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Correlation of the expression of the nuclear photosynthetic gene ST-LS1 with the presence of chloroplasts

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A detailed analysis of the expression of a chimeric gene, consisting of the upstream region of the nuclear photosynthetic gene ST-LS1, encoding a component of the water-oxidizing complex of photosystem II, fused to the coding sequence of β-glucuronidase (GUS) as a reporter, is described. The expression of this chimeric gene at the cellular level was detected by histochemical methods and shows that the expression of this gene is correlated with the presence of chloroplasts. Interestingly, the GUS activity was not only detected in typical photosynthetic tissues, e.g. leaves and stems, but also in green roots containing chloroplasts. In contrast no activity was detected in neighbouring white root tissue which was devoid of chloroplasts. One can therefore separate the relative importance of the (morphological) differentiation steps responsible for the formation of tissues normally involved in photosynthesis, from the importance of the developmental stage (characterized by the presence of chloroplasts), for the expression of this nuclear photosynthetic gene. Our data strongly suggest that the developmental stage of the plastids is the primary determinant for the activity of this nuclear photosynthetic gene, although they do not yet allow the exclusion of the reverse type of control, i.e. control of the differentiation of the plastid by the expression of certain nuclear genes. A chimeric gene, consisting of the promoter of the 35S cauliflower mosaic virus (CaMV) gene and the GUS coding sequence, was used as a control throughout the experiments, confirming that the observed differential ST-LS1 - GUS gene expression reflects the particular transcriptional regulation impacted on this gene by its *cis*-acting regulatory sequences.

Key words: cell-specific expression/chloroplast dependent expression/photosynthetic gene/35S promoter

Introduction

One important feature of eukaryotes is the fact that their genetic information is divided between two or, in the case of higher plants and algae, three different organelles—the nucleus, the mitochondria and the plastids. One important task for the cell is the coordination of the expression of genes present in these different cell compartments. This is of special importance in view of the fact that many plastidic and mitochondrial proteins are encoded by nuclear genes. Yeast mutants which are devoid of mitochondrial DNA, but nevertheless form organelles which structurally resemble mitochondria, are examples for the importance of the nuclear genome.

The photosynthetic apparatus of higher plants consists of several large protein complexes. As these complexes are encoded by both nuclear and plastidic genes, the plant cell therefore is faced with the problem of coordinating the expression of a large number of genes present in both compartments.

The molecular mechanisms which lead to this coordinated expression are unknown. In addition to light irradiation, which triggers the expression of several nuclear photosynthetic genes (Tobin and Silverthorne, 1985), the developmental stage of the cell is also important for their expression. In mature plants these genes are highly expressed in leaf mesophyll cells, whereas under natural growth conditions no expression is detectable in, for example, roots.

Several recent observations indicate that a 'plastidic factor' might be involved in the regulation of nuclear photosynthetic genes. It has been reported by Oelmüller and Mohr (1986) that the photo-oxidative damage of chloroplasts in mustard seedlings grown on a medium containing the herbicide Norfluorazon, leads to a severe reduction of the amount of translatable RNA, encoding the small subunit ribulose biphosphate carboxylase (RBCS) or the chlorophyll a/b binding protein (CAB). After a partial recovery of the chloroplasts, the amount of translatable mRNA increases again (Schuster *et al.*, 1988). Similar effects have been observed for the accumulation of CAB mRNA in carotenoid deficient tissue of maize seedlings where the carotenoid deficiency was due either to a mutation or to treatment with a herbicide (Mayfield and Taylor, 1984). Chlorophyll deficient maize seedlings, however, which contain plastids arrested in a developmental stage prior to chloroplast formation, accumulate normal levels of CAB mRNA (Mayfield and Taylor, 1984).

In the cases described above, the photo-oxidative damage of the chloroplasts did not affect the expression of several genes encoding cytoplasmic proteins (Reiß *et al.*, 1983; Mayfield and Taylor, 1984). These and other observations (Eckes *et al.*, 1985; Simpson *et al.*, 1986; Börner, 1986; Stockhaus *et al.*, 1987a) can be taken as indicative of a so-called 'plastidic factor', produced by the chloroplasts at a certain stage of development and which is essential for the expression of nuclear encoded chloroplastidic proteins. The observations summarized above are, however, hampered by the fact that all these data are based either on the use of inhibitors or of mutants leading to a photo-oxidative damage of the chloroplasts. With these experiments it is difficult to prove that the photo-oxidation will only influence the expression of the photosynthetic genes studied by the different authors and not result in any side effects. Furthermore these data are all based on the analysis of tissue homogenates. An analysis of the expression of these

genes at the single cell level would undoubtedly allow the establishment of a firmer correlation.

In order to overcome these limitations we decided to use an alternative approach which allows us to follow the expression of a well-characterized gene encoding a protein involved in photosynthesis at the single-cell level in intact 'wild-type' plants. Following this approach, we hoped to answer the question whether or not a close correlation of the activity of this gene with the developmental stage of the plastids could be established.

To this end we used β-glucuronidase (GUS) (Jefferson *et al.*, 1987) as a marker enzyme for the analysis of the cell specificity of ST-LS1 gene expression. ST-LS1 is a nuclear gene of potato, which was originally isolated by differential screening of cDNA libraries from leaves. This gene is expressed in a leaf/stem specific manner (Eckes *et al.*, 1985, 1986) and encodes a 10.8 kd protein associated with the oxygen evolving complex of photosystem II (Lautner *et al.*, 1988). For the experiments described below, a 1600 bp long upstream fragment of the ST-LS1 gene containing *cis*-elements sufficient both for high and specific expression of a chloramphenicol acetyltransferase (CAT) gene (Stockhaus *et al.*, 1987b) was fused to the GUS coding sequence. As a positive control we used a construct composed of the cauliflower mosaic virus (CaMV) 35S transcript (35S) promoter and the GUS coding sequence. These chimeric genes were introduced into potato (homologous system) and tobacco (heterologous system). The cell specificity of their expression was analysed in various organs and at various developmental stages at the cellular level. The observed correlation between ST-LS1−GUS gene expression and the presence of chloroplasts is discussed.

Results

Construction of ST-LS1−GUS and 35S−GUS genes and integration into the plant genome

The ST-LS1 gene upstream sequences from position −1600 to +11 (Stockhaus *et al.*, 1987b) were fused to the GUS coding sequence, followed by a polyadenylation signal of the T-DNA gene encoding the nopalinesynthase (abbreviated as ST-LS1−GUS) (see Figure 1A). A chimeric gene, consisting of the 35S promoter from position −526 to +4, GUS coding sequences and a polyadenylation signal derived from the CaMV 35S gene (see Figure 1B) (abbreviated as 35S−GUS) served as a positive control. By using the 35S promoter, we wanted to demonstrate that the absence of staining in some tissues (cf. below) observed for the ST-LS1−GUS construct is not due to artefacts caused by the lack of substrate in these cells, but rather to differential activity of the ST-LS1 promoter.

These constructs were inserted into the binary vector BIN19 (Bevan, 1984) and were used for the transformation of potato and tobacco plants via *Agrobacterium* strain pGV2260. The substrate 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) was used for the histochemical analysis of transgenic plants. This results in a blue staining of cells expressing the GUS enzyme (Jefferson *et al.*, 1987; Jefferson, 1987). In untransformed plants there was no GUS enzyme activity detectable in any of the tissues described here (data not shown). The data described below are based on the analysis of a number of independent transgenic potato and tobacco plants.

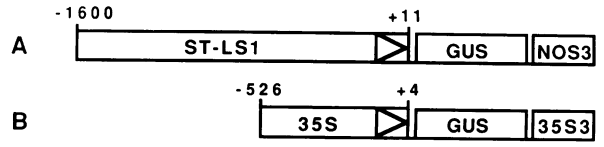

Fig. 1. Representation of the chimeric genes used for the analysis of the cell-specific expression patterns. (**A**) The ST-LS1 upstream sequences from position −1600 to +11 were fused to the GUS coding region and a polyadenylation signal of the nopalinesynthase gene (NOS3). (**B**) A chimeric gene consisting of the 35S upstream sequences from position −526 to +4, the GUS coding sequence (GUS) and the 35S polyadenylation signal (35S3) was used as a positive control.

ST-LS1−GUS and 35S−GUS gene expression in photosynthetic organs

The high level of steady-state ST-LS1 mRNA in leaves (Eckes *et al.*, 1985) indicated that this gene is strongly expressed in this organ. In order to identify the cells expressing the ST-LS1−GUS gene, the staining reaction was performed with transverse potato leaf sections. Very intensive staining of spongy mesophyll, palisade mesophyll and cells associated with the vascular bundles was observed (see Figure 2A). By electron microscopy it was demonstrated that parenchymatic cells which are associated with the vascular tissue contain chloroplasts. Parenchymatic cells of the middle rib of the leaf do not express the ST-LS1−GUS gene and do not contain chloroplasts.

This expression pattern is very similar to the one observed for the 35S−GUS gene which is also expressed to high levels in the palisade and spongy mesophyll cells as well as in parenchymatic cells associated with the vascular tissue (see Figure 2A).

The leaf epidermis is mainly composed of epidermal cells, stomata guard cells and trichomes. This tissue allows the comparison of the expression of the GUS fusions in photosynthetically inactive epidermal cells, containing rudimentary plastids, and photosynthetically active guard cells, which do contain chloroplasts. In contrast to the 35S−GUS gene, which is expressed both in epidermal and in guard cells (see Figure 2C), the ST-LS1−GUS gene expression is restricted to the chloroplast containing guard cells (see Figure 2B). This observation demonstrates that the substrate is not the limiting factor in epidermal cells, but that the staining pattern reflects the differential expression of the ST-LS1−GUS gene. It is furthermore remarkable that in tobacco trichomes both genes are expressed. In the small cells at the trichome tip which contain many chloroplasts the ST-LS1−GUS gene is however expressed to a higher level than the 35S−GUS gene (see Figure 2D and E).

In stem tissue of transgenic ST-LS1−GUS plants we detected low GUS enzyme activities in cells associated with the phloem tissue. In parenchymatic cells of either the pith or the stem-cortex respectively there was no detectable GUS enzyme activity (see Figure 2F). The 35S−GUS gene is highly expressed in parenchymatic cells of the phloem tissue (see Figure 2G). The analysis of longitudinal sections of the shoot apex confirmed the results obtained with the cross-sections. The ST-LS1−GUS gene expression is restricted mainly to the axillary buds and to parts of the apical meristem (see Figure 2H), whereas the 35S−GUS construct is highly expressed in the vascular tissue, axillary buds and in certain cells of the apical meristem (see Figure 2I).

