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Organ-specific and dosage-dependent expression of a leaf/stem specific gene from potato after tagging and transfer into potato and tobacco plants

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ABSTRACT

ST-LS1, a single copy gene from potato displaying a leaf/stem specific gene expression, was tagged by an exon modification and introduced into both potato and tobacco cells using Agrobacterium vectors. After regeneration of whole plants, the expression of the tagged gene was analyzed with respect to its organ specifity and compared to the expression of the corresponding resident gene. The expression of the transferred gene in transgenic plants closely followed the expression of the resident gene. No marked influence of the plant species serving as host was observed. The level of expression of the introduced gene varied by a factor of at least 100 in independent transformants when normalized to the expression of the resident gene. Southern analysis performed on the transformed plants indicated a correlation between copy number of the introduced gene and its expression level. The activity of the tagged gene as well as of the resident gene was significantly inhibited by treatment of the transgenic plants with the herbicide norfluorazon, indicating that this gene activity is dependent on the presence of functional chloroplasts in the leaves.

INTRODUCTION

We are interested in qualitative as well as quantitative aspects of the expression of transferred genes in transgenic plants. To this end we have isolated and characterized several genes from potato displaying a developmental-specific expression (1-3). One of these genes (named ST-LS1) displays a leaf/stem specific, light-dependent and chloroplast associated type of expression (4). In contrast to the small subunit of the ribulose-1,5 bisphosphate carboxylase and the light-harvesting chlorophyll a/b binding protein, which are both encoded by multi-gene families, the ST-LS1 gene is present as a single copy in the potato genome resulting in a considerable advantage for its functional analysis.

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In a series of experiments described below we wanted to answer the following questions:

-a) How does the expression of the introduced gene compare to the expression of the resident gene in its qualitative and quantitative characteristics in the homologous host (i.e. potato)

-b) What is the influence of the homologous versus heterologous host on both qualitative and quantitative aspects of the expression of the introduced gene.

In order to be able to answer these questions, we modified the ST-LS1 gene by an exon-modification.

A DNA segment derived from the IAA H gene of the T-DNA of Agrobacterium tumefaciens (5,6) with a length of 470 nucleotides was inserted into the last exon of the ST-LS1 gene shortly before the poly-A addition site (3). This modification results in the following two advantages:

- The expression of the newly introduced gene can be directly compared in independent transformants. Normally this comparison is difficult to do as it can not be ruled out that different plants are under different physiological conditions. Because of the tagging procedure the newly introduced gene can be assayed in parallel with the resident gene therefore allowing an internal normalization of environmental influences on expression.
- The tagged gene can be reintroduced in homologous as well as heterologous backgrounds thus allowing us to determine the importance of host-specific factors on the expression of these genes.

MATERIALS AND METHODS

Recombinant DNA techniques

Standard procedures were used for recombinant DNA work (7).

Isolation and analysis of nucleic acids

Isolation of RNA from potato and tobacco was performed according to (8); isolation of DNA from Agrobacteria as well as from potato and tobacco plants was performed according to (9), analysis of nucleic acids by southern and northern blot type hybridization was performed essentially as described previously (3).

Transfer of the ST-LS1/470 gene into tobacco and potato plants

The ST-LS1/470 gene present in the intermediate vector pMPK

110 (3) was transferred into the T-DNA of the disarmed strain Agrobacterium C58C1 (pGV 3850 Kan^R) (10) by a triparental mating using GJ 23 as the mobilizing strain (11). Agrobacteria containing the cointegrate structure were checked for the presence of the non-rearranged gene and used for transformation. Tobacco (var. N. tabacum cv Wisconsin 38) was transformed following established procedures (12) using kanamycin (100 µg/ml) for selection of transformants. Potato was transformed by inoculating surface sterilized tuber discs with the respective Agrobacterium strain and leaving the discs on basic Murashige and Skoog Medium (13) complemented with 2 % sucrose and 1 mg/ml of indole acetic acid for two days. Subsequently the discs were transferred to a shoot induction medium composed of basic Murashige and Skoog components supplemented with 2 % sucrose, 0.1 mg/ml indoleacetic acid, 0.5 mg/ml zeatin riboside and 100 µg/ml of kanamycin sulfate. In addition this medium contained 500 µg/ml of carbenecillin and 500 µg/ml of claforam (Hoechst) in order to inhibit bacterial growth. When shoots reached a size of 2-3 cm (after about 2 months) they were cut off and transferred to hormone free medium (13) for root induction. Rooted plantlets were screened for nopaline (14) and nopaline positve plants were transferred to soil and grown in greenhouses.

