

Vicilin with carboxy-terminal KDEL is retained in the endoplasmic reticulum and accumulates to high levels in the leaves of transgenic plants

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Summary

Gene constructs were designed to test the effect of the endoplasmic reticulum (ER)-targeting signal, KDEL, on the level of accumulation of a foreign protein in transgenic plants. The gene for the pea seed protein vicilin was modified by the addition of a sequence coding for this tetrapeptide at its carboxyl terminus. The altered gene was placed under the control of a CaMV 35S promoter and its expression in the leaves of both tobacco and lucerne (alfalfa) was compared with that of an equivalent vicilin construct lacking the KDEL-coding sequence. The presence of the ER-targeting signal led to a greatly enhanced accumulation of the heterologous protein. In lucerne and tobacco leaves, the level of vicilin–KDEL protein was 20 and 100 times greater than that of the unmodified vicilin, respectively. These differences in expression level could not be explained by corresponding differences in the steady-state levels or the translatability of the mRNAs. However, when the stability of vicilin and vicilin–KDEL proteins was compared in their respective transgenic hosts, unmodified vicilin was found to be degraded with a half-life of 4.5 h while vicilin–KDEL was much more stable with a half-life of more than 48 h.

Immunogold labelling of leaf tissues from transgenic lucerne and tobacco showed the presence of vicilin associated with large aggregates within the ER lumen of vicilin–KDEL plants. No such aggregates were detected in transgenic plants expressing wild-type vicilin.

It is concluded that the carboxy-terminal KDEL caused the retention of the modified vicilin in the ER, and that this retention led to the increased stability and higher level of accumulation of vicilin–KDEL in leaves of transgenic plants.

Introduction

Apart from its value as a tool for answering questions about basic cellular processes such as gene expression, protein targeting or enzyme function, plant transformation has great potential as a means of introducing agronomically useful characters into crop and pasture plants. Although the number of commercially important plants that can be transformed and regenerated is still small, the list is increasing rapidly (Gasser and Fraley, 1989). For some applications of this technology, such as the expression of a protein toxic to insect pests, low levels of heterologous protein may suffice (Perlak *et al.*, 1990), but to alter the nutritional quality of plants it is necessary to maximize the level of gene expression and protein accumulation. We wish to introduce foreign proteins, with high nutritional value for animals, into the leaves of pasture plants. In order to alter the nutritional quality of these organs significantly, the foreign proteins must constitute 1–10% of the total protein of the leaf. To achieve these levels of protein accumulation we anticipate that we will need to maximize transcription and translation as well as protein stability in transgenic plants.

The proteins that occur in the protein storage vacuoles (protein bodies) of seeds (e.g. storage proteins, lectins, enzyme inhibitors) constitute a potentially useful class for the improvement of pasture plants if they could be expressed at a sufficiently high level in the leaves and stems of their transgenic hosts. Some, such as the 2S albumins of brazil nut and sunflower seed (Ampe *et al.*, 1986; Kortt *et al.*, 1991), are unusually high in the nutritionally important amino acids cysteine and methionine, while others, such as the protease inhibitors and α -amylase inhibitors (Hilder *et al.*, 1987; Moreno and Chrispeels, 1989), may have useful plant protection properties. The amino acid sequence of these seed-specific proteins includes signals that direct them firstly into the lumen of the endoplasmic reticulum (ER) and thence into the vacuolar protein bodies. The genes for these proteins can be modified by removing the 5' upstream regions that confer seed specificity and replacing them with a leaf-specific promoter or a strong constitutive promoter such as the CaMV 35S promoter, so that the modified gene will be expressed in the leaves and other non-seed tissues of their transgenic host. Provided the protein coding sequence is unchanged, the transgenic storage protein would be targeted to the vacuoles of the leaves in their new host (Chrispeels, 1991; Dorel *et al.*, 1988; Hunt and Chrispeels, 1991). However,

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experience to date shows that seed storage proteins only accumulate to very low levels in the leaves of a transgenic host, even when the transgene contains the CaMV 35S promoter (Higgins and Spencer, 1991; Lawton *et al.*, 1987). This may be because they undergo continuous breakdown in the protease-rich environment of leaf vacuoles (Van der Valk and Van Loon, 1988). This interpretation is supported by the findings of Lawton *et al.* (1987) that all the conglycinin (a seed protein from soybean), when expressed in transgenic tobacco leaves, was found to be associated with vesicles and membranes (ER and Golgi) and none was present in the soluble fraction of a leaf homogenate, as might be expected from a vacuolar localization (R. Beachy, personal communication).

In an effort to circumvent this problem of instability of seed proteins expressed in leaves, we attempted to redirect the heterologous protein to a different intracellular location by the use of the ER-targeting signal, the tetrapeptide lys-asp-glu-leu (KDEL). This sequence of amino acids, which is found at the carboxyl terminus of mammalian, fungal and plant proteins that reside in the lumen of the ER, has been shown to be necessary and sufficient for the retention of luminal and other proteins in the ER (Pelham, 1990). Its presence on the carboxyl terminus of vicilin could therefore be expected to cause vicilin to be retained in the lumen of the ER in transgenic plants. The results reported here show that this is indeed the case. Vicilin-KDEL, but not unmodified vicilin, was found in large aggregates in the ER of transgenic tobacco and lucerne leaves. The presence of the carboxy-terminal KDEL sequence also resulted in a marked increase in the level of vicilin accumulation both in tobacco (about 100-fold) and lucerne (about 20-fold) leaves. While vicilin mRNA levels and vicilin mRNA translatability were similar in plants expressing vicilin and vicilin-KDEL, degradation of newly synthesized protein was much slower in the case of the vicilin-KDEL construct. These data are consistent with the interpretation that the high level of accumulation of the modified vicilin is due to its localization in the ER and its consequent lack of exposure to the proteolytic environment of the leaf vacuole.

Results

Modification of the vicilin gene

In order to introduce an ER retention signal at the carboxyl terminus of pea vicilin, an *Xho*I site was first created near the end of the coding sequence and a short nucleotide sequence was then spliced into this position. This resulted in the replacement of the two carboxy-terminal amino acids (ser-val) of vicilin with arg-gly and the addition of six new amino acids, namely, ser-glu-lys-asp-glu-leu (Figure 1). Because of the extensive evidence of the key role of the

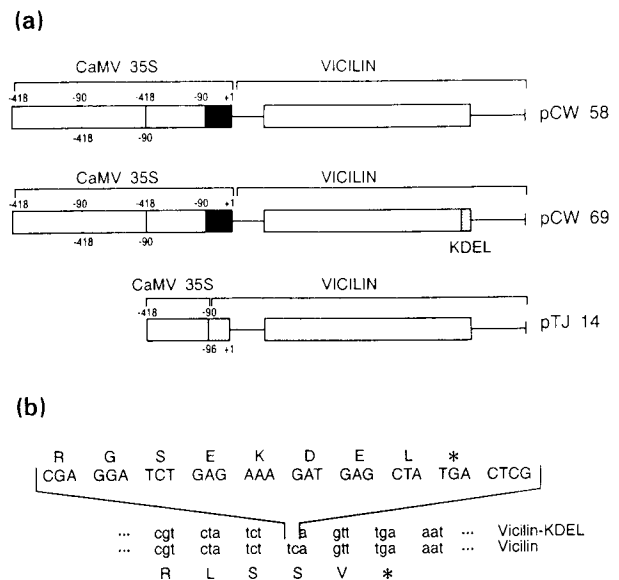


Figure 1. Structure of the chimeric CaMV 35S–vicilin and vicilin–KDEL genes.

