 1988), and pEN5 (6.4 kb; M. De Loose, unpublished results), respectively. The *psam-uidA* gene is carried by pCRSGUS1 (6 kb; Peleman et al., 1989a). The *p2'-uidA* construct resides on pGluc1 (14.2 kb; Plant Genetic Systems NV) and the *p35S-uidA* gene on p260 (15 kb; P. Breyne, unpublished results). The promoter fragments used in these chimeric genes are similar to those used in the *p2'-nptII* and *p35S-nptII* genes.

### Electroporation of Leaf Bases

Twenty-five 7-day-old seedlings were used for each electroporation experiment. After removing the coleoptile, the leaf base region (4 mm) or the mixed sheath and lamina tissue (4 mm) (Figure 1) was isolated. The explants were transversely divided into 2-mm fragments and incubated for 3 hr in electroporation (EPR) buffer (10% glucose, 4 mM $CaCl_2$, 10 mM Hepes, pH 7.2) with 0.2 mM spermidine. We then removed the EPR buffer, washed the explants twice with EPR buffer, and transferred them to a disposable spectrophotometer cuvette containing 0.2 mL of EPR buffer containing 0.2 mM spermidine. Twenty micrograms of pLD1 DNA, or equimolar concentrations of the other plasmids, was incubated with the explants for 1 hr. Next, 11 $\mu$L of a 3 M NaCl stock was added and the cuvette was put on ice for 10 min. Parallel stainless steel electrodes, 2 mm thick and 6 mm apart, were inserted in such a way that the leaf bases were gathered between them. One pulse with an electrical field strength of 375 V/cm was discharged from a 900-$\mu$F capacitor. The homemade electroporation unit consisted of an ISCO power supplier connected with an array of capacitors arranged in a circuit, as described by Fromm et al. (1985). After 15 min on ice, explants were rinsed in KpR medium, pipetted to 5 mL of KpR medium (Thompson et al., 1986) with 2 mg/L 2,4-D, and incubated in the dark for 4 days. The explants from green seedlings were incubated in the light. The protocol was identical for the other species mentioned in this article, except that for maize only 17 explants were used. To test the frequency of callus induction of electroporated leaf bases, the concentration of glucose in the liquid KpR medium was reduced by 50% every day, and on the 4th day leaf bases were transferred to Linsmaier and Skoog (1965) medium with 3% sucrose, 0.4% agarose (type II, Sigma), and 2.5 mg/L 2,4-D (LS2.5).

### Enzymatic Assays

NPTII assays were performed according to Dekeyser et al. (1989), except that leaf bases were crushed in 70 $\mu$L of 2 × ice-cold extraction buffer (1% $\beta$-mercaptoethanol, 50 mM Tris, pH 6.8, 0.13 mg/mL leupeptin). Quantification of the NPTII activity has been described by Reiss et al. (1984).

Histochemical localization of GUS activity in electroporated leaf bases was performed essentially as described by Jefferson et al. (1987). Four days after electroporation, leaf bases were immersed in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-$\beta$-glucuronic acid (Research Organics Inc., Cleveland, OH). The reaction proceeded for 18 hr in the dark at 37°C. To stop the reaction, the tissue was rinsed three times in 0.1 M sodium phosphate buffer. Leaf bases were subsequently incubated in a 0.1 M sodium phosphate-buffered 1% glutaraldehyde solution for 3 hr and rinsed once more in phosphate buffer. Tissues were then embedded in 4% (w:v) agarose and sliced in 40-$\mu$m sections with a vibrocutter (Campden Instruments, London). Examination of the slices was performed with bright-field microscopy.

### Electron Microscopy

Electron microscopy and immunochemistry were performed according to De Clercq et al. (1990). The pea LHCP antiserum was a generous gift of A. Cashmore and L. Szabo.

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