RESULTS

Tagging of the ST-LS1 gene, transfer into potato and tobacco cells and subsequent regeneration of intact plants

For the reasons outlined in the introduction this series of experiments was performed with a tagged derivative of the ST-LS1 gene. The type of tagging chosen was the insertion of a foreign DNA fragment into the last exon of the gene. It has already been shown that his modification does not interfere with the production of a stable chimaeric RNA composed of sequences derived from the ST-LS1 gene itself as well as from the tag (3). Furthermore the stability of the chimaeric RNA does not seem to differ significantly from the stability of the non-modified RNA based upon the observation that both RNA's disappear in the darkness with about the same kinetics (data not shown). As the chimaeric RNA exceeds the length of the RNA made from the resident gene by exactly the

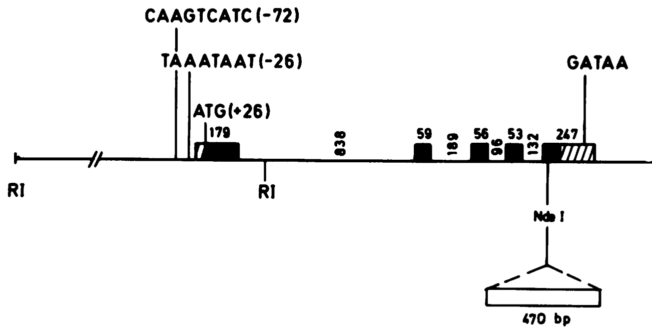

Figure 1: Schematic structure of the ST-LS1/470 gene. Exons are indicated by filled boxes, the numbers given indicate the size of the exons and introns in nucleotides. The 470 bp fragment was inserted into a single NdeI site of the last exon (3).

size of the tag (470 nucleotides) both the RNA made from the resident gene as well as the RNA originating from the introduced tagged gene can be followed simultanously on a Northern blot.

Figure 1 shows the schematic structure of the ST-LS1 gene and of its tagged derivative ST-LS1/470. The ST-LS1 gene and 1.6 kb of 5'-upstream and 1.2 kb of 3'-downstream sequences were first cloned into the intermediate vector pMPK 110 (3) and subsequently mobilized into the Agrobacterium host strain (C58 C1 (pGV 3850 Km^R) (10,11). The structure of the cointegrates formed was checked by Southern blot hybridization (data not shown). In order to transfer this gene to tobacco cells, leaf discs of *Nicotiana tabacum cv.* W38 were infected with the respective Agrobacterium strain (12). For transformation of potato, tuber discs were infected with the Agrobacterium strains. Plantlets were screened for transformation by assaying for nopaline. Nopaline positive potato and tobacco plants were subsequently transferred to the greenhouse for further analysis.

The modified gene ST-LS1/470 is expressed in an organ-specific way in transgenic potato plants and closely follows the expression of the resident gene ST-LS1

To analyze the organ-specific expression of the ST-LS1/470 gene, RNA was isolated from leaves, stem, root and tuber of five independently derived transformed potato plants and analyzed by

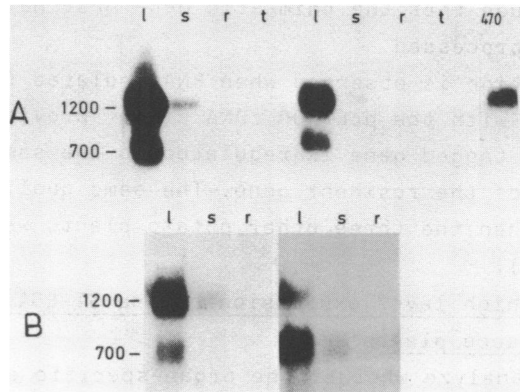

Figure 2: Organ-specific expression of the ST-LS1/470 gene in transformed potato (a) and tobacco (b) plants.
Figure 2a: Northern blot analysis of two transformed potato plants (no. 2 and no. 3) for the expression of the transferred ST-LS1/470 gene. Total RNA (50 µg each) isolated from leaf(l), stem(s), root(r) and tuber(t) tissue of transformed plants growing in the greenhouse was separated on 1.2 % agarose gels in the presence of formaldehyde. Hybridization was performed against cDNA pcL 700 containing part of the coding region of the ST-LS1 gene after transfer onto nitrocellulose filters. The size of the RNA's is given in nucleotides. Lane "470" shows the result of a hybridization using only the 470 bp fragment used to tag the ST-LS1 gene as a probe.
Figure 2b: Northern blot analysis of two transformed tobacco plants (no. 4 and no. 5) for the expression of the transferred ST-LS1/470 gene. Total RNA (50 µg each) isolated from leaf(l), stem(s) and root(r) tissue of transformed plants growin in the greenhouse was separated on 1.2 % denaturing formaldehyde gels. Further processing was as described in fig. 2a.

Northern type experiments for the expression of the transferred as well as of the resident gene.

The results for two of the plants are shown in figure 2a. In both cases, two RNA's of 700 and 1200 nucleotides length hybridizing to the cDNA clone pcL 700, which is homologous to the coding region of ST-LS1 (3) are visible in the leaf and the stem RNA. The higher molecular weight RNA shows specific hybridization to the 470 bp fragment used to tag the last exon of the ST-LS1 gene (fig. 2a, lane "470") proving that this RNA originates from the transferred gene. Since the size of the chimaeric RNA is in agreement with the expected increase due to the tag (470 nucleo-

tides) we conclude that the chimaeric gene must have been correctly spliced and processed.

No hybridization is observed when RNA isolated from tubers or roots is probed with the pcL 700 cDNA clone, proving that the expression of the tagged gene is regulated in the same manner as the expression of the resident gene. The same qualitative results were obtained when the three other potato plants were tested (data not shown).

Organ-specific high level expression of the ST-LS1/470 gene in transformed tobacco plants

In order to analyze whether the organ-specific expression of the modified ST-LS1 gene is significantly influences by the host plant, experiments similar to the ones described above were performed using transgenic tobacco plants. The result of the analysis of the expression of the ST-LS1/470 gene in different organs of two tobacco plants is shown in figure 2b. Tobacco has at least one endogenous gene highly homologous to the potato ST-LS1 gene which, upon hybridization with the cDNA clone pcL 700, gives rise to an RNA with a length of about 700 nucleotides (3). The second hybridizing RNA of about 1200 nucleotides length again originates from the transferred ST-LS1/470 gene as shown by its specific hybridization with the 470 nucleotide tag (data not shown). Most remarkable the expression of the modified potato gene closely follows the expression of the resident tobacco gene in all organs tested. Thus the highest level of expression is seen in leaves followed by stem tissue whereas no expression can be detected in roots.

Variation in level of expression of the ST-LS1/470 gene is correlated with copy number in both tobacco and potato plants

The results shown in figure 2a and 2b demonstrate that the ST-LS1/470 gene qualitatively follows the expression of the respective endogenous gene in potato as well as in tobacco. However the quantitative expression of the ST-LS1 gene differs in each of the transformants. Thus the level of expression of the transferred gene varies by at least a factor of 100 relative to the level of expression of the resident gene when comparing different transformants. Because this relative level of expression is normalized to the level of the resident gene, the observed differences cannot be explained by differences in the physiological state of

individual transgenic plants. The phenomena of varying gene expression in independent transformants is not an uncommon observation and has been seen in a number of other cases where genes have been introduced into a new chromosomal background by using artificial gene transfer methods (10,21,24-26). The most common explanation for this phenomenon is the assumption of a dominant position effect. Due to the random integration of the transferred DNA the newly introduced genes will most probably be in a different chromosomal position which might influence the level of expression of the transferred gene. Though this explanation is probably true in a number of cases the data shown in figure 3 suggest that in the case of the five potato plants as well as in the case of four of the five tobacco plants transformed with the ST-LS1/470 gene, the copy number of the transferred gene is the major factor determining the level of expression. Figure 3a, upper part shows the result of a Northern analysis of five independent transgenic potato plants, clearly showing the significant variation in the relative expression level of the ST-LS1/470 gene compared to the resident gene. In figure 3a, lower part the result of the Southern blot analysis of the same transformed potato plants is shown. After digestion with HindIII two prominent bands running at 3 and 3.5 kb hybridized to the cDNA pcL 700. The lower band at 3.0 kb represents the resident gene (4) whereas the upper band at 3.5 kb is derived from the transferred gene ST-LS1/470. The identity of the upper band was proven by its specific hybridization with the 470 bp fragment used as tag (data not shown).

By using the 3.0 kb band as internal reference for copy number and the 700 nucleotides long RNA as internal reference for expression level, these data allow a direct comparison of copy number and expression level in each transformant. Visual inspection of figure 3a indicates a correlation between copy number and expression level in the case of five transformed potato plants. This impression is substantiated by scanning the different autoradiograms. Such a semi-quantitative measurement indicated that the average level of expression of a single copy of the reintroduced gene amounts to 20 - 30 % of that of the resident gene. Similar data were obtained for the tobacco plants analyzed as shown in figure 3b. Again comparison of the RNA data shown in the

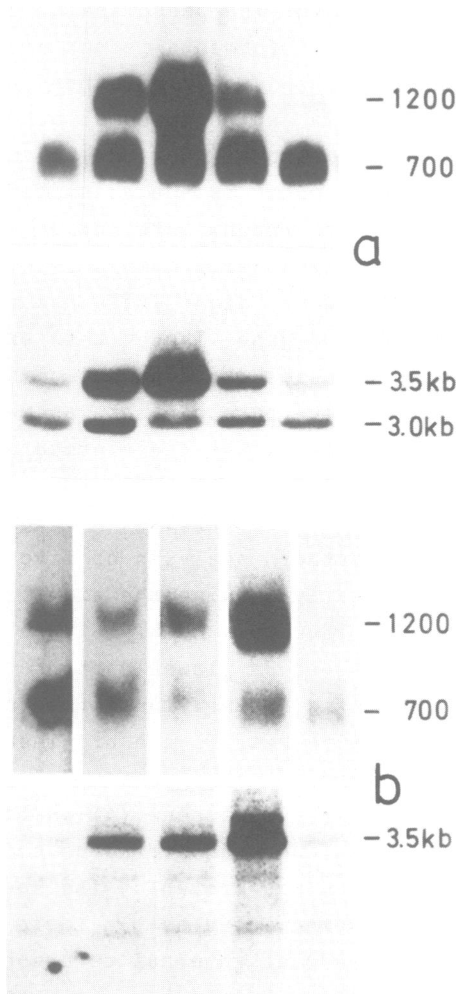

Figure 3: Comparison of expression level and copy number of the ST-LS1/470 gene in transformed potato and tobacco plants.
a) upper part: Northern blot analysis of RNA (50 µg each) isolated from leaves of five independently transformed potato plants (plant no. 1 - 5). Further details are as described in fig. 2.
lower part: Southern blot analysis of total DNA isolated from the five potato plants (plant no. 1 - 5) for the ST-LS1 and the ST-LS1/470 gene. Total DNA (15 µg each) isolated from leaves of transformed plants was digested with HindIII and separated by gel electrophoresis on 0.8 % agarose gels. After transfer onto nitrocellulose filters the DNA was probed for the presence of the ST-LS1/470 as well as the ST-LS1 gene by hybridization with the cDNA clone pcL 700. The size of the bands hybridizing is given in kb.

b) upper part: Northern blot analysis of RNA (50 μg each) isolated from leaves of five independently transformed tobacco plants (plant no. 1 - 5) for the expression of the ST-LS1 and the ST-LS1/470 gene. Further details are as described in fig. 2.
lower part: Southern blot analysis of total DNA isolated from five tobacco plants (plant no. 1 - 5) for the ST-LS1 and the ST-LS1/470 gene. Total DNA (15 μg each) isolated from leaves of transformed plants was digested with HindIII and separated by gel electrophoresis on 0.8 % agarose gels. After transfer onto nitrocellulose filters the DNA was probed for the presence of the ST-LS1/470 gene by hybridization with the cDNA clone pcL 700.

lower part indicate at least a qualitative correlation between expression level and copy number with the exception of plant no. 2.

Expression of the ST-LS1 gene and its modified derivative ST-LS1/470 is dependent upon the presence of intact plastids

The expression of several nuclear genes encoding plastid proteins such as the small subunit of the ribulose-1,5-Bisphosphate carboxylase and the light harvesting chlorophyll a/b binding protein of photosystem II is dependent upon light as well as upon the presence of intact plastids (15-17) suggesting that the presence of a plastid derived signal is necessary for the induction of these genes. This is also the case for the ST-LS1 gene (4). We were therefore interested to see whether or not the modified ST-LS1/470 gene would also be dependent for its expression in transgenic plants on the presence of functional chloroplasts. Plastids of plants treated with the herbicide Norfluorazon, an inhibitor of carotenoid biosynthesis, are severely damaged by photooxidation processes by exposure to strong white light (18).

Shoots of the transformed potato plant were grown in tissue culture for two weeks in the presence or absence of Norfluorazone in strong white light. Subsequently RNA was isolated from these shoots and analyzed for expression of the modified as well as of the resident ST-LS1 gene. The results of figure 4 show that the expression of both genes is significantly reduced in shoots grown in white light in the presence of 1 μM of Norfluorazon . This can be interpreted as an indication for the presence of sequences on the ST-LS1/470 carrying DNA fragment which might be responsible for its chloroplast-dependent expression.

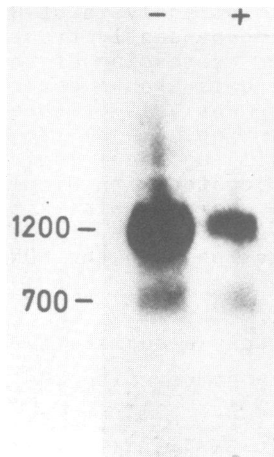

Figure 4: Effect of norfluorazone upon the expression of the ST-LS1/470 and the ST-LS1 gene.
Transformed potato plants (plant no. 3) were put onto basic MS-medium (13) in the presence (+) or absence (-) of norfluorazon and illuminated with strong (3000 lux) white light for 3 weeks. Subsequently RNA was isolated and analyzed for the expression of the ST-LS1 and the ST-LS1/470 gene as described in fig. 2.

DISCUSSION

ST-LS1, a single-copy gene from potato which displays a leaf/stem specific and light- and chloroplast-dependent pattern of expression, has been tagged by an exon-modification and introduced into both potato and tobacco cells using Ti-plasmid vectors. After regeneration of intact potato and tobacco plants, the expression of the tagged derivative of the ST-LS1 gene was analyzed at the RNA-level. After transfer into its homologous host, potato, it was expressed in a strict organ-specific manner. Its expression was highest in leaves and lower in stem and no expression was detectable in either roots or tubers of potato. This qualitative result was obtained in five independent transformants indicating that the developmental specific expression of this gene is controlled by cis-regulatory sequences located on the transferred DNA segment.

Analogous results were obtained when this gene was transferred to tobacco. The expression was highest in leaves followed by stem tissue whereas no expression was observed in roots of transfor-

med tobacco plants. Again this qualitative result was obtained with several independent transformants.

Organ-specific expression of genes in transgenic plants has been described for several seed-specific genes such as the phaseolin gene from bean (19) and the ß-conglycinin gene from soybean (20) and also for several genes encoding either the small subunit of the ribulose 1,5 bisphosphate carboxylase or the light harvesting chlorophyll a/b binding protein (21-24). Our data therefore confirm the conclusion that organ-specific expression is controlled by cis-acting sequences and provide the first direct comparison of the expression of transferred genes versus resident genes in both homologous and heterologous hosts.

No significant influence of host-specific factors on the qualitative characteristics of the expression of this gene were observed.

Despite the fact that the transferred gene was expressed in parallel to the resident gene in terms of its developmental-specific expression, significant differences were observed with respect to level of expression. As evident from figure 3 the relative expression of the introduced gene varied by at least 100 fold in independent transformants, the highest level being reached in the transformed potato plant no. 3 where the combined expression of the different copies of reintroduced gene produced at least 15 fold more RNA than the resident gene.

Variability in the expression level has been observed in several other cases in transgenic plants (3,10,21,24) as well as transgenic mice (25) and Drosophila (26).

It was suggested that the dominant influence on expression level is exerted by a "position-effect" (3,10,21). However most of these analysis were hampered by the fact that it could not be specified whether or not the observed variation in expression level was simply due to differences in the physiological state of the different plants analyzed. By using the expression of the resident gene as standard it can be assured that the differences observed in fig. 3 are indeed true differences in expression level and not simply the consequences of different physiological conditions. Furthermore due to the tagging, the copy number of the introduced gene and the resident gene can be directly com-

pared and internally standardized thus eliminating potential mistakes resulting from differences in the amount of DNA loaded per slot. Within the small sample size analyzed, data shown in figure 3a and b present evidence for the presence of a dose effect for genes artificially introduced into higher plants. We do not know, whether this is a gene-specific finding. If however this observation is of a more general nature in higher plants it would allow a new strategy for the overproduction of certain products besides using strong promoters, i.e. trying to increase the copy number of the gene of interest by e.g. multiple or tandem transformation.

The expression of the ST-LS1 gene has been shown to be dependent on the presence of intact chloroplasts (4). A significantly reduced expression of the ST-LS1/470 gene and of the resident gene was observed in plants where the chloroplast structure had been destroyed by exposing norfluorazone treated plants to strong white light. This observation can be taken as first indication that the supposed chloroplast-derived signals necessary for full expression of the ST-LS1 gene have to be recognized by sequences present in the DNA segment used in the gene transfer experiments.

Future experiments will be devoted to a refined analysis of the cis-acting sequences controlling the developmental-specific, light-dependent and chloroplast-associated expression of the ST-LS1 gene.

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