(a) In pCW58 and pCW69, the vicilin 5′-upstream sequence from +1 to −2500 was replaced with one full copy of the CaMV 35S promoter (+1 to −418) together with two copies of the CaMV 35S enhancer (−90 to −418). The black boxes refer to the TATA-containing region of the CaMV 35S promoter (+1 to −90). In pCW69, an *Xho*I site was introduced upstream of the stop codon (see b) and a short oligonucleotide sequence was ligated into the restriction site. The mutated region encoded the amino acids, RGSEKDEL (abbreviated here to KDEL) in place of SV at the carboxyl terminus of vicilin (dotted box). In pTJ14, the TATA-containing region (hatched) was supplied by the vicilin gene (+1 to −96). The vicilin 5′-upstream region from −97 to −2500 was replaced by one copy of the CaMV 35S enhancer (−90 to −418).

(b) Protein and nucleic acid sequences of the carboxyl terminus of vicilin and vicilin–KDEL. The *Xho*I site (see legend to a) was introduced into the codon (tca) for the penultimate amino acid. This mutagenesis, together with the inserted oligonucleotide (described in Experimental procedures), resulted in a transcript for a mutant form of vicilin with two amino acids deleted and replaced by eight new residues terminating in KDEL. Upper case lettering in the DNA sequence has been used to denote the additional 31 bases derived from the oligonucleotide insertion and the mutagenesis step required to introduce the *Xho*I site. The asterisks indicate the stop codon.

terminal peptide, lys-asp-glu-leu (KDEL) or some variation thereof, in ER retention (see Pelham, 1990), we refer throughout to our modified vicilin as vicilin–KDEL, but would emphasize that in fact the carboxy-terminus has been modified by the removal of two amino acids and addition of an octapeptide that terminates in SEKDEL. This hexapeptide was initially found to confer ER retention on lysozyme, which is normally secreted (Munro and Pelham, 1987), but later work showed that the tetrapeptide KDEL alone was sufficient to relocate a number of proteins to the ER (Pelham, 1990). The other sequence changes were necessary to create and utilize an *Xho*I site during gene reconstruction.

The 3′ flanking region used in all constructs was a 280 bp portion of the vicilin gene, while the 5′ region consisted

of single or multiple copies of the CaMV 35S promoter/enhancer which were introduced upstream of the cap site. The use of different promoter regions was part of a study to test their effect on gene expression. We found that the use of different untranslated regions and single or multiple copies of the CaMV 35S promoter/enhancer caused only minor changes (2–4-fold) in mRNA and protein accumulation (Wandelt *et al.*, 1991). The specific constructs used for transformation of tobacco and lucerne in this study are shown in Figure 1.

Accumulation of vicilin and vicilin-KDEL in leaves of transgenic plants

The vicilin (pCW58 or pTJ14) and vicilin-KDEL (pCW69) constructs were introduced into tobacco and the resultant transformants analysed on immunoblots after Na-SDS-PAGE of leaf extracts obtained from small plants grown in lidded containers in a growth chamber. These transformed plants are referred to as vicilin or vicilin-KDEL plants.

Figure 2 shows a representative immunoblot of two highly expressing tobacco plants transformed with either vicilin or vicilin-KDEL gene constructions. Vicilin accumulated in the leaves as full-length polypeptides with molecular mass of 50 000. In the plants transformed with the unmodified vicilin gene (Figure 2, lane 1) there were, in addition, two smaller vicilin-related polypeptides with

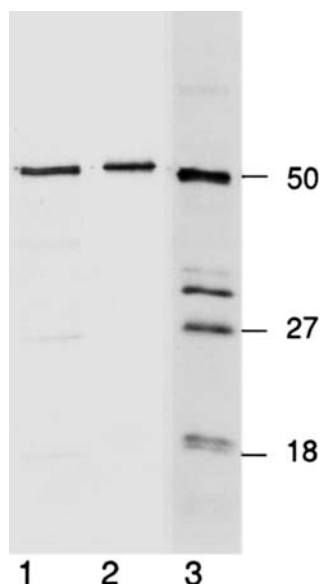


Figure 2. Vicilin levels in the leaves of transgenic tobacco plants transformed with vicilin and vicilin-KDEL genes.

In lane 1, vicilin was immunoselected from 500 μg of total leaf protein from a pTJ14-transformed plant prior to fractionation on SDS-PAGE and immunoblotting. Lane 2 represents 2.5 μg of total leaf protein from a vicilin-KDEL (pCW69) plant and lane 3 shows vicilin from 2.0 μg of total pea cotyledon protein. Numbers on the vertical axis represent molecular mass in kDa.

molecular masses of 27 000 and 18 000. These coincided with normal post-translational processing products found in vicilin from pea seeds (Figure 2, lane 3). In Figure 2, the sample loaded in lane 1 (from a vicilin plant) was derived from 200 times more total protein than the sample in lane 2 (from a vicilin-KDEL plant), but it produced a similar strength of signal in the immunoblot.

The level of vicilin and vicilin-KDEL accumulation showed considerable variability between individual transformants. Quantification by densitometry of immunoblots from 15 transformants, using a range of levels of pea vicilin to generate a standard curve, showed that the content of vicilin-related protein in young tobacco leaves ranged from 26 to 500 ng per mg leaf protein for vicilin and from 860 to 37 300 ng per mg leaf protein for vicilin-KDEL plants. The averages for the vicilin and vicilin-KDEL tobacco plants were 250 and 25 300 ng per mg of protein, respectively. Thus, on average, carboxy-terminal KDEL caused a 100-fold increase in the accumulation of vicilin in tobacco leaves.

The vicilin content of transgenic tobacco was influenced by the manner in which the plants were grown and the position of the leaf on the plant. The levels of vicilin and vicilin-KDEL were both lower (by about 20-fold) in the young leaves of tobacco plants grown in the glasshouse than in the leaves of plants grown in tissue culture boxes. It is also noteworthy that there were more low molecular weight vicilin-KDEL cleavage products in the leaves of glasshouse-grown plants than in the leaves of tissue culture-grown plants (compare lane 2 in Figure 2 with lane 1 in Figure 3a). However, none of these cleavage products was the same size as the cleavage products of unmodified vicilin in pea seeds or tobacco leaves, indicating that new cleavage sites were used that were different from those recognized in pea seed vacuoles. This result is evidence that vicilin-KDEL is accumulated in a different cell compartment to unmodified vicilin in transgenic tobacco. The influence of leaf position on vicilin content is shown in Figure 3a, an immunoblot of protein extracts from different leaves on a single glasshouse-grown tobacco plant. All lanes were loaded with vicilin derived from 1 mg of leaf protein. There was a clear decline in the concentration of vicilin-KDEL in the leaf extracts as the leaves matured.

Vicilin (pTJ14) and vicilin-KDEL (pCW69) constructs were also used to transform lucerne, and protein levels in the leaves of glasshouse-grown plants were analysed. The variability in the leaves of the various transformants was considerably less than in the tobacco plants and the levels were, on average, comparable to those in tobacco grown in the glasshouse. The difference between the vicilin and the vicilin-KDEL lucerne plants was about 20-fold. The average for six vicilin plants was 50 ng mg^{-1} of leaf protein, while six vicilin-KDEL plants had an average of 1020 ng mg^{-1} of leaf protein with a range from 806 to 1368 ng. This

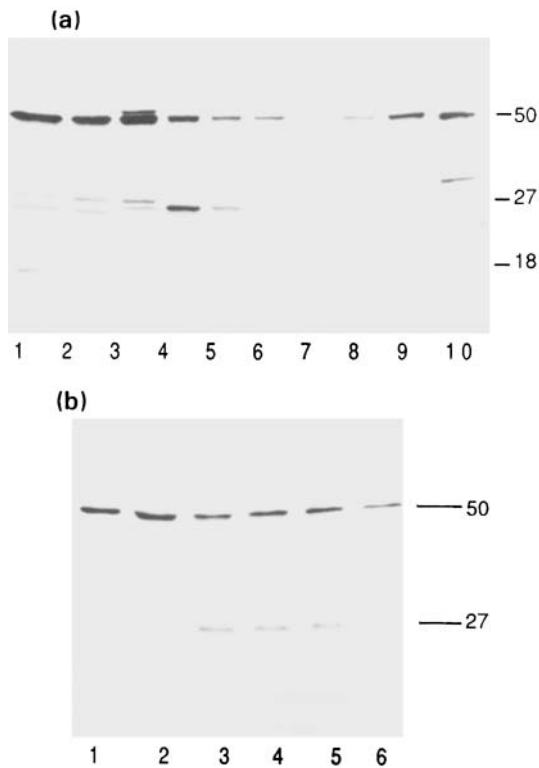


Figure 3. Vicilin levels in leaves of vicilin-KDEL transgenic tobacco and lucerne plants grown in a glasshouse.

Vicilin was immunoselected from total leaf protein prior to fractionation by SDS-PAGE and detection by immunoblotting. Numbers on the vertical axis represent molecular mass in kDa.

(a) Lanes 1–9 represent successive leaf positions on a tobacco plant, the youngest leaf being shown in Lane 1. Lane 10 contained 2 μ g of pea seed protein while lanes 1–9 contained vicilin derived from 1 mg leaf protein.

(b) Lanes 1–6 represent successive leaf positions on a lucerne plant as described in (a). Each lane contained 30 μ g leaf protein.

means that the presence of KDEL caused a 20-fold increase in the abundance of vicilin in the glasshouse-grown lucerne leaves.

In transgenic lucerne, the vicilin-KDEL content per unit of total protein also declined with leaf position from the younger leaves at the top to older leaves at the bottom (Figure 3b), as was the case for tobacco. Unlike the situation in tobacco, however, there was no evidence of accumulation of vicilin cleavage products in the young leaves of vicilin-KDEL plants, although one cleavage product (molecular mass 27 000) became apparent in lower leaves. This could arise from proteolytic activity in the lumen of older lucerne leaves or from the transport of

a fraction of the vicilin-KDEL to the vacuole and its subsequent cleavage in that organelle.

Vicilin-KDEL accumulated in the ER

To find out if the carboxy-terminal KDEL on vicilin was effective in retaining the protein in the ER, we examined the distribution of vicilin in the leaves of transformed lucerne and tobacco plants by immunocytochemistry combined with electron microscopy. The results, shown in Figure 4a, indicated that in vicilin-KDEL lucerne plants, immunoreactive material was present as relatively large aggregates (<0.5 μ m) in the lumen of the ER. In this example, which is not unique, the ER lumen widened and the limiting membranes were continuous with the outer leaflet of the nuclear envelope. Similar aggregates were not found in the ER of lucerne leaves transformed with the unmodified vicilin gene (pTJ14) and no immunostaining was observed. It is likely that the level of vicilin in these leaves (0.005% of total protein) was below the sensitivity of the immunocytochemical method used here. We cannot be sure that the aggregates of vicilin-KDEL exist *in situ*. They may have been formed during the fixation procedure, although we have previously observed similar condensed protein aggregates in vacuoles of unfixed protoplasts from immature pea cotyledons (Goodchild and Craig, 1982). It is also not clear whether the vicilin-KDEL aggregates contain only vicilin-KDEL or also include other proteins. Figure 4(b and c) shows typical vicilin-KDEL-containing inclusions in the ER of tobacco leaves. Many of the inclusions are larger than those seen in lucerne and this results in a greater distortion of the ER membranes in tobacco. As a result, the ER membrane is not well contrasted around much of the larger tobacco deposits because the plane of the membrane is not normal to the plane of the section. One can, however, clearly follow groups of ribosomes ringing each deposit, mirroring the ER membrane (see arrowheads, Figure 4b). In Figure 4c, a smaller ER inclusion lies adjacent to the plasma membrane and the ER cisternae appear to be continuous with the plasmodesmata.

Vicilin and vicilin-KDEL differ in their stability

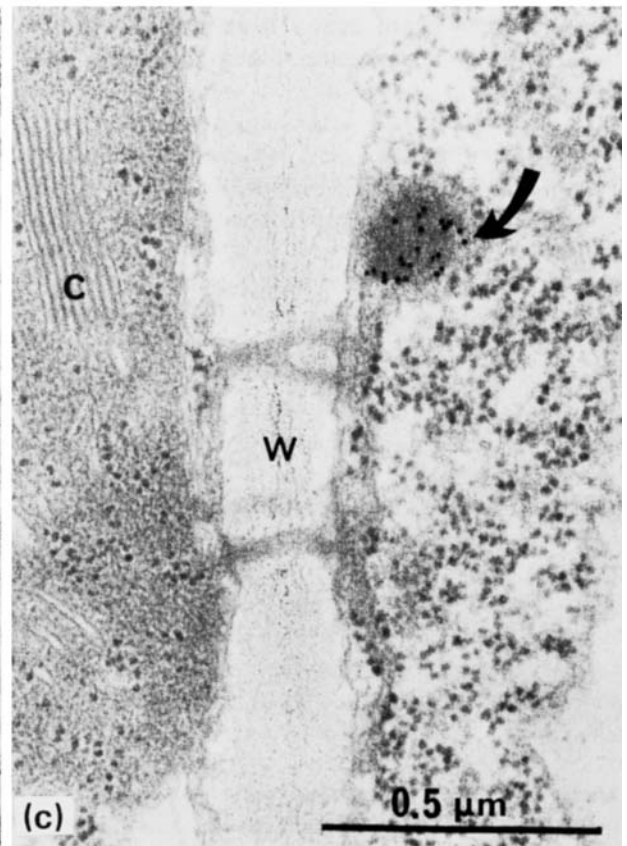
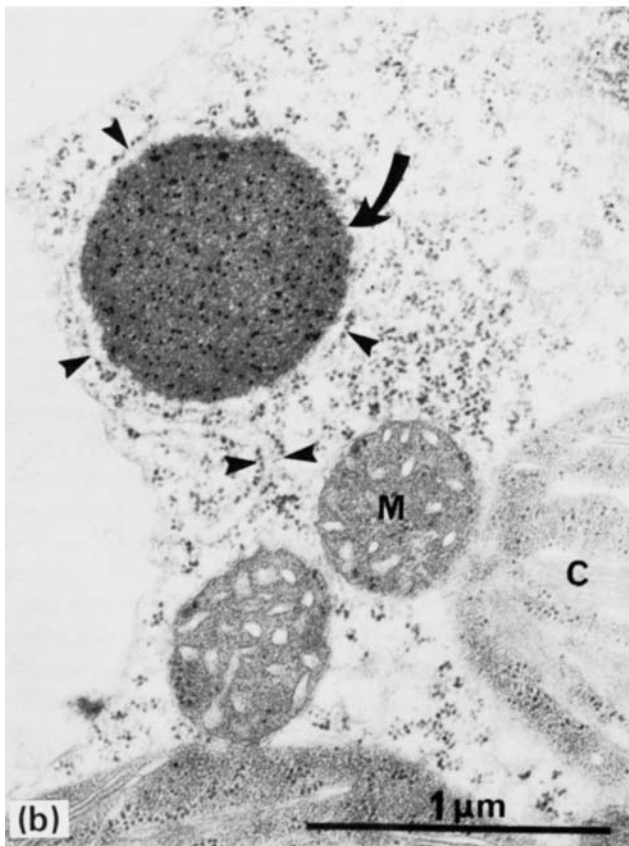
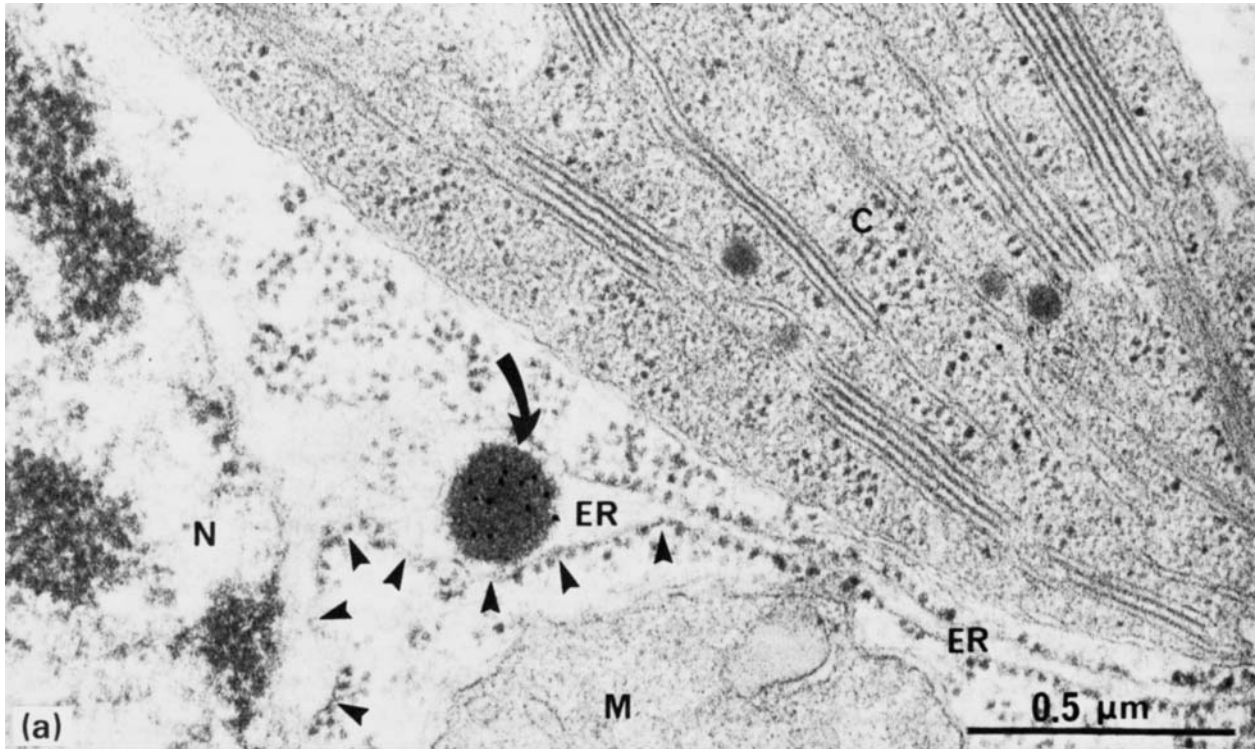
The above results indicate that the addition of the carboxy-terminal KDEL sequence to vicilin results in its retention in

Figure 4. Detection of vicilin-KDEL by immunogold labelling and electron microscopy.

(a) A section of immature lucerne leaf showing immunogold labelling of vicilin-KDEL (arrow) within the ER lumen. The ER membrane is continuous with the nuclear envelope (arrowheads).

(b) A section of a fully expanded tobacco leaf showing a body containing vicilin-KDEL detected by immunogold labelling (arrow). These bodies are larger than in lucerne making identification of the surrounding ER membrane more difficult. The presence of the ER membrane is indicated by the chain of ribosomes (arrowheads) surrounding the protein deposit.

(c) A fully expanded tobacco leaf showing gold-labelled small ER inclusions (arrow) containing vicilin-KDEL. These ER cisternae are continuous with plasmodesmata.



the ER where it accumulates to a high level. Unmodified vicilin, with its vacuolar targeting signal, would be expected to be transported to the leaf vacuole. Its low level of accumulation implies that it is then exposed to the action of proteolytic enzymes in the vacuole. To test the assumption that the marked difference in accumulation of vicilin and vicilin-KDEL is due to differential levels of degradation, we have carried out pulse-chase experiments to determine the half-life of vicilin and vicilin-KDEL in transgenic lucerne leaves.

The tops of lucerne plants (leaf bud with one or two additional leaves) were fed ¹⁴C-amino acids for 4 h and the radioactive pulse was chased with non-radioactive amino acids for 7, 20, 48 and 140 h. Plants transformed with either unmodified vicilin (pTJ14) or vicilin-KDEL (pCW69) were used. The leaf proteins were extracted and vicilin was isolated by immunoadsorption with antibodies coupled to Sepharose 4B. The polypeptides were separated by SDS-PAGE, and a fluorograph was prepared. The results (Figure 5) showed that in the vicilin plant at the end of the chase period, label was incorporated into the expected 50 kDa polypeptides and smaller cleavage products. During the chase there was a rapid disappearance of both the full-length vicilin and the smaller polypeptides. In the vicilin-KDEL plants most of the incorporation was into the full length 50 kDa polypeptide, with no evidence of cleavage products. During the chase there

was relatively little change for up to 48 h, with a decrease at the 140 h time point (Figure 5, lane 9). Vicilin levels in the fluorographs were quantified using a scanning densitometer and the half-life was calculated. The results from three independent experiments were averaged and it was found that the t_{1/2} for vicilin was 4 h and for vicilin-KDEL it was over 48 h. Vicilin-KDEL appeared to be completely stable for at least 30 h and breakdown after that time varied in different experiments. These data indicated at least a 12-fold increase in protein stability as a result of adding carboxy-terminal KDEL to vicilin.

Accumulation and translatability of the vicilin and vicilin-KDEL mRNAs

The foregoing results provide strong evidence that the greatly increased level of accumulation of vicilin-KDEL relative to unmodified vicilin is largely the result of its targeting to the ER and consequent enhanced stability. However, rates of protein synthesis and accumulation often reflect the abundance of mRNA and the efficiency with which it is translated. A contributing factor to this enhanced accumulation could therefore be an increase in level and/or translatability of vicilin-KDEL mRNA. Significant changes were introduced into the carboxyl terminus of the coding region in the course of preparing the vicilin-KDEL gene (Figure 1b). Bases coding for two amino acids were

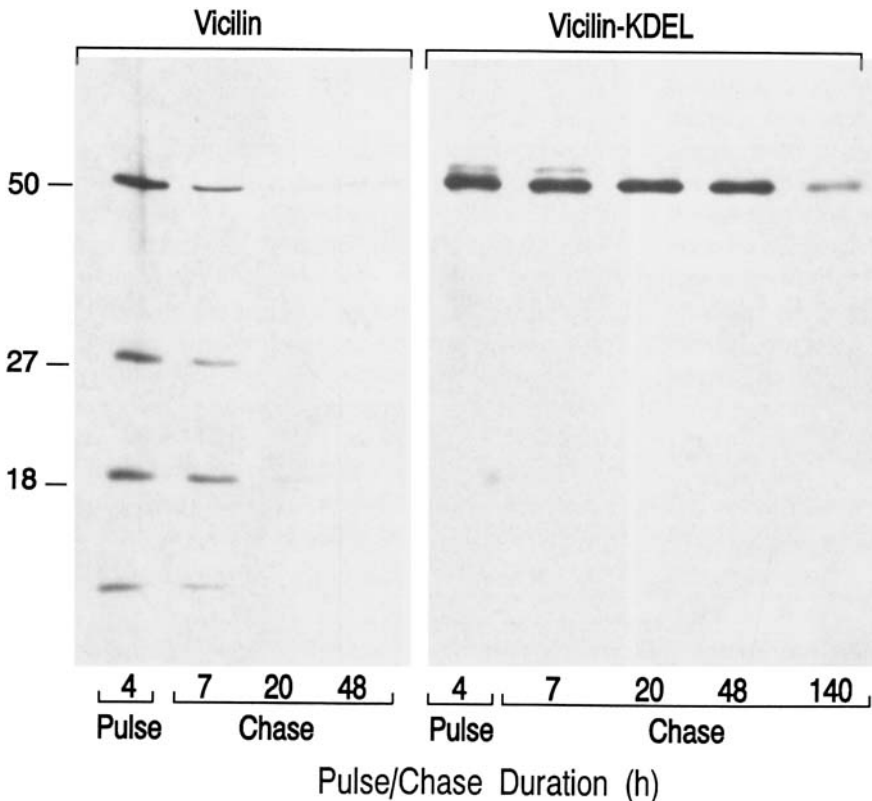


Figure 5. Stability of vicilin and vicilin-KDEL proteins in lucerne plants. Shoots were pulse-labelled for 4 h with ¹⁴C-amino acids, followed by chase periods of 7, 20 or 48 h in the case of vicilin (pTJ14) plants. Vicilin-KDEL (pCW69) plants were also pulse-labelled for 4 h followed by chase periods of 7, 20, 48 or 140 h. After each period, shoots were harvested and total soluble proteins were extracted. Vicilin was immunoselected and fractionated by SDS-PAGE and detected by fluorography. Numbers on the vertical axis represent molecular mass in kDa.

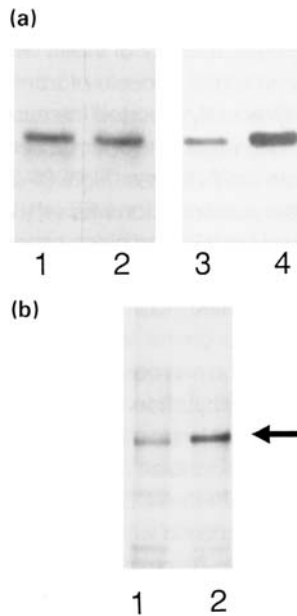


Figure 6. Levels of vicilin mRNA from vicilin-KDEL transgenic plants. (a) Vicilin mRNA levels measured by RNA blot hybridization. Each lane contained 5 μ g of total RNA. Lane 1: vicilin (pCW58) in tobacco; lane 2: vicilin-KDEL (pCW69) in tobacco; lane 3: vicilin (pTJ14) in lucerne; lane 4: vicilin-KDEL (pCW69) in lucerne. (b) Vicilin mRNA levels measured by *in-vitro* translation. Total RNA from the leaves of vicilin and vicilin-KDEL lucerne plants was translated in a rabbit reticulocyte cell-free translation system containing [35 S]methionine. Vicilin and vicilin-KDEL were immunoselected from cell-free reaction mixtures containing equal amounts of trichloroacetic acid-insoluble radioactivity and were fractionated by SDS-PAGE. The radioactive vicilins (arrow) were detected by fluorography; lane 1, vicilin, and lane 2, vicilin-KDEL.

deleted and a total of eight new amino acids were introduced. To check whether these changes affected mRNA level or translatability, we compared steady-state levels and *in-vitro* translatability of vicilin mRNA in transgenic plants expressing either vicilin or vicilin-KDEL.

RNA was extracted from one pair of tobacco plants and one pair of lucerne plants. We chose two tobacco plants transformed with pCW58 (vicilin) and pCW69 (vicilin-KDEL) that differed 100-fold in the accumulation of vicilin protein. Northern blot analysis showed that these two plants contained similar amounts of vicilin mRNA (Figure 6, lanes 1 and 2). When lucerne plants transformed with pTJ14 (vicilin) and pCW69 (vicilin-KDEL) and differing 20-fold in their accumulation of vicilin protein were analysed, the vicilin-KDEL lucerne leaves were found to contain about four times more vicilin mRNA than the vicilin leaves (Figure 6, lanes 3 and 4). This is within the range of variation in mRNA levels found between individual transformants containing the same vicilin gene construct and no greater than the average effect found earlier due to multiple CaMV promoters (2–4-fold; Wandelt *et al.*, 1991). We consider it most likely that this fourfold difference can be explained

by the effects of position of the transgene's insertion on its transcriptional activity.

The translatability of the vicilin mRNA in total RNA from transgenic lucerne leaves was tested by *in-vitro* translation experiments using [35 S]methionine. The total translation products were first immunoselected with vicilin antibodies linked to Sepharose 4B prior to separation by SDS-PAGE (Figure 6). The data clearly show that translation of the same amount of total RNA from each plant resulted in a 4–5-fold greater abundance of vicilin-KDEL relative to vicilin, as might be expected from the fourfold greater abundance of its mRNA (Figure 6). We conclude from this experiment that any alteration in the structure of the mRNA of vicilin caused by the addition of the sequence encoding the modified carboxyl terminus did not change its translational efficiency.

Discussion

The level of accumulation of a foreign protein in a transgenic plant is dependent on many different factors. These include the rate of transcription of the introduced gene, the stability of the resultant mRNA, its translatability by the host's protein synthesizing machinery and the stability of the protein in the particular intra- or extracellular compartment to which it is targeted. In many applications of genetic engineering to plant improvement, it is important to maximize the level of accumulation of the transgenic protein. For example, in order to make a significant impact on the sulphur-containing amino acid content of a ruminant diet, it would be necessary to achieve an expression level of the sunflower seed 2S albumin (which contains 23% cys plus met) of approximately 4% of total leaf protein. This is one or two orders of magnitude higher than the level of all but a few of the transgenic proteins reported in plants so far.

Experience to date indicates that generalizations cannot be made about the effect of the factors listed above on the final level of accumulation of any particular transgenic protein. For example, the CaMV 35S promoter has now been used in a large number of studies and the levels of protein accumulation that have been obtained have varied greatly. Eckes *et al.* (1989) showed that replacing the normal promoter of glutamine synthetase (GS) with the CaMV 35S promoter resulted in an increased level of GS mRNA and in the production of GS amounting to 5% of total soluble leaf protein. We have used the 35S promoter from CaMV to express a vicilin gene with an unmodified coding region (Wandelt *et al.*, 1991) and also found a direct correlation between mRNA level and vicilin accumulation. However, the absolute level of accumulated vicilin remained low (less than 0.01% of total soluble protein) due presumably to the low stability of vicilin in leaves. Similarly, Kay *et al.* (1987) reported a strong enhancing effect (up to

100-fold) of multiple copies of the CaMV 35S promoter on transcription of the neomycin phosphotransferase II gene and octopine-type T-DNA genes 5 and 7 in transgenic tobacco but, using analogous vicilin constructs, we have found no consistent increases in levels of vicilin mRNA or protein in tissues of transgenic plants (Wandelt *et al.*, 1991).

These variable results from experiments aimed at increasing the expression of introduced genes through increased transcription led us to consider the possibility of directing the transgenic protein to specific intracellular locations. Once again, experience to date has been somewhat variable. A number of vacuolar seed proteins have been expressed in transgenic hosts. These proteins, in their homologous host, are normally synthesized on the rough ER and transported via the endomembrane system to be stored in the vacuolar protein bodies in the developing seed. These proteins contain vacuolar targeting signals within their amino acid sequence and these signals are generally recognized in heterologous tissues and species (Chrispeels, 1991). When genes for vacuolar seed proteins are expressed in leaves of transgenic plants it is assumed that they will be targeted to the vacuoles of leaf cells. Hilder *et al.* (1987) found that cowpea trypsin inhibitor, a vacuolar seed protein, accumulated to 1% of the total leaf protein, but other seed vacuolar proteins, such as soybean conglycinin (Lawton *et al.*, 1987) and pea vicilin (Higgins and Spencer, 1991) accumulated at only very low levels (<0.01%).

Equally variable results have been obtained when proteins that are normally secreted are expressed in transgenic plants. When genes for immunoglobulin oligomers were expressed in tobacco, the resultant protein accumulated up to 1.3% of the total leaf protein (Hiatt *et al.*, 1989). In contrast, when potato plants were transformed with the gene for human serum albumin using the CaMV 35S promoter, human serum albumin was secreted and accounted for only 0.02% of the total leaf protein (Sijmons *et al.*, 1990). Clearly, targeting to cells walls does not, *per se*, guarantee a high level of accumulation of a transgenic protein.