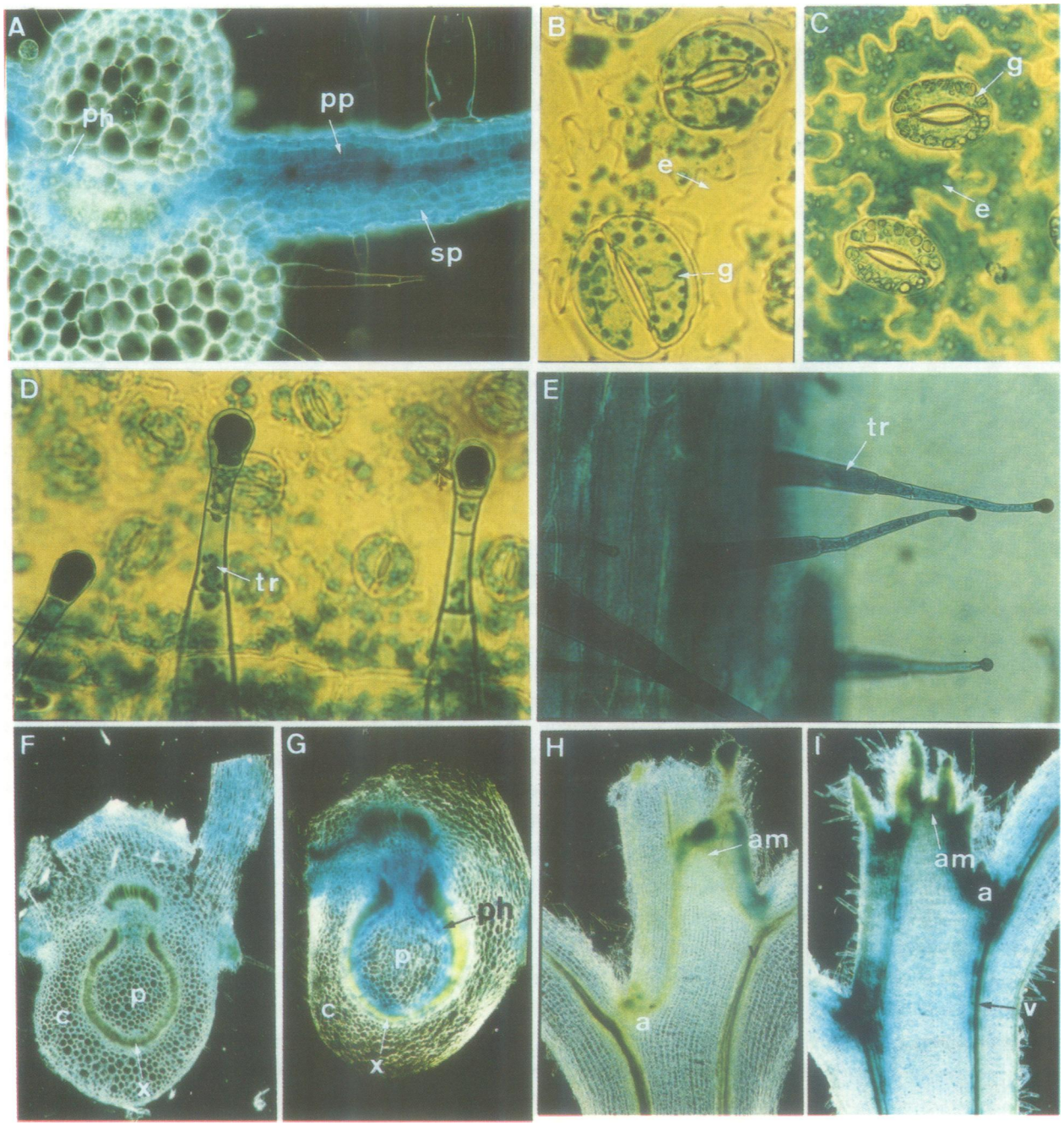

Fig. 2. Histochemical localization of the GUS enzyme activity in leaves and stem of tobacco plants transformed with the ST-LS1−GUS or the 35S−GUS gene. (**A**) Dark-field photograph of a transverse leaf section of a 35S−GUS plant. The dark blue staining represents high levels of GUS enzyme activity. Bright-field photographs of leaf epidermis of a ST-LS1−GUS plant (**B**) and a 35S−GUS plant (**C**); trichomes of a ST-LS1−GUS plant (**D**) and a 35S−GUS plant (**E**). Dark-field photographs of transverse stem sections of a ST-LS1−GUS plant (**F**) and a 35S−GUS plant (**G**); longitudinal sections of the shoot apex of a ST-LS1−GUS plant (**H**) and a 35S−GUS plant (**I**). a, axillary bud; am, apical meristem; c, cortex parenchyma; e, epidermal cell; g, guard cell; p, pith parenchyma; ph, phloem; pp, palisade parenchyma; sp, spongy parenchyma; tr, trichomes; v, vascular tissue; x, xylem.

Expression pattern in non-photosynthetic organs, e.g. roots and tubers of transgenic potato plants

In a second series of experiments we analysed the expression of the GUS fusions in organs characterized by the lack of chloroplasts under normal growth conditions.

The histochemical analysis of potato tuber cross-sections demonstrates that the ST-LS1−GUS gene is not expressed in tubers under normal growth conditions. In tubers exposed to white light for a few days, however, weak ST-LS1−GUS gene expression is detectable in rudimentary leaves of sprouting green buds (see Figure 3A) and the outer layer of chloroplast containing parenchymatic cortex cells (see Figure 3C). The 35S−GUS gene is expressed in parenchymatic cells associated with the vascular tissue in the pith (see Figure 3D) and in germinating buds (see Figure 3B). There was no expression detectable in the starch containing parenchymatic cells in the pith and in the periderm tissue of the tuber. Using transversal sections of roots of transgenic potato plants grown in soil we detected no ST-LS1−GUS gene expression (see Figure 3E), whereas the 35S−GUS

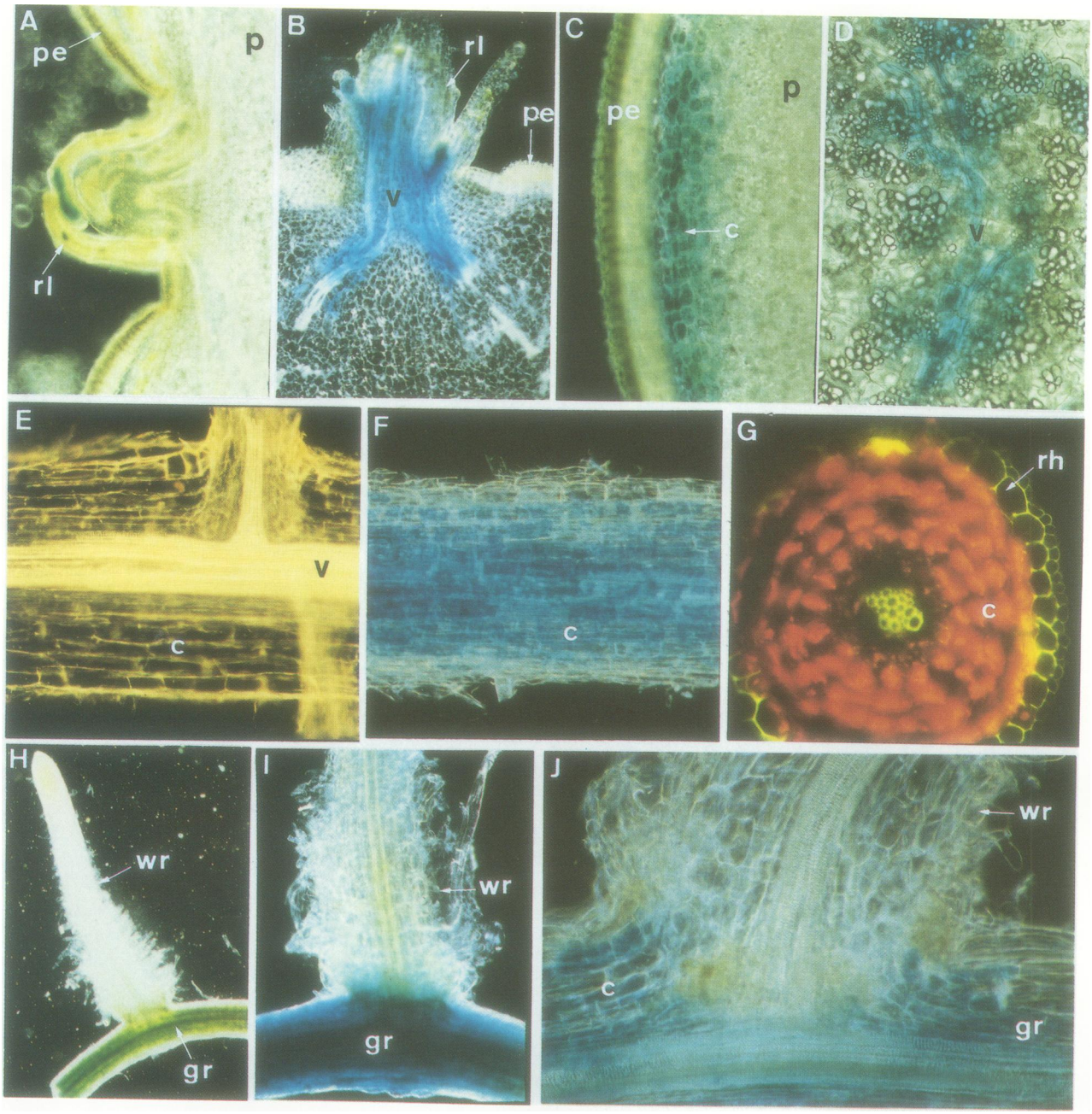

Fig. 3. Histochemical localization of GUS enzyme activity in roots and tubers of potato plants transformed with the ST-LS1−GUS or the 35S−GUS gene. Dark-field photographs of transverse sections of tubers of ST-LS1−GUS plants (**A**,**C**) and tubers of 35S−GUS plants (**B**,**D**); longitudinal section of a root of a ST-LS1−GUS plant grown in soil (**E**) and a 35S−GUS plant grown in soil (**F**); fluorescence photograph of a cross-section of a root of a potato plant grown in tissue culture (**G**). Dark-field photograph of an intact root of a tissue culture ST-LS1−GUS plant before (**H**) and after the GUS reaction (**I**); longitudinal section of a root of a ST-LS1−GUS plant grown in tissue culture (**J**). c, cortex parenchyma; gr, green root; p, pith parenchyma; pe, periderm; rh, rhizodermis; rl, rudimentary leaves; v, vascular tissue; wr, white root.

gene is highly expressed in the parenchymatic tissue of the root (see Figure 3F).

Roots of potato plants grown in tissue culture and which are therefore exposed to light do contain chloroplasts in parenchymatic cells (Eckes *et al.*, 1985) (see Figure 3G). The redifferentiation of these parenchymatic cells to chloroplast containing cells starts at a certain distance from the root tip. White side roots, growing out of older green roots (see Figure 3H), therefore represent a unique system allowing a direct comparison between ST-LS1−GUS gene expression in green roots, which contain chloroplasts, and young whitish roots which do not.

In parenchymatic cells containing chloroplasts a strong GUS enzyme activity is detectable (see Figure 3J), whereas there is no GUS enzyme activity detectable in the young outgrowing roots (see Figure 3I and J). In whitish roots of tobacco plants grown in tissue culture exposed to white light, we also observed chloroplasts by fluorescence microscopy, though their number is much lower. In these tobacco roots the ST-LS1−GUS gene is expressed, albeit at a rather low level (data not shown). In contrast to this highly differential expression of the ST-LS1−GUS gene in correlation to the

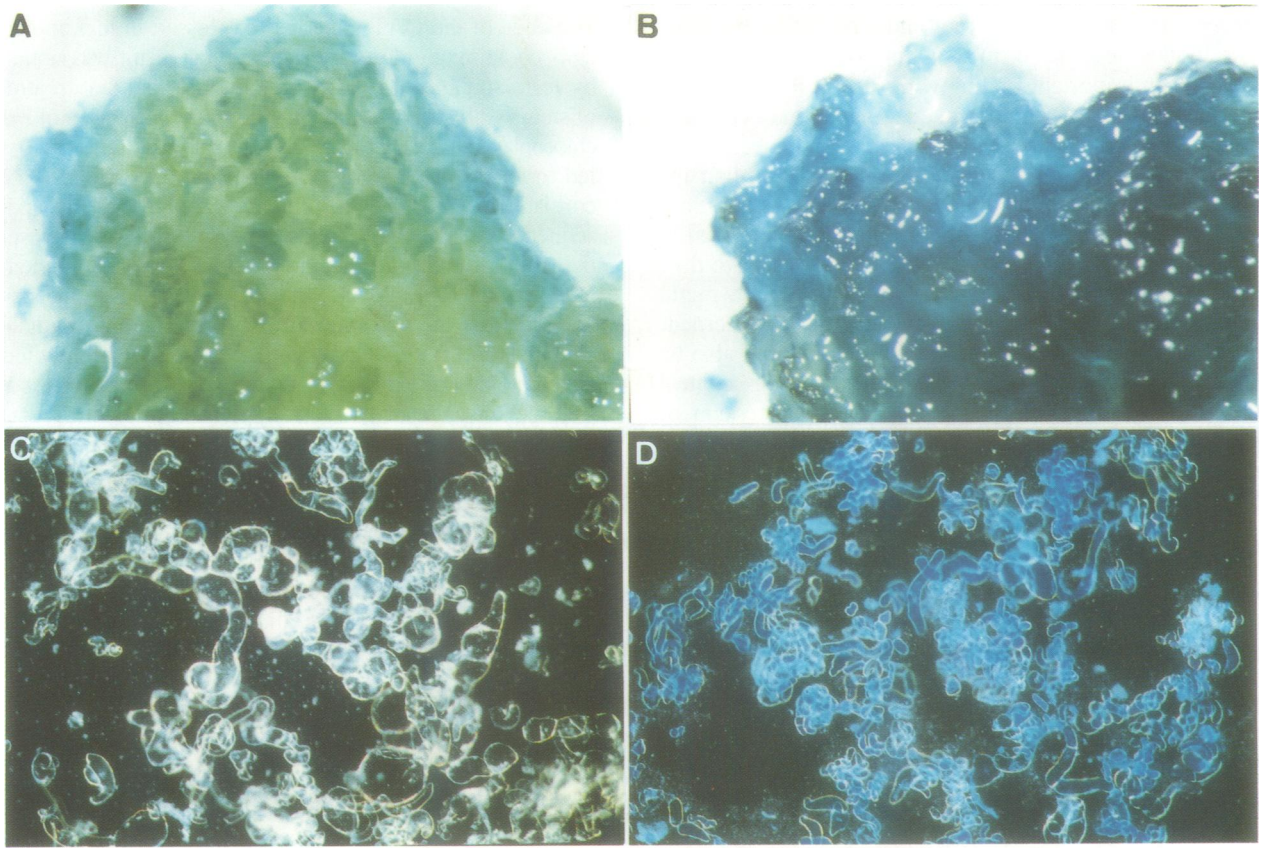

Fig. 4. Histochemical analysis of GUS enzyme activity in callus and suspension culture cells derived from potato plants transformed with the ST-LS1−GUS or the 35S−GUS gene. Bright-field photograph of a ST-LS1−GUS callus (**A**) and 35S−GUS callus (**B**). Dark-field photograph of ST-LS1−GUS suspension culture cells (**C**) and 35S−GUS suspension culture cells (**D**).

presence of chloroplasts, the 35S−GUS gene is expressed in white as well as in green parenchymatic root cells (data not shown).

Expression in potato callus and suspension culture cells

As a final step in our analysis, the expression pattern of both genes in undifferentiated callus and suspension culture cells was determined. A weak expression of the ST-LS1−GUS gene was detected in green callus cells (see Figure 4A). In callus cells representing a different developmental stage characterized by the lack of chloroplasts, no GUS activity was detected. The callus used for these experiments was derived from transgenic potato plants displaying high levels of GUS activity in leaves. The 35S−GUS gene is expressed to much higher levels in callus cells (see Figure 4B).

In tobacco as well as potato suspension culture cells grown under heterotrophic conditions and devoid of chloroplasts we again did not detect any ST-LS1−GUS gene expression (see Figure 4C). This contrasts with the high expression of the 35S−GUS gene in these cells (see Figure 4D).

Discussion

The photosynthetic apparatus localized in the chloroplasts of higher plants contains protein complexes which are encoded by the nuclear and the plastidic genome. In view of the central importance of the photosynthetic activity for the survival of the plant, it is obvious that the expression of the genes of both compartments must be interlinked and tightly controlled. Whereas post-transcriptional control appears to be especially important for the regulation of a number of plastidic genes (reviewed by Gruissem, 1989), the expression of nuclear photosynthetic genes appears to be regulated primarily at the transcriptional level. Light signal transducing systems in which phytochrome is involved play an essential role in this regulation (Tobin and Silverthorne, 1985). The coordinated expression of both nuclear and plastidic genes has, however, received less attention.

The data described in the Results point to a very strong correlation between the expression of a defined nuclear gene from potato (called ST-LS1), encoding a component of the water oxidizing complex of photosystem II, and the presence of chloroplasts. The three most striking examples for the correlation of the presence of chloroplasts with the expression of this nuclear photosynthetic gene are the data obtained for the leaf epidermis, root tissue and the potato tuber. In the epidermis of leaves, the ST-LS1−GUS gene is expressed in guard cells and trichomes which contain chloroplasts, whereas in epidermal cells which are devoid of chloroplasts there was no detectable ST-LS1−GUS gene expression. This result also indicates that, irrespective of the nature of the signal which is responsible for the induction of the ST-LS1 gene, it most likely has to be created within the cell itself and does not have any dominant influence on neighbouring cells. This signal therefore is unlikely to be able to diffuse or to be transported to other cells.

Our observation that the ST-LS1−GUS gene can be expressed in parenchymatic root and tuber cells, provided these tissues are made to contain green chloroplasts, represents an important finding with respect to the relative importance of the morphological differentiation of cells and the developmental stage of the plastids with regard to expression of the ST-LS1 gene. The observation that the ST-LS1−GUS gene is actively expressed in root and tuber cells containing chloroplasts whereas it is not expressed in neighbouring cells of the same type which are devoid of chloroplasts suggests that the presence of chloroplasts might be a prerequisite for the expression of the gene concerned. It should, however, be mentioned that they would also be compatible with an inverse type of control, i.e. control of the differentiation of the plastid by the expression of certain nuclear genes. These results also demonstrate that light—another factor often connected with the expression of photosynthetic nuclear genes—while essential, is not sufficient for induction of ST-LS1 gene expression. All further data described in the Results are in agreement with the main conclusion described above, i.e. the importance of the presence of chloroplasts for expression of the ST-LS1 gene. This result was obtained from the analysis in the homologous system (potato) as well as in the heterologous system (tobacco) for all tissues analysed.

The approach used in this study, i.e. the histochemical detection of β-glucuronidase activity from a chimeric gene transcriptionally driven by the promoter region of the ST-LS1 gene, was used for several reasons.

Firstly we wanted to know whether or not the postulated plastidary signal acts at the level of transcription. The chimeric gene used as a reporter consisted of regulatory sequences derived from a photosynthetic gene and of a coding sequence derived from a prokaryotic gene. We assumed that a prokaryotic mRNA would not be influenced markedly by plant specific post-transcriptional regulation mechanisms.

As outlined in the Introduction the importance of a plastidic factor for the expression of nuclear photosynthetic genes has been implied by several studies. These studies relied on the oxidative damage of chloroplasts by either the use of inhibitors of carotenoid biosynthesis or on the analysis of albino mutants. These previous data cannot with certainty exclude the possibility that the suppression of the activity of photosynthetic genes is due to a non-specific side effect of photo-oxidative damage. Our data, in contrast, were obtained in a 'wild-type' situation and in addition allowed us to monitor the expression on the cellular level.

It is important to examine whether or not the observed differential expression of the GUS enzyme is exclusively due to the specificity impacted by the ST-LS1 promoter. The expression of a chimeric 35S−GUS gene was therefore analysed in parallel and the expression patterns obtained for both genes were compared. This kind of analysis showed that the observed differential expression of the GUS gene results from ST-LS1 promoter activity and not, for example, from accessibility of the substrate or differences of GUS mRNA and protein stability.

Two other reports have to some extent described in a similar way the correlation between expression of another photosynthetic gene and the presence of chloroplasts. Using immunocytochemical methods, Aoyagi *et al.* (1988) showed that a chimeric gene consisting of the promoter of the nuclear photosynthetic small subunit RBCS gene fused to the coding sequence of the CAT gene was expressed in leaf and stem cells containing chloroplasts. A similar result was obtained by Jefferson *et al.* (1987) who demonstrated that treatment of stems with strong white light led to the formation of many chloroplasts in cortical parenchyma cells (chlorenchyma) and led to an increased level of expression of a chimeric gene consisting of a RBCS gene promoter fused to the GUS coding sequence. In these two cases the expression of the respective photosynthetic gene could not be separated from the formation of the typical photosynthetic tissues (leaves and stem). Nevertheless the observation that the *cis*-acting regulatory elements of different photosynthetic genes apparently led to the same kind of expression pattern as described for the ST-LS1 gene suggests that the hypothesized control of the expression of the ST-LS1 gene by the chloroplast could be a general phenomenon and might be relevant for a number of nuclear photosynthetic genes. The identification of tissues which, except for the difference of the developmental stage of their plastids, are very similar (tissue of green and white roots for example) will be very useful for the characterization of the signal(s) controlling the activity of nuclear photosynthetic genes.

Materials and methods

Recombinant DNA techniques

Standard procedures were used for recombinant DNA work (Maniatis *et al.*, 1982).

Transformation of tobacco and potato plants and tissue culture techniques

The chimeric genes were inserted in the vector BIN19 (Bevan, 1984) and introduced into the *Agrobacterium tumefaciens* strain pGV2260 (Deblaere *et al.*, 1985) by direct transformation according to Höfgen and Willmitzer (1988). In order to transfer the chimeric genes to tobacco cells, leaf discs of *Nicotiana tabacum* cv. SNN were infected with the respective *Agrobacterium* strain and subsequently regenerated (Horsch *et al.*, 1985). The transformation and regeneration of *Solanum tuberosum* cv. Desiree plants was performed as described by Rocha-Sosa *et al.* (1989).

Potato and tobacco callus was cultivated on MS medium (Murashige and Skoog, 1962) supplemented with 2% sucrose and 3 mg/l 2,4D (potato) or 1 mg/l 2,4D (tobacco) in a 16 h light/8 h dark rhythm. Suspension cultures were cultivated in liquid MS medium containing 2% sucrose and 1 mg/l 2,4D in continuous dim white light.

Histochemical localization

The histochemical reactions were performed as described by Jefferson (1987) using X-Gluc as substrate. For the sections of plant material a cryo-microtome was used. The staining reactions were performed with either unfixed cuttings or with cuttings fixed for 5−15 min in ice-cold 2% formaldehyde, 1 mM EDTA in 100 mM Na−phosphate (pH 7.0). The fixed cuttings were washed extensively before the staining reaction. The reaction times varied between 2 and 16 h.

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