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Expression of a tuber-specific storage protein in transgenic tobacco plants: demonstration of an esterase activity

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A chimaeric gene composed of the 5′ upstream region of ST-LS1, a leaf/stem specifically expressed gene from *Solanum tuberosum*, and the RNA-coding as well as the 3′ downstream region of patatin, the major storage protein of potato tubers, has been transferred into tobacco plants using the *Agrobacterium* system. The introduction of this gene led to a leaf/stem specific expression of a 42-kd large protein which immunocrossreacts with patatin antiserum. Only low amounts of immunoreacting protein of smaller size could be detected in transgenic tobacco leaves indicating that the patatin protein is fairly stable in this heterologous environment. The size of the protein as well as the size of the RNA detected in transgenic tobacco leaves using a patatin-specific probe indicates that the patatin RNA was accurately processed in both leaf and stem tissue of tobacco. The expression of the patatin gene led to the appearance of a new esterase activity in the transformed tobacco which co-migrated with a protein immunoreacting with patatin antiserum. These data therefore demonstrate that patatin in addition to serving as a storage protein displays an enzymatic activity.

Key words: patatin/gene transfer/storage protein/esterase/potato

Introduction

Patatin is the trivial name for a 40-kd glycoprotein representing the dominant storage protein of potato tubers where it can account for up to 40% of total tuber protein (Racusen and Foote, 1980). In several instances it was found that storage proteins of plants had a function in addition to serving as a protein reserve. Thus several seed proteins act as proteinase inhibitors or have an antifungal or antibacterial activity (Croy and Gatehouse, 1985).

In the case of patatin it was proposed that this storage protein might have a lipid acyl hydrolase (LAH) activity. This proposal was based on the fact that patatin co-purified with the LAH activity in a number of biochemical assays (Racusen, 1984). As this biochemical evidence cannot rule out the possibility that the LAH activity of patatin is due to a contamination by a different protein co-purifying with patatin we decided to set up a genetic test to see whether patatin actually has an LAH activity. As the inactivation of the patatin genes in potato and thus the construction of a patatin null mutant is not possible we decided to transfer the gene encoding patatin in a foreign host devoid of the patatin gene and furthermore decided to express it in a foreign organ. We therefore chose to try to express the patatin gene in tobacco leaves. In view of the fact that patatin has a fairly complicated structure with six introns it was also of interest to see whether the precise processing of the patatin transcript could be achieved in a heterologous host and in heterologous tissue. Finally we were also interested in the stability of a storage protein in a foreign environment.

Results

Construction of a chimaeric gene containing the patatin gene under the control of a leaf/stem specific promoter and transfer into tobacco plants

As outlined above we planned to express the patatin in a host plant devoid of any endogenous patatin gene. Previous experiments had shown that the tobacco genome does not contain any homology to patatin sequences, therefore making this an ideal host plant (Rosahl, 1986).

As we were also interested in studying the processing of a fairly complex precursor RNA as well as the stability of the patatin protein in a heterologous organ, we used a 1350-nucleotide-long fragment of a leaf/stem specifically expressed gene (ST-LS1) from potato previously isolated in our group (Eckes *et al.*, 1986) to drive the expression of the patatin in leaves of transgenic tobacco plants. This fragment which contains the complete 5′ upstream region of the ST-LS1 gene including 13 nucleotides of the 5′ untranslated leader has been shown to be sufficient for a light inducible, leaf/stem specific and chloroplast-associated expression of chimaeric genes in transgenic tobacco plants (Stockhaus, 1986).

The RNA coding part as well as the 3′ downstream sequences of the patatin gene were taken from the gene *pgt5* previously isolated and characterized in our group (Rosahl *et al.*, 1986b). They were fused to the promoter of the ST-LS1 gene by a transcriptional fusion; the 5′ untranslated leader of the chimaeric gene containing 13 nucleotides of 5′ untranslated leader of the ST-LS1 gene followed by seven nucleotides of the polylinker sequences of pUC 18 and 29 nucleotides of the 5′ untranslated leader of the patatin gene. A more detailed description of this construction is given in Figure 1.

The construction was cloned into the intermediate vector pMPK110 and subsequently mobilized into the *Agrobacterium* host strain C58C1 (pGV 3850 Km^r) (Jones *et al.*, 1985) using a triparental mating (van Haute *et al.*, 1983). The structure of the co-integrate was checked by Southern blot hybridization (data not shown). Subsequently leaf disks of *Nicotians tabacum* cv. W38 were infected with the *Agrobacterium* strain containing the chimaeric patatin gene.

Transformed cells were selected on kanamycin-containing medium and regenerated plantlets were transferred into the glasshouse for further analysis.

Analysis of the expression of the chimaeric patatin gene in transgenic tobacco plants

Six independently derived tobacco plants (nos 14, 22 and 29–32) were analysed for the presence of the chimaeric patatin gene as well as for its expression in leaves. The results are summarized in Figure 2.

To check the integrity of the transferred segment, total DNA

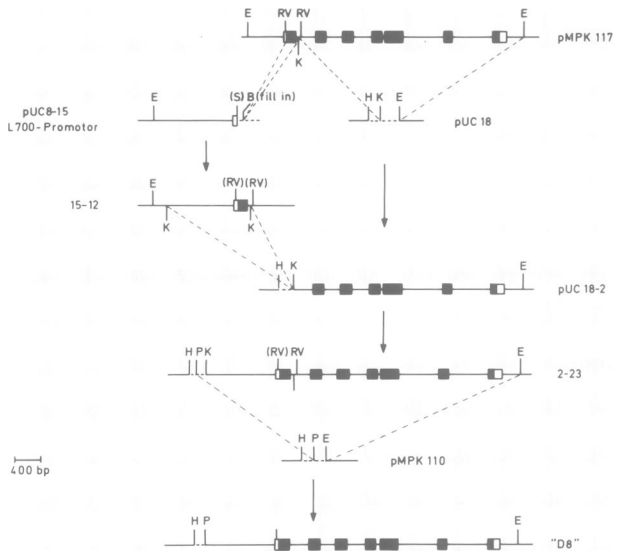

Fig. 1. Construction of a chimaeric gene containing the RNA-coding region of the patatin gene pgT5 (pMPK 117) and the 5′ upstream region of the gene ST-LS1 (L700 promotor). A 295-bp *Eco*RV−*Eco*RV fragment from the patatin gene including 29 bp of the 5′ untranslated region, 168 nucleotides of the first exon and 98 bp of the first intron was cloned into the plasmid pUC8-15 which contains 1600 nucleotides from the 5′ upstream region of ST-LS1 including the promoter region and 11 bp of the 5′ untranslated leader. Cleavage of plasmid 15-12 with *Kpn*I released a 1.6-kb fragment which was cloned into the *Kpn*I site of the plasmid pUC18-2 containing a 4.35-kb *Kpn*I−*Eco*RI fragment of the patatin gene. The resulting plasmid 2-23 contained 1.35 kb of the promoter region of ST-LS1, 11 bp of the 5′ untranslated region and 7 bp of polylinker sequences fused to 29 bp of the patatin 5′ untranslated leader and the seven exons of the patatin gene pgT5. The chimaeric gene was transferred to the intermediate vector pMPK110 as a 6-kb-long *Pst*I−*Eco*RI-fragment.

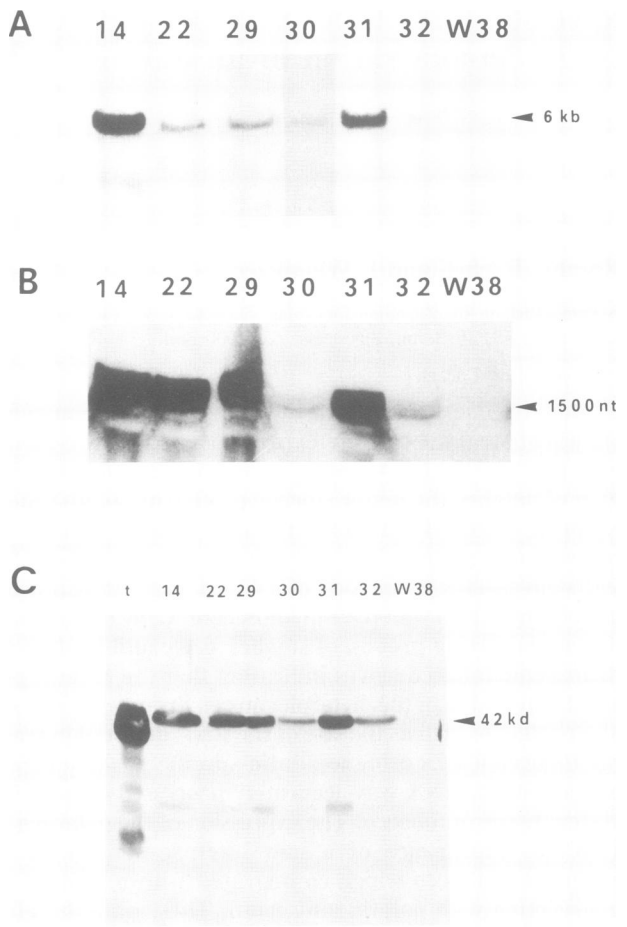

Fig. 2. Analysis of six tobacco plants transformed with the chimaeric gene D8. **A** Southern analysis. DNA (10 μg) isolated from each transformed plant (14,22,29−32) as well as from untransformed tobacco (W38) was digested with *Eco*RI and *Pst*I, run on 0.8% agarose gels and transferred onto nitrocellulose. Filters were hybridized using the radioactively labelled 5.3-kb *Eco*RI fragment of the patatin genomic clone pgT5 as a probe. The size of the *Pst*I−*Eco*RI fragment containing the chimaeric gene is indicated. **B** Northern analysis. Total RNA (70 μg) isolated from leaf tissue of the transformed plants and untransformed tobacco (W38) was separated on 1% formaldehyde denaturing gels and transferred onto nitrocellulose. Filters were hybridized against the labelled 5.3-kb *Eco*RI fragment of the patatin genomic clone pgT5. **C** Western analysis. Total protein (500 μg each) from leaves of the transformed tobacco plants (14−32) as well as total protein from potato tubers (t) and from untransformed tobacco leaves (W38) was run on 12% denaturing SDS gels, blotted onto nitocellulose and incubated with patatin-specific antiserum.

isolated from the different plants was digested with *Eco*RI and *Pst*I and after transfer onto nitrocellulose filters hybridized with the patatin genomic clone pgT5 as a probe (Figure 2A) (Rosahl *et al.*, 1986b). Whereas no hybridization is visible with DNA extracted from untransformed tobacco plants, a band of 6 kb appears in the digests of all DNAs isolated from transformed plants, which is the size to be expected in the case of a non-rearranged chimaeric patatin gene. It is also obvious from the varying intensity of this band that the copy number of this gene differs in the different transformed plants.

The expression of the chimaeric gene in leaves of transgenic tobacco plants was tested at the RNA as well as at the protein level. The results of a Northern blot analysis are shown in Figure 2B. Upon hybridization with the genomic clone pgT5 an RNA of 1500 nucleotides is visible in the transformants (14, 22 and 29−32) which is not present in untransformed tobacco (W38). This RNA co-migrates with authentic patatin mRNA from potato tubers (cf. Figure 3) indicating that the transcription as well as the processing of the complex patatin pre-mRNA is occurring faithfully in leaves of transformed tobacco plants. To analyse the protein content of the plants total soluble protein was separated on SDS-containing denaturing polyacrylamide gels and after electrophoretic transfer onto nitrocellulose filters analysed for patatin using patatin specific antiserum. The results are shown in Figure 2C. In all plants a protein immunologically crossreacting with the patatin antibody is detectable. This protein migrates with a slightly higher mol. wt than the majority of the patatin protein isolated from tubers (lane t). Furthermore there is variation in the level of patatin protein present in different transformants. A comparison of B and C, Figure 2, reveals that the amount of protein follows the amount of RNA found in different transformants.

The ST-LS1 promoter is sufficient for organ-specific expression of the patatin gene

The promoter used for the expression of the patatin gene is derived from a single copy gene from potato which displays a leaf/stem and chloroplast-associated type of expression (Eckes *et al.*, 1985, 1986). The patatin gene on the other hand is only expressed in tubers of potato (Rosahl *et al.*, 1986a). Run-off transcription experiments performed in isolated nuclei have indicated that the developmental-specific expression of both genes is most likely controlled at the level of transcription (Eckes *et al.*, 1985; Rosahl *et al.*, 1986a) suggesting that the 5′ upstream region of the genes is involved in their controlled expression. However, these data do not rule out the possibility that the RNA coding part of the

Fig. 3. Tissue specific expression of the chimaeric patatin gene. Total RNA (**A**) (100 μg each) and protein (**B**) (500 μg each) was isolated from leaf, stem and root tissue of the transformed tobacco plant 14 and analysed for the presence of patatin transcripts or protein by Northern and Western blot experiments. Further details are as in Figure 2. **Lane t** contains 40 μg of total RNA from potato tubers.

patatin gene is also involved in expression control. For that reason we analysed the expression of the patatin gene in leaf/stem and root tissue at the RNA as well as at the protein level in transformed tobacco plants. The data shown in Figure 3 demonstrate that the expression of the patatin gene clearly follows the specific expression of the ST-LS1 gene indicating that the RNA coding part of the patatin gene does not exert any dominant effect on the expression of the chimaeric gene.

Expression of the patatin protein leads to the appearance of a new esterase activity in leaves of transgenic tobacco plants

Patatin has long been supposed to contain an esterase activity (Galliard, 1971; Racusen, 1984). In view of our observation that patatin can be stably expressed in leaves of transgenic tobacco plants we decided to test whether these tissues would, as a result of the transfer and expression of the patatin gene, contain a new esterase activity. Since tobacco leaf cells contain numerous esterase activities and a possible change in these esterase activities could also be due to the transformation event *per se* we decided to construct a control which should be as isogenic to the transformed tobacco as possible. A derivative of the chimaeric ST-LS1 patatin gene was therefore constructed where the open reading frame of the protein-coding region of the patatin gene was interrupted by the insertion of a 470-nucleotide-long DNA segment originally derived from gene 2 of the T-DNA (cf. Eckes *et al.*, 1986). No protein immunoreacting with the patatin antiserum could be detected in leaves of tobacco plants transformed with this construction as shown in Figure 4B, lanes 2 and 4. Tobacco plants containg the inactivated patatin gene therefore served as a control in the experiments described below.

Figure 4A shows the results of an *in situ* esterase activity stain performed on protein extracts from leaves of transgenic plants containing either the active patatin gene (lane 1, plant no. 29, and lane 3, plant no. 14) or the inactivated form (lane 2, plant

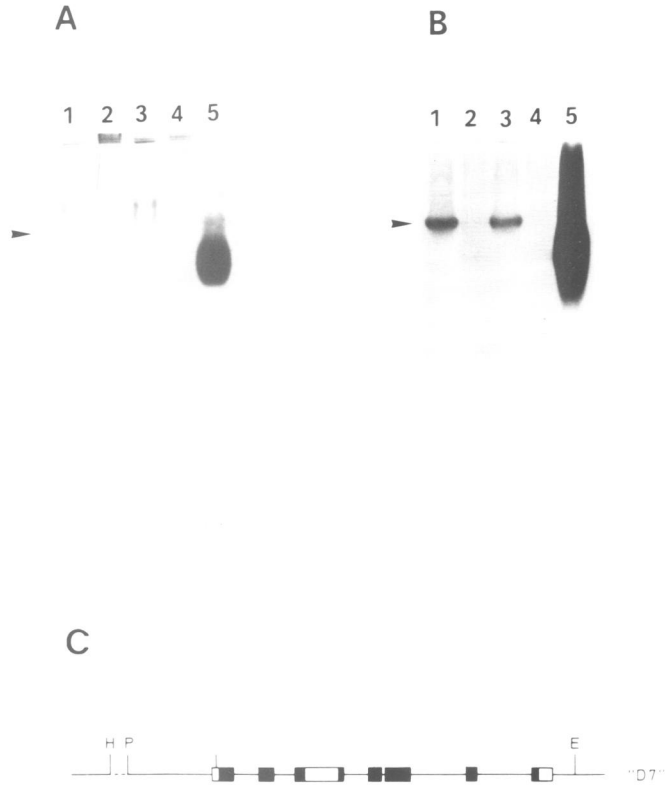

Fig. 4. Esterase activity of patatin in transgenic tobacco. Total protein (500 μg each) isolated from leaf tissue of tobacco plants transformed with the chimaeric patatin gene D8 (**lane 1**, plant 29 and **lane 3**, plant 14) or the inactivated form D7 of the chimaeric gene (**lane 2**, plant 36 and **lane 4**, plant 40) as well as total tuber portein (**lane 5**) was separated on 10% native polyacrylamide gels. Identical gels were stained for esterase activity (**A**) using α-naphtyl-acetate as a substrate or analysed for the presence of patatin protein by Western blot experiments (**B**). A schematic drawing of the inactivated form D7 of the chimaeric patatin is shown in (**C**). The 470-bp fragment represented by a white box was inserted into the third exon of the patatin gene. The cloning was analogous to the construction of D8 described in Figure 1.

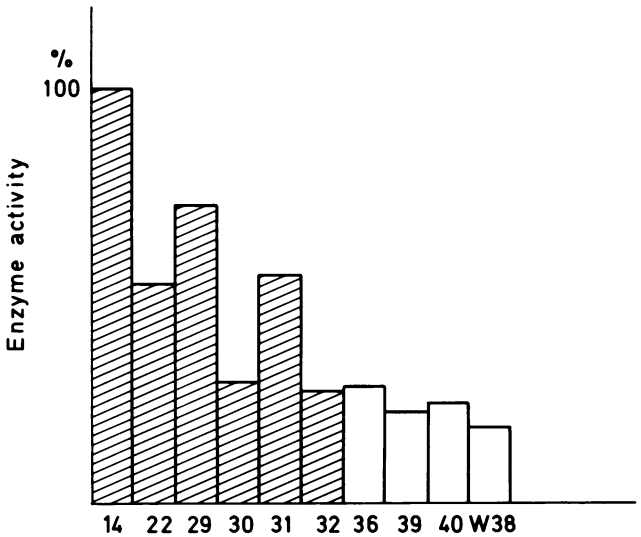

Fig. 5. Relative PNP-laurate esterase activity measured in crude extracts prepared from plants transformed with the chimaeric gene D8 (14–32), D7 (36 and 40) or from untransformed tobacco (W38). Enzyme activity was determined as described in Materials and methods. The 100% value indicated on the axis corresponds to a specific activity of 0.01 μmol PNP/(mg protein)(min.).

no. 36, and lane 4, plant no. 40). Upon incubation of the native gel with α-naphthyl-acetate a new esterase activity appears only in the extracts derived from the patatin-expressing plants whereas there is no activity in the control. Furthermore this new esterase band co-migrates with a protein immunoreacting with patatin antiserum which again can only be seen in leaves of tobacco plants containing the active patatin gene (Figure 4B, lanes 1 and 3). Similar results were obtained with plants 22 and 31 (data not shown). These data therefore strongly suggest that the patatin expressed in leaves of transgenic tobacco plants does actually exhibit an esterase activity.

This conclusion is furthermore supported by the fact that a para-nitrophenyl(PNP)-laurate esterase activity could be detected in cell-free extracts prepared from transgenic tobacco leaves. Testing different fatty acid esters it was found that the esterase activity was highest using PNP-laurate as a substrate. Same amounts of protein from tobacco plants either transformed with the active patatin gene D8 (14−32) or the inactivated gene D7 (36−40) were used to measure PNP-laurate esterase activity. As shown in Figure 5 extracts from four tobacco plants (14, 22, 29 and 31) contain a significantly higher enzyme activity than the control extracts prepared from the plants 36, 39 and 40 and the untransformed tobacco W38. The esterase activity detected in plants 30 and 32 is similar to the activity measured for the control plants. Comparison of the activity of the PNP-laurate esterase in the different transformed tobacco plants with the amount of patatin RNA and protein (Figure 2B and C) shows a strong correlation between the amount of patatin protein present and enzyme activity.

Discussion

The protein-coding part as well as the 3′ downstream region of a patatin-encoding gene has been fused to the 5′ upstream region of ST-LS1, a leaf/stem specifically expressed gene from potato. The fragments were connected in the respective 5′ untranslated region of both genes leading to a transcriptional fusion. This chimaeric gene was transferred into tobacco cells using the *Agrobacterium* system and whole plants were regenerated and analysed for the expression of the transferred gene. Examination of root, stem and leaf tissue shows that the chimaeric gene was expressed in a leaf/stem specific manner. This indicates that its expression is governed by the promoter of the ST-LS1 gene and that the RNA-coding part of the patatin gene does not exert any dominant negative control on its expression in e.g. leaves of tobacco. It is therefore unlikely that the tuber-specific expression of the native patatin gene is negatively controlled by sequences contained either in the 3′ downstream or in the RNA-coding part of the patatin gene.

The introduction of the chimaeric patatin gene into leaves leads to the appearance of a new RNA which co-migrates with authentic patatin mRNA from potato tubers and hybridizes specifically with patatin probes.

Furthermore a new protein of ~42 kd mol. wt which immunoreacts with patatin antiserum is found in leaves of transformed tobacco plants. Both these observations show that the patatin gene under the control of the ST-LS1 promoter is not only transcribed in an organ-specific way but that the splicing and processing events necessary to create the functional patatin mRNA from its precursor occur with high fidelity not withstanding the fact that this gene contains six introns varying in length from 88 to 816 nucleotides (Rosahl *et al.*, 1986b). In most other cases where a correct expression of a foreign gene was observed in transgenic plants these genes were found to be expressed in the corresponding organ of a heterologous plant (cf. e.g. Sengupta-Gapalan *et al.*, 1985; Beachy *et al.*, 1985; Nagy *et al.*, 1985). The only other example of a rather complex gene expressed in a heterologous environment is the expression of the phaseolin gene from bean in sunflower tumour tissue (Murai *et al.*, 1983). In this case too it was found that the five introns of the phaseolin gene were faithfully removed in sunflower tumour tissue. Thus it seems that the correct splicing and processing of the precursor RNAs do not always require developmental specific factors.

One remarkable feature of the expression of the patatin gene in tobacco leaves is its high stability. The amount of low mol. wt peptides immunoreacting with patatin antiserum represents only a low percentage of the intact patatin protein. Analogous experiments where seed storage proteins were transferred into heterologous plants have often led to a more prominent degradation. Thus only degradation products of the phaseolin protein were found in transformed sunflower as well as tobacco callus cells (Murai *et al.*, 1983; Chee *et al.*, 1986). Upon the transfer of zein genes into sunflower tumour cells no immunoreacting polypeptides were found despite the presence of a functional (i.e. *in vitro* translatable) zein mRNA (Matzke *et al.*, 1984; Goldsbrough *et al.*, 1986). Expression of seed proteins such as phaseolin and β-conglycinin in seeds of heterologous plants such as tobacco or petunia also led to some instability of the proteins (Sengupta-Gopalan *et al.*, 1985; Beachy *et al.*, 1985).

The patatin protein formed in tobacco leaves has a lower mobility on denaturing as well as on native polyacrylamide gels than the majority of the patatin protein present in potato tubers. As patatin contains a signal peptide this shift in mobility could be either due to the fact that the signal peptide is not recognized in tobacco leaves or to differences in the way the patatin protein is post-translationally modified in tobacco leaves as compared with potato tubers. Binding studies using Con-A Sepharose columns show a nearly 90% retention of the tobacco-made patatin indicating that the patatin is glycosylated in tobacco leaves (Sonnewald, U., personal communication) indicating that the signal peptide must be functional. Thus the shift in mobility is probably due to differences in post-translational modifications like glycosylation.

Storage proteins accumulate to high levels in seeds or tuber tissues. It is widely accepted that their most important function is to serve as a means of storing nitrogen for germination and early seedlings growth. However, in addition to serving as a nitrogen-deposit a number of storage proteins also fulfill other roles which are very often connected to protection against pathogens, as in the case of proteinase inhibitors (cf. Richardson, 1976) or certain seed proteins displaying antibacterial activity (Ponz *et al.*, 1983). In the case of patatin it has been speculated that in addition to representing the main storage protein of potato tubers it might have a LAH activity (Racusen, 1984). By transferring the patatin gene into a background devoid of any endogenous patatin we were able to prove this assumption. This of course raises the question about the possible function of such massive amounts of LAH activity in potato tubers. One plausible assumption is that the LAH activity of patatin is important for the transition of the tuber from dormancy to vegetable growth where a high LAH activity could be important for the rapid degradation of cell membranes and thus rapid liberation of certain metabolites. In addition patatin might also be involved in protection against microbial invasion as the fatty acids liberated by the LAH activity might be used for the formation of wax or certain cytotoxic acid derivatives (Racusen, 1984). By

transferring the patatin gene into tobacco and getting stable expression and accumulation of the patatin protein in tobacco leaves new approaches have become possible which should be helpful in elucidating the possible biological role of this plant storage protein.

Materials and methods

Recombinant DNA techniques

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

Isolation and analysis of nucleic acids

Isolation of DNA from both Agrobacteria as well as higher plants was carried out according to Murray and Thompson (1980); total RNA was isolated from plants according to Logemann *et al.* (1986). Gel electrophoretic separation of the nucleic acids and subsequent analysis of blot hybridization was performed as described earlier (Rosahl *et al.*, 1986a).

Transfer of the chimeric patatin gene into tobacco cells and regeneration of intact plants

The chimaeric patatin gene D8 (cf. Figure 1) as well as the derivative D7 (cf. Figure 4) present in the intermediate vector pMPK 110 (Eckes *et al.*, 1986) were transferrred into the T-DNA of the disarmed strain *Agrobacterium* C58C1 [pGV 3850 Kan^R (Jones *et al.*, 1985] by a triparental mating using GJ 23 as the mobilizing strain (van Haute *et al.*, 1983). Co-integrates obtained were selected in the presence of spectinomycin (100 μg/ml) chloramphenicol (25 μg/ml) streptomycin (300 μg/ml) and erythromycin (50 μg/ml). Agrobacteria growing in the presence of these antibiotics were subsequently checked for the presence of the non-rearranged gene and used for transformation of tobacco leaf disks (Horsch *et al.*, 1985). Shoots arising on 100 μg/ml of kanamycin were rooted on hormone-free medium containing the same amount of kanamycin and subsequently tested for nopaline synthase activity. Only those plants which were both kanamycin resistant and nopaline synthase positive were transferred into the glasshouse and cultivated further.

All manipulations were performed according to the 'Richtlinien für den Umgang mit neukombinierten Nukleinsäuren' of the Bundesministerium für Forschung und Technologie.

Protein analysis

Western blot analysis. Total protein was extracted from tobacco leaf tissue with 80 mM Tris−HCl pH 6.8, 2% SDS, 10% glycerol and separated on 12.5% denaturing polyacrylamide gels (Laemmli, 1970) by gel electrophoresis. The subsequent transfer onto nitrocellulose membranes and incubation with patatin antiserum was performed according to Gershoni and Palade (1983). Detection of immunoreactive protein was carried out using alkaline phosphatase coupled to anti-rabbit IgG offering 5-bromo-4-chloro-3-indolyl-phosphate as substrate.

In situ esterase stain. Total protein extracted from tobacco was separated on 10% native polyacrylamide gels. Gels were subsequently stained for esterase activity using α-naphthyl-acetate and fast blue RR in 50 mM Tris−HCl pH 7.4.

Enzyme assays

Total protein was extracted with 25 mM sodium phosphate buffer pH 7.0 containing 0.1 g/g Dowex and 0.1 g/g Polyvinyl pyrrolidone, filtered through Miracloth and centrifuged. The supernatant was passed through a Sephadex G50 column. Protein content was determined according to Bradford (1976). Equal amounts of protein extracted from different tobacco plants were assayed for PNP-laurate esterase activity as described by Racusen (1984).

Acknowledgements

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