The discovery of the ER-retention signal (KDEL) on soluble, resident, luminal proteins of the ER (Munro and Pelham, 1986; Pelham, 1990) opened the option of targeting transgenic proteins to another specific intracellular location. Our results reported in this paper indicate that by adding KDEL to vicilin and thus causing it to be accumulated in the ER, we achieved a 20- to 100-fold increase in its accumulation in the leaves of transgenic lucerne and tobacco and maximum levels of accumulation of 3.7% of total protein. The accumulation of vicilin-KDEL in the ER was confirmed by immunocytochemistry, and measurements of mRNA levels and *in-vitro* translatability indicated that the enhanced accumulation was not due to increases

in either of these parameters. Pulse-chase experiments pointed to a far greater stability of vicilin when located in the ER as compared to the vacuole of transgenic leaves. Ohtani *et al.* (1991) recently reported the case of an α -zein gene from maize expressed in tobacco seeds. Although the level of α -zein mRNA was high (1–2.5% of total mRNA), zein protein accumulation was very low (0.00001–0.003% of total seed protein) and pulse-chase experiments indicated that the zein had a half-life of less than 1 h, even less than that of unmodified vicilin in tobacco leaves (4.5 h, see Figure 5).

The KDEL modification has been used to target another vacuolar seed protein, phytohaemagglutinin (PHA), to the ER of transgenic plants (Herman *et al.*, 1990). In this case there was no apparent increase in the level of the foreign protein. However, the PHA-KDEL construct retained its original 5' upstream sequence which confers seed-specific expression on the gene. Since the seed vacuolar protein bodies are already a very stable environment for storage of such proteins there may have been little scope for enhanced accumulation by re-targeting to the ER.

Herman *et al.* (1990) noted that not all of the PHA-KDEL was retained in the ER and that up to 50% was transported to the storage vacuoles. A similar partial retention in the ER was reported for two mammalian secretory proteins (growth hormone and chorionic gonadotropin) which were modified by the addition of KDEL (Zagouras and Rose, 1989). Although there was a significant reduction in the rate of secretion of these modified proteins, it was not abolished entirely. Similarly, in the present experiments the appearance of a cleavage product of 27 kDa in the older leaves of lucerne plants containing vicilin-KDEL (Figure 3b) could be taken to reflect the passage of some of this protein from the ER to the less stable environment of the leaf vacuole.

There are several possible explanations for partial retention of KDEL-modified proteins in the ER. Recently, Pelham and colleagues (Semenza *et al.*, 1990) reported that in yeast, retention of KDEL proteins involves interaction with an integral membrane protein whose abundance determines the efficiency and capacity of the KDEL retention system. Increased levels of this membrane protein, which is part of the cis-Golgi system (Pelham, 1990), led to increased retention of KDEL proteins. A failure of transgenic cells to retain all the KDEL proteins could be attributed to a saturation of the endogenous KDEL receptor protein. An alternative explanation may be that there are other sequences in the polypeptide chain of the KDEL protein that enhance ER retention. For example, Munro and Pelham (1987) have suggested that a negatively charged region near the carboxyl terminus is needed, in addition to KDEL, for efficient ER retention. Protein disulphide isomerase (Edman *et al.*, 1985) and immunoglobulin heavy chain binding protein (Munro and Pelham, 1986) are resident

ER proteins and they contain glu/asp clusters near their carboxyl termini. There are two known plant proteins with KDEL at their carboxyl termini. They are the auxin-binding protein from maize (Hesse *et al.*, 1989; Inohara *et al.*, 1989) and the sulphhydryl-endopeptidase of *Vigna mungo* (Akasofu *et al.*, 1989). The auxin-binding protein is known to be located in the ER and consistent with this, it contains a cluster of acidic residues in the terminal region. The precise cellular compartment for the endopeptidase is unknown but it contains only three acidic residues in the final 20 carboxy-terminal amino acids, unlike all of the other ER residents which contain at least six acidic residues near the terminus. Vicilin-KDEL also contains six acidic residues in the terminal 20 amino acids which may account for its efficient targeting to the ER. PHA-KDEL, on the other hand, contains only four acidic residues in the same region and this may account for the incomplete retention of the protein in the ER (Herman *et al.*, 1990).

Our results indicate that the final level of protein accumulation also depends on the growth conditions of the plants (small plants grown in sterile boxes in a growth chamber compared with plants grown in the glasshouse) as well as on leaf position. We achieved levels of up to 3.7% in the young leaves of tobacco plants grown in sterile boxes, but when these same plants were grown in the glasshouse the level of accumulation dropped considerably. The reasons for these changes in relative abundance of vicilin with change in plant growth conditions and with position on the plant are not understood. One possibility is that the foreign proteins are less stable under glasshouse conditions and in older leaves; another is that in each of these situations the synthesis of the foreign protein does not keep pace with the synthesis of the leaf proteins of the host and vicilin is simply diluted out. Besides culture conditions and leaf position, the calculated abundance of the protein is also influenced by its extractability, which may change with time.

Another way of enhancing foreign gene expression in plants has been described by Perlak *et al.* (1990). They found that by changing codon usage in a truncated gene for *Bacillus thuringiensis* toxin to a codon usage that was more typical of highly expressed genes in the leaf, they were able to raise the level of the toxin by 100-fold, to about 0.1% of total soluble protein. Preliminary inspection of codon usage in major seed and leaf proteins indicates that there may be scope for using this strategy to obtain enhanced translation of mRNA for seed-specific proteins such as vicilin in leaves.

Experimental procedures

Vicilin and vicilin-KDEL constructs

A vicilin gene (pEN2) described earlier (Higgins *et al.*, 1988) was modified for expression throughout the plant by removing 2.5 kb

of 5' flanking DNA sequence from +1 to -2500 and replacing it with one, two or three copies of the CaMV 35S promoter sequence. The viral promoter was duplicated and triplicated essentially as described earlier (Kay *et al.*, 1987). This involved cloning nucleotides 7017-7435, corresponding to -418 to +1 of the CaMV 35S promoter (Guilley *et al.*, 1982), into the *Bam*HI site of pUC8 to generate pLW 1 (Walker and Llewellyn, unpublished data). The orientation was such that the CaMV 35S cap site (+1) was just upstream of the *Sma*I site. The promoter was duplicated by excising the CaMV sequence corresponding to -418 to -90 of pLW1 with *Hinc*II and *Eco*RV and inserting this into the unique *Eco*RV site of pLW1 to generate pCW2. The triplicate CaMV 35S promoter was made by inserting the same *Hinc*II/*Eco*RV fragment into the unique *Eco*RV site of pCW2 to yield pCW3.

The vicilin gene was modified so that the 5' upstream sequences could be removed precisely from the cap site to -2500. This was achieved by introducing a *Cla*I restriction site at +2 using *in-vitro* mutagenesis (Amersham kit) to change positions +4 and +6 to G and T, respectively. The resultant plasmid (pCW43) was digested with *Cla*I, treated with DNA polymerase I (Klenow fragment) to blunt-end the site and was then digested with *Eco*RI to release the vicilin gene from +3 to +2691. This fragment was cloned down-stream of the single and triple CaMV 35S promoters by digesting pCW3 with *Ava*I (+10 relative to CaMV cap site), blunt-ending as described above and digesting with *Eco*RI. The resulting chimeric CaMV 35S/vicilin gene containing three copies of the 35S promoter was called pCW53. In order to introduce this gene into tobacco and lucerne, it was transferred from the pUC8 recombinant by digestion with *Nar*I and *Eco*RI and inserted into pGA492 (An, 1986) cut with *Cla*I and *Eco*RI to yield pCW58 (Figure 1a). In addition to this derivative of the vicilin gene, a second, described earlier as pTJ14 (Higgins and Spencer, 1991) was also used in these studies. This gene contains one copy of the CaMV 35S promoter from -90 to -418 placed upstream of the vicilin 'TATA' box at -96 (Figure 1a).

To introduce the amino acids, ser-glu-lys-asp-glu-leu (SEKDEL) at the carboxyl terminus of vicilin we made a unique *Xho*I site seven nucleotides upstream of the vicilin stop codon in pCW53. The following two oligonucleotides, 5' TCGAGGATCTGAGAAAGATGAGCTATGAC 3' and 5' TCGAGTCATAGCTCATCTTTCTCAGATCC 3' were synthesized (Applied Biosystems), annealed and cloned into the *Xho*I mutant of pCW53. The correct orientation was determined by double-stranded DNA sequencing and was called pCW67. The *Xho*I site changed the penultimate amino acid from S to R while the oligonucleotide insertion replaced the final SV with RGSEKDEL. Thus, in the vicilin-KDEL mRNA there were 31 bases replacing the first two bases in the codon for the penultimate amino acid (Figure 1b). The vicilin-KDEL gene was transferred to the binary vector, pGA492 as described above to yield the plasmid pCW69 (Figure 1a).

Plant transformation and regeneration

All recombinant binary plasmids were transferred to *Agrobacterium tumefaciens*, strain LBA4404, by triparental mating as described earlier (Ditta *et al.*, 1980). Tobacco (*Nicotiana tabacum* cv. Wisconsin 38) was transformed using the leaf disc method (Higgins *et al.*, 1988; Horsch *et al.*, 1985). Lucerne (*Medicago sativa* cv. Rangelander) was transformed using leaf and stem segments as described by Schroeder *et al.* (1991). Briefly, the lucerne genotype 24/1 used in this study was selected for its embryogenic potential *in vitro* from the cultivar Rangelander. Leaves of actively growing glasshouse plants were sterilized (5 sec in 70% ethanol followed by 12 min in 1% sodium hypochlorite) and washed three times

with sterile distilled water. Leaf explants (4 mm²) were submerged in the bacterial suspension for 10 min, then lightly blotted and placed on B₅h medium (Brown and Attanosov, 1985) with a 12 h photoperiod of fluorescent light at 26°C for 3 days of co-cultivation. Leaf segments were then washed three times with sterile water and excess water was shaken off before the segments were transferred to selective medium. Callus was induced on B₅h medium containing 50 mg l⁻¹ kanamycin and 100 mg l⁻¹ cefotaxime. Callus was transferred to fresh B₅h medium after 4 weeks. Kanamycin-resistant embryos emerged within the next 3 weeks. The embryos were transferred to SHe medium (Brown and Attanosov, 1985) without selection to allow embryo maturation and plantlet formation.

Developed roots on established plantlets were cut back to 5 mm in length and plantlets were cultured on SHe medium containing 100 mg l⁻¹ kanamycin. At the same time, leaves from individual plantlets were placed on B₅h containing 50 mg l⁻¹ kanamycin to induce callus formation. Putative transgenic plants were established by transferring only well-rooted plantlets to peat cups containing a sand:peat:perlite (1:1:1) mixture in a glasshouse misting bed for 3 weeks.

Immunoblotting

Protein isolation from plant tissues, their estimation and immunoblotting was carried out following the procedures outlined in Higgins *et al.* (1988).

Densitometry

Negatives and fluorographs were scanned using a Joyce-Loebl double beam microdensitometer (MK111 CS). The relative contributions of individual bands were monitored by excising and weighing the peaks.

Pulse and chase labelling

Shoot tips from transgenic lucerne plants, containing one bud and two leaves were harvested and divided into lots of three shoot tips. Each lot of shoot tips was fed with 50 µCi of ¹⁴C-labelled amino acid mixture in 30 µl solution kept in the wells of a microtitre plate. The shoots took up the label within 30 min and this was followed by feeding with distilled water for 4 h (pulse period). One lot was harvested and the rest fed with MS salts, iron and vitamins (Murashige and Skoog, 1962) supplemented with 117 mM asparagine, 30 mM glutamine and 2% sucrose. The other lots were harvested at various time intervals (7, 20, 48 and 140 h). Harvested lots were extracted and the specific activity of the protein was determined. Equal quantities of radioactivity in proteins from each treatment were immunoprecipitated with vicilin antibodies, separated by SDS-PAGE (Higgins *et al.*, 1988) and a fluorograph prepared.

RNA isolation and Northern blotting

Total RNA from the transgenic tobacco and lucerne leaves was isolated following the procedure outlined by Verwoerd *et al.* (1989). RNA concentration was measured spectrophotometrically assuming that one A₂₆₀ unit represents 40 µg RNA ml⁻¹ using a 1-cm pathlength. Total RNA (5 µg) was analysed using Northern blot analysis after fractionation of RNA on a 1.4% agarose gel containing 1.9% deionized formaldehyde (Higgins and Spencer,

1991). RNA was blotted for 18 h onto a nylon membrane (Hybond N, Amersham (Aust) Ltd). Hybridization was carried out using a 740-bp fragment (*Xba*I-*Hpa*I) of the vicilin gene. The fragment was labelled by oligo-labelling using a kit from Bresatec (Adelaide, South Australia). The hybridization was performed at 42°C using Khandjian hybridization buffer (Khandjian, 1987). Blots were washed with 2× SSC at room temperature for 10 min, then with 2× SSC + 0.1% SDS at 68°C until the background radioactivity dropped to 100–200 c.p.m. Blots were exposed to X-ray film at –80°C using a Hi-Plus (Du Pont) intensifying screen.

In-vitro translation of vicilin mRNAs

The *in-vitro* translation of vicilin mRNA (from transgenic lucerne plants) was conducted in the presence of rabbit reticulocyte lysate (Amersham) and [³⁵S]methionine. Thirty micrograms of total RNA (preheated at 67°C for 10 min) were translated with 80 µl lysate and 500 µCi [³⁵S]methionine in a 100 µl reaction mixture at 30°C for 1 h. A protein aliquot containing 3 × 10⁷ c.p.m. was immunoprecipitated with preimmune immunoglobulin conjugated to Sepharose beads, to reduce the subsequent non-specific binding to Sepharose. The same protein sample was then immunoprecipitated with vicilin antibody conjugated to Sepharose beads and the bound polypeptides separated by gradient SDS-PAGE and a fluorograph prepared. The total *in-vitro* translation products were also fractionated by SDS-PAGE followed by fluorography to ascertain that the two mRNA populations were intact (data not shown).

Immunocytochemistry

Lucerne leaflets (from <1 cm to fully expanded) and young to fully expanded tobacco leaves (from 2 to 23 cm long) of glasshouse-grown plants were collected and prepared for immunocytochemical analysis as described (Craig and Miller, 1984). Small tissue pieces (1–2 mm²) were fixed in 2% *p*-formaldehyde, 1% glutaraldehyde in 25 mM sodium phosphate, pH 7.1, postfixed in 1% OsO₄, dehydrated in a graded ethanol series and embedded in LR White resin at 55°C. Sections were collected on parlodion carbon-coated nickel grids and immunolabelled as described previously (Craig and Goodchild, 1984) with affinity-purified rabbit antibodies against pea vicilin followed by protein A coupled to 12-nm colloidal gold particles. After immunolabelling, the sections were contrasted with lead and uranyl salts and viewed in a JEOL 100 CX transmission electron microscope. When the antivicalin was omitted, or replaced by anti-leghaemoglobin, negligible gold was bound, indicating the specificity of the labelling.

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References

- Akasofu, H., Yamauchi, D., Mitsuhashi, W. and Minamikawa, T. (1989) Nucleotide sequence of cDNA for sulfhydryl-endo-peptidase (SH-EP) from cotyledons of germinating *Vigna mungo* seeds. *Nucl. Acids Res.* **17**, 6733.

- Ampe, C., Van Damme, J., deCastro, L.A.B., Sampaio, M.J.A.M., Van Montague, M. and Vanderkerchove, J.** (1986) The amino acid sequence of the 2S sulphur-rich proteins from seeds of Brazil nut (*Bertholletia excelsa* H.B.K.). *Eur. J. Biochem.* **159**, 597–604.
- An, G.** (1986) Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cells. *Plant Physiol.* **81**, 86–91.
- Brown, D.C.W. and Attanosov, A.** (1985) Role of genetic background in somatic embryogenesis in *Medicago*. *Plant Cell Tissue Organ Cult.* **4**, 111–122.
- Chrispeels, M.J.** (1991) Sorting of proteins in the secretory system. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 21–53.
- Craig, S. and Goodchild, D.J.** (1984) Periodate-acid treatment of sections permits on-grid immunolocalisation of pea seed vicilin in ER and Golgi. *Protoplasma*, **122**, 35–44.
- Craig, S. and Miller, C.** (1984) LR White resin and improved on-grid immunogold detection of vicilin, a pea seed storage protein. *Cell Biol. Int. Rep.* **8**, 879–886.
- Ditta, G., Stanfield, S., Corbin, D. and Helinski, D.R.** (1980) Broad host range DNA cloning system for gram-negative bacteria: construction of a genebank of *Rhizobium meliloti*. *Proc. Natl Acad. Sci. USA*, **77**, 7347–7351.
- Dorel, C., Voelker, T.A., Herman, E.M. and Chrispeels, M.J.** (1989) Transport of proteins to the plant vacuole is not by bulk flow through the secretory system and requires positive sorting information. *J. Cell Biol.* **108**, 327–337.
- Eckes, P., Schmitt, P., Daub, W. and Wengenmayer, F.** (1989) Overproduction of alfalfa glutamine synthetase in transgenic tobacco plants. *Mol. Gen. Genet.* **217**, 263–268.
- Edman, J.C., Ellis, L., Blacher, R.W., Roth, C.A. and Rutter, W.J.** (1985) Sequence of protein disulphide isomerase and implications of its relationship to thioredoxin. *Nature*, **317**, 267–270.
- Gasser, C.S. and Fraley, R.T.** (1989) Genetically engineering plants for crop improvement. *Science*, **244**, 1293–1299.
- Goodchild, D.J. and Craig, S.** (1982) Structural aspects of protein accumulation in developing pea cotyledons. IV Effects of preparative procedures on ultrastructural integrity. *Aust. J. Plant Physiol.* **9**, 689–704.
- Guilley, H., Dudley, R.K., Jonard, G., Balazs, E. and Richards, K.E.** (1982) Transcription of cauliflower mosaic virus DNA: detection of promoter sequences and characterization of transcripts. *Cell*, **30**, 763–773.
- Herman, E.M., Taqul, B.W., Hoffman, L.M., Kjemtrup, S.E. and Chrispeels, M.J.** (1990) Retention of phytohemagglutinin with carboxyterminal tetrapeptide KDEL in the nuclear envelope and the endoplasmic reticulum. *Planta*, **182**, 305–312.
- Hesse, T., Feldwisch, J., Bashusemann, D., Bauw, G., Puype, M., Vandekerckhove, J., Löbler, M., Klämbt, D., Schell, J. and Palme, K.** (1989) Molecular cloning and structural analysis of a gene from *Zea mays* (L.) coding for a putative receptor for the plant hormone auxin. *EMBO J.* **8**, 2453–2461.
- Hiatt, A.R., Cafferkey, R. and Bowdish, K.** (1989) Production of antibodies in transgenic plants. *Nature*, **342**, 76–78.
- Higgins, T.J.V. and Spencer, D.** (1991) The expression of a chimeric cauliflower mosaic virus (CaMV 35S)-pea vicilin gene in tobacco. *Plant Sci.* **74**, 89–98.
- Higgins, T.J.V., Newbigin, E.J., Spencer, D., Llewellyn, D.J. and Craig, S.** (1988) The sequence of a pea vicilin gene and its expression in transgenic tobacco plants. *Plant Mol. Biol.* **11**, 683–695.
- Hilder, V.A., Gatehouse, A.M.R., Sheerman, S.E., Barker, R.F. and Boulter, D.** (1987) A novel mechanism of insect resistance engineered into tobacco. *Nature*, **300**, 160–163.
- Horsch, R.B., Fry, J.E., Hoffman, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T.** (1985) A simple and general method for transferring genes into plants. *Science*, **227**, 1229–1231.
- Hunt, D.H. and Chrispeels, M.J.** (1991) The signal peptide of a vacuolar protein is necessary and sufficient for the efficient secretion of a cytosolic protein. *Plant Physiol.* **96**, 18–25.
- Inohara, N., Shimomura, S., Fukui, T. and Futai, M.** (1989) Auxin-binding protein located in the endoplasmic reticulum of maize shoots: molecular cloning and complete primary structure. *Proc. Natl Acad. Sci. USA*, **86**, 3564–3568.
- Kay, R., Chan, A., Daley, M. and McPherson, J.** (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science*, **236**, 1299–1302.
- Khandjian, F.W.** (1987) Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. *Bio/Technol.* **5**, 165–167.
- Kortt, A.A., Caldwell, J.B., Lilley, G.G. and Higgins, T.J.V.** (1991) Amino acid and cDNA sequences of a methionine-rich 2S protein from sunflower seed (*Helianthus annuus* L.). *Eur. J. Biochem.* **195**, 329–334.
- Lawton, M.A., Tierney, M.A., Nakamura, I., Anderson, E., Komeda, Y., Dube, P., Hoffman, N., Fraley, R.T. and Beachy, R.N.** (1987) Expression of a soybean β -conglycinin gene under the control of the cauliflower mosaic virus 35S and 19S promoters in transformed petunia tissues. *Plant Mol. Biol.* **9**, 315–324.
- Moreno, J. and Chrispeels, M.J.** (1989) A lectin gene encodes the α -amylase inhibitor of the common bean. *Proc. Natl Acad. Sci. USA*, **86**, 7885–7889.
- Munro, S. and Pelham, H.R.B.** (1986) An Hsp 70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell*, **46**, 291–300.
- Munro, S. and Pelham, H.R.B.** (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell*, **48**, 899–907.
- Murashige, T. and Skoog, F.** (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–477.
- Ohtani, T., Galili, G., Wallace, J.C., Thompson, G.A. and Larkins, B.** (1991) Normal and lysine-containing zeins are unstable in transgenic tobacco seeds. *Plant Molec. Biol.* **16**, 117–128.
- Pelham, H.R.B.** (1990) The retention signal for soluble proteins of the endoplasmic reticulum. *Trends Biochem. Sci.* **15**, 483–486.
- Perlak, F.J., Deaton, R.W., Armstrong, T.A., Fuchs, R.L., Sims, S.R., Greenplate, J.T. and Fischehoff, D.A.** (1990) Insect resistant cotton plants. *Bio/Technol.* **8**, 939–943.
- Schroeder, H.E., Khan, M.R.I., Knibb, W.R., Spencer, D. and Higgins, T.J.V.** (1991) The expression of a chicken ovalbumin gene in three lucerne cultivars. *Aust. J. Plant Physiol.* **18**, 495–505.
- Semenza, J.A., Hardwick, K.G., Dean, N. and Pelham, H.R.B.** (1990) ERD2, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell*, **61**, 1349–1357.
- Sijmons, P.C., Dekker, B.M.M., Schrammeijer, B., Verwoerd, T.C., Van Den Elzen, P.J.M. and Hoekema, A.** (1990) Production of correctly processed human serum albumin in transgenic plants. *Bio/Technol.* **8**, 217–222.
- Van der Valk, H.C.P.M. and Van Loon, L.C.** (1988) Subcellular localization of proteases in developing leaves of oats (*Avena sativa* L.). *Plant Physiol.* **87**, 536–541.
- Verwoerd, T.C.V., Dekker, B.M.M. and Hoekema, A.** (1989) A

small scale procedure for the rapid isolation of plant RNAs. *Nucl. Acids Res.* **17**, 2362.

Wandelt, C., Knibb, W., Schroeder, H.E., Khan, M.R.I., Spencer, D., Craig, S. and Higgins, T.J.V. (1991) The expression of an ovalbumin and a seed protein gene in the leaves of transgenic

plants. In *Plant Molecular Biology 1990* (Hermann R.G. and Larkins, B.A., eds). New York: Plenum Press, in press.

Zagouras, P. and Rose, J.K. (1989) Carboxy-terminal SEKDEL sequences retard but do not retain two secretory proteins in the endoplasmic reticulum. *J. Cell Biol.* **109**, 2633–2640.