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Fruit developmental regulation of the kiwifruit actinidin promoter is conserved in transgenic petunia plants

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Abstract

We have examined the expression of actinidin, a cysteine protease found in kiwifruit, over the course of fruit development. Protease activity was first seen in fruit that had reached about half their final weight, and rose to high levels at harvest. The 5′-flanking region (nucleotides −1301 to +58) of a kiwifruit actinidin gene was fused to the β-glucuronidase (GUS)-coding region, and the chimaeric gene was introduced into transgenic petunia plants. Induction of the GUS gene was observed during the later stages of seed pod development, closely resembling the pattern of actinidin induction in fruit tissues of kiwifruit. Some GUS expression was also detected in the vascular system of the receptacle, leaves, stems and roots. A shorter promoter fragment consisting of nucleotides −115 to +58 conferred similar spatial and temporal regulation in some of the transgenic plants.

Introduction

Actinidin from kiwifruit belongs to the papain family of cysteine proteinases, which in plants includes papain from papaya, bromelain from pineapple, C14 protease from tomato, and aleurain from barley [2, 19]. Plant cysteine proteinases that occur in fruits are often present in high abundance, but their physiological roles remain to be established.

In ripe kiwifruit, actinidin is the most abundant soluble fruit protein [17]. The actinidin mRNA is rare in leaves and roots and increases in abundance during later stages of fruit development, suggesting that regulation of transcription is important in controlling protease levels [18, 17]. These features make actinidin an attractive model for studying gene expression in late fruit development.

Other genes known to be expressed late in fruit development commonly represent enzymes with a role in fruit ripening, enzymes of the ethylene

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number L07552.

biosynthetic pathway [21], and enzymes involved in cell wall degradation [9]. In some cases expression of these ripening-related genes is known to be under the direct control of ethylene while in others ethylene appears not to be involved [15]. The expression pattern of actinidin differs from those of ripening enzymes, because its mRNA is already abundant in fruit at time of harvest (mature green) [18]. Actinidin expression also differs from other genes expressed at earlier times in fruit development. For example, Houck and coworkers isolated a tomato fruit-specific clone which is expressed at low levels throughout fruit development and which increases to high levels during ripening [16, 22]. Another gene, for hydroxymethyl-glutaryl-CoA reductase, is expressed in tomato fruit only during the first half of development [14]. Recent progress has been made in the delineation of the promoter sequences involved in fruit-specific transcriptional regulation of two fruit-specific genes [22, 23, 6], but precise details of their regulatory mechanism(s) remain to be elucidated.

In this report, we show that actinidin protease activity first appears when fruit are about half of their final weight, and subsequently increases as fruit develop. In kiwifruit actinidin is encoded by a fairly large gene family [12]. An actinidin gene has been cloned and sequenced in our laboratory [20]. Here we construct fusions between promoter fragments isolated from this gene and the GUS reporter gene [11], and introduce the chimaeric gene into petunia plants. This heterologous system was chosen primarily because of the long life cycle of kiwifruit plants (3–4 years to fruiting). We demonstrate that a 1.36 kb actinidin promoter is able to direct late 'fruit' induction of the GUS reporter gene in transgenic petunia plants.

Methods

Measurement of cysteine protease activity in kiwifruit tissues

Actinidin activity was measured spectrophotometrically using the assay of Boland and Hardman [3]. Ten ml of extract was assayed in 3 ml of cysteine buffer (0.1% cysteine, pH 6.0), using the artificial substrate N-a-CBZ-l-lysine *p*-nitrophenyl ester (0.1 M). Absorbance measurements were taken at 25 °C to monitor the reaction.

Kiwifruit samples were collected from mature plants grown on DSIR research orchards at Kumeu. In New Zealand, kiwifruit vines flower in late November or early December. Fruit development occurs over the next five months and they are harvested when fruit have reached a soluble solids content of >6.2 Brix (early May). For the fruit developmental series, Hayward or Bruno fruit were hand pollinated with pollen from fresh flowers of Matua-type males. Ten hand-pollinated fruit were sampled every two weeks thereafter on a random basis. For Hayward sampling was continued through September, several months after the fruit are normally picked. For each time point, ten fruit were weighed after removal of the stalk and calyx, and either stored frozen or assayed immediately. For enzyme activity, two of the fruit were peeled, reweighed to obtain fresh weight measurements, blended in ca. 0.5 volume of cysteine buffer, and assayed as above.

Construction of binary vectors

Two binary vectors, pKIWI503 and pKIWI603, were constructed for *Agrobacterium* transformation. Both were derived from pKIWI103 (B. J. Janssen, unpublished). pKIWI103 is a binary vector containing a selectable kanamycin gene between the T-DNA border sequences. It also contains a GUS gene driven by the mannopine synthase (mas) promoter. The GUS-coding region was derived from pRAJ275 [11], which contains a Kozak's consensus sequence around the ATG initiation codon. pKIWI103 was constructed by inserting the *Xho* I fragment of pKIWI100 [10], containing the mas-GUS gene construct, into the *Sal* I site of pEND4K [13]. The structure of the T-DNA region of pKIWI103 is shown in Fig. 3.

The two actinidin fusion genes were con-

structed by replacing the mas promoter in pKIWI103 with 5′-flanking regions from the actinidin gene. A fragment containing 173 bp of the actinidin promoter region was first obtained by a polymerase chain reaction (PCR) from a subclone of IKIWI44 [20]. The oligonucleotide primer introduced a *Sal* I site immediately after nucleotide + 58 of the actinidin promoter sequence, and the amplified sequence extended back to the *Pst* I site (nucleotides −115 to −110 in the promoter sequence, Fig. 2). This *Sal* I-*Pst* I fragment from the PCR product was cloned into pKIWI100 to form pKIWI201, which served to provide a *Bam* HI site adjacent to the *Pst* I site. To form pKIWI603, the 173 bp of promoter was excised from pKIWI201 using *Bam-Sal* and substituted for the mas promoter in pKIWI103. To form pKIWI503, the 1.2 kb *Pst* I fragment from the actinidin promoter in IKIWI44 was inserted in the correct orientation in the *Pst* I site of pKIWI201, and then the 1.36 kb *Bam-Sal* fragment again used to replace the mas promoter of pKIWI103. The promoterless construct, pKIWI702, was constructed by deleting the mas promoter (a *Bam* HI fragment) from pKIWI103. Some of the sites relevant to the constructions are indicated in Fig. 3.

The constructs were verified by restriction mapping, PCR, and by DNA sequencing across their *Bam* HI and *Sal* I junctions. For nucleotide sequencing of the actinidin promoter, the *Bam-Sal* fragment from pKIWI503 was subcloned into a plasmid vector, and the subclones used as templates for dideoxy sequencing. The sequences were determined on an ABI model 373A sequencer.

Transgenic plants and GUS assays

Transgenic 'Mitchell' petunia plants were generated as described by Deroles and Gardner [8]. Genomic DNA was prepared from kanamycin-resistant plants as described by Dellaporta *et al.* [7] and screened for the presence of the GUS gene by PCR using GUS gene-specific primers [1]. Kanamycin-resistant plants that contained the GUS gene were grown on in a controlled-environment chamber [see ref 1] for further analysis.

Histochemical GUS staining of whole organs or manual sections of transgenic plants, as well as quantitative fluorimetric GUS assays of cell extracts, were carried out as described by Jefferson [11]. Manual sections of plant samples were stained for GUS activity at 37 °C overnight, and the sample was cleared by treating with ethanol at 65 °C. For fluorimetric assays, the GUS activity was normalized against total soluble protein, measured by the dye binding method [4].

Results

Actinidin protease activity during kiwifruit development

Cysteine protease activity was measured in extracts from various parts of kiwifruit vines, including fruit, leaves and stems. Only fruit showed levels of activity significantly above background (D. Burns, unpublished data). The developmental pattern of actinidin expression was measured in kiwifruit harvested throughout the course of a growing season. Figure 1 shows the results for two cultivars: Hayward, the industry standard, and Bruno. For both varieties, actinidin activity was first detected when fruit reached about half their normal size. The level of activity steadily increased as the fruit developed, reaching a plateau when fruit reached harvest stage (around 150 days after pollination for Bruno, 160 for Hayward).

DNA sequence of the actinidin promoter

Actinidin is encoded by a large gene family in kiwifruit [12]. The DNA sequence of the coding region of an actinidin gene (IKIWI44), together with 173 bp of 5′-flanking region, has been previously published [20]. To study the transcriptional regulation of this gene, we have completed sequence analysis of the 5′-flanking region up to the *Pst* I site located 1359 bp upstream from the

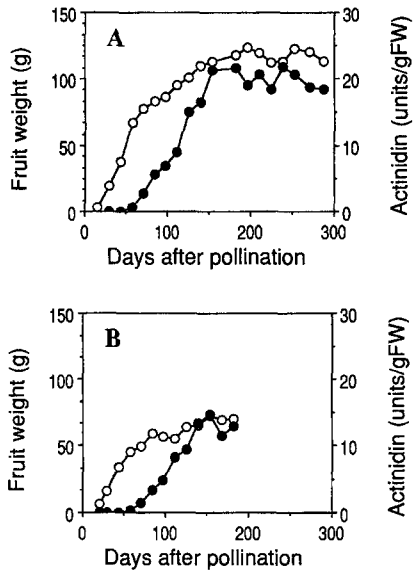


Fig. 1. Actinidin protease activity during fruit development. A. Data for cv. Hayward; B. Data for cv. Bruno. Open circles stand for fruit weight, filled circles for actinidin activity. Actinidin activity was measured as described in Materials and methods. Values are the average of measurements from two fruit, except on five occasions when two additional fruit were measured. Fruit weight is the average of ten fruit for each time point.

initiation codon ATG. The sequence is shown in Fig. 2. The major transcription initiation site of the gene, designated + 1 in Fig. 2, was determined by primer extension analysis using RNA prepared from fruit tissues of kiwifruit (data not shown). Although there are several sequence motifs in the 5′-flanking region matching the consensus TATA box, only the most proximal motif appears to be utilised, as it is located between −31 and −24 relative to the start site.

Tissue specificity of the 1.36 kb actinidin promoter in transgenic petunia plants

To determine the role of the actinidin promoter in gene regulation, we constructed a reporter plasmid (pKIWI503) by joining the actinidin promoter region from (−1301 to + 58 onto the ATG codon of the GUS coding region in a binary vector (Fig. 3, see Materials and methods for details of construction). A shorter promoter construct, pKIWI603, contains upstream sequences from −115 to + 58, while a control plasmid pKIWI702 contains only the linker region in front of the GUS gene.

Agrobacterium-mediated DNA transfer was used to generate transgenic petunia plants containing each construct. The mannopine synthetase (mas) promoter fused to the GUS-coding region (pKIWI103, see Fig. 3) served as a comparison. All primary transformants were analysed by PCR (and in a few cases Southern hybridization as well) to confirm that they contained the gene fusion. The number of positive plants generated for each construct was as follows: pKIWI503, 14 plants; pKIWI603, 11; pKIWI702, 10; pKIWI03, 13. For each construct, all of these positive plants were analysed for their GUS expression pattern by histochemical and quantitative methods.

Histochemical staining of tissue sections showed that the 1.36 kb actinidin promoter effected a consistent expression pattern in transgenic petunia plants. GUS staining was found primarily in the vascular bundles and in seed pods.

In the stems, GUS activity was present in the xylem tissues, predominantly in xylem parenchyma cells (Fig. 4A). In the leaves, GUS activity was present in the major veins of all transgenic plants (see Fig. 4B) and also in the epidermal layer among half of the plants (data not shown). In the root system, GUS activity was present in the central cylinder and pericycle cells, and such expression was either exclusively or preferentially detected at the root branching points (Fig. 4C). GUS induction occurred at sites of root branching before any external protrusion was visible (not shown, but see Fig. 4M). After branching the GUS activity remained associated with the branching point rather than the newly formed root tip, but subsequently declined and eventually vanished.

Some transgenic plants showed limited GUS staining in the pollen, above the level of background staining observed in non-transgenic plants (data not shown). Unpollinated flowers also contained strong GUS activity in flower receptacles.

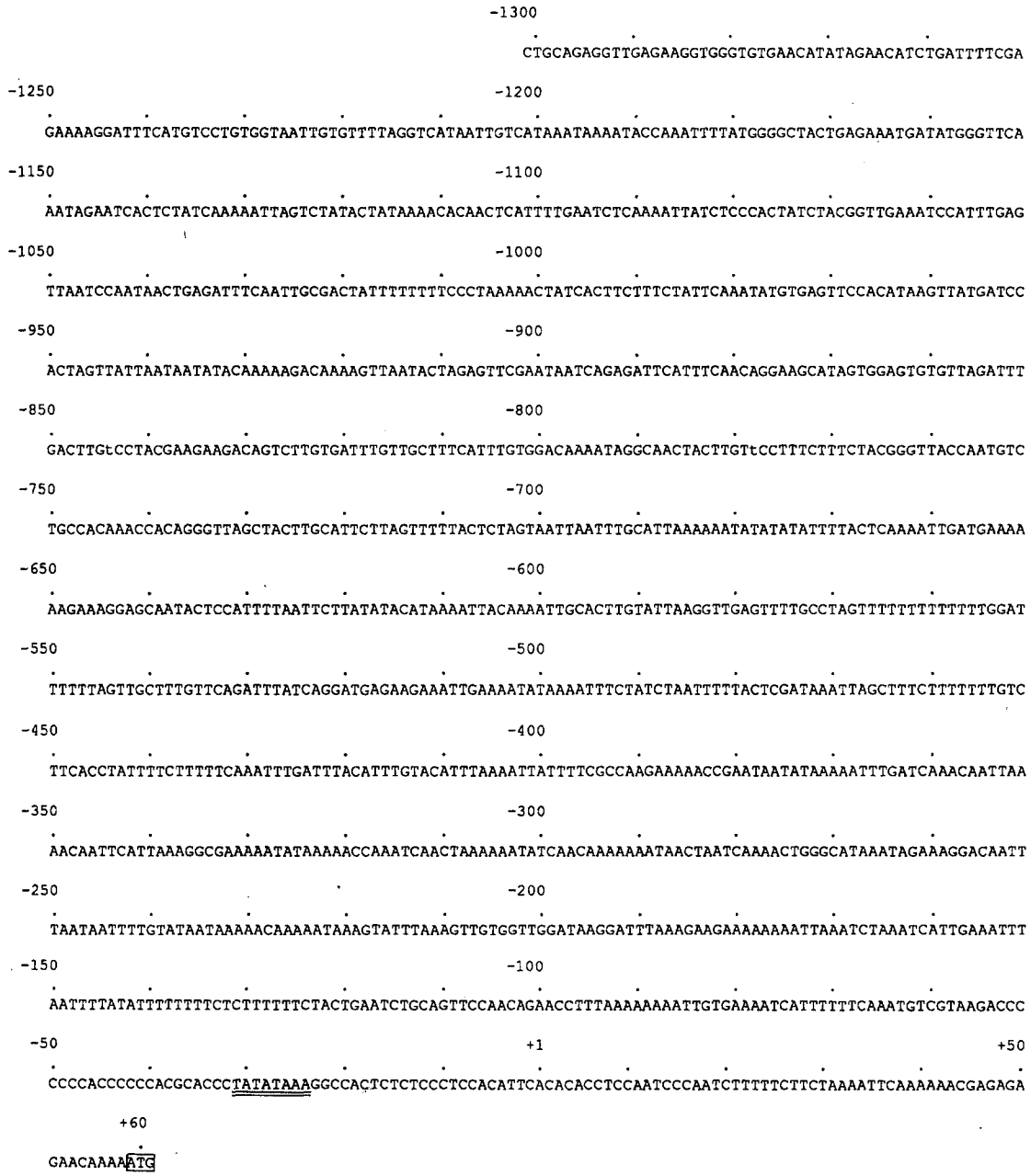


Fig. 2. DNA sequence of kiwifruit actinidin promoter. The sequence downstream from nucleotide −115 has been published previously [20]. The major transcription initiation site is designated + 1. Two minor initiation sites are located at + 3 and + 5. The TATA box is double-underlined. The translation initiation codon ATG is boxed.

A cross section of the receptacle revealed GUS expression in all tissues of the receptacle, including cortex and epidermis, though the predominant site of expression was vascular tissues (Fig. 4D). GUS activity was weak or undetectable in the placenta of unpollinated flower ovaries, petals and sepals, except in the tissues adjacent to the receptacle. When the seed pods began to change colour, they manifested consistent GUS activity in cells throughout the pla-

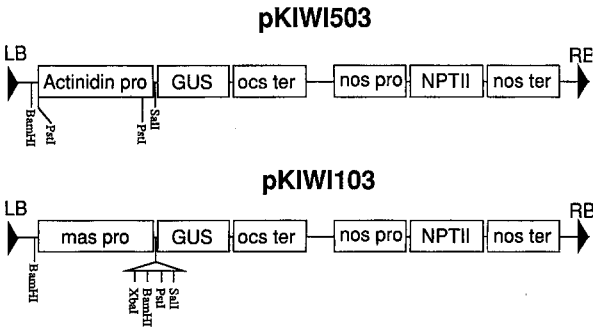


Fig. 3. Structure of the actinidin-GUS binary vector. The T-DNA of the vectors pKIWI503 and pKIWI103 are shown (not to scale). The 1.36 kb actinidin promoter was fused to the GUS-coding region followed by the ocs terminator. The selection marker NPT II coding region was under control of the nos promoter and terminator. In pKIWI103 the GUS coding region was fused to the 1.4 kb mas promoter. pKIWI702 (not shown) contained a promoterless GUS coding region. Otherwise the three vectors are identical, and all have the same *Sal* I linker immediately in front of the GUS ATG codon. Some restriction sites relevant to the construction are indicated (see Materials and methods).

centa, as shown by both longitudinal and cross sections (Fig. 4E, F).

In contrast, the mas promoter-GUS fusion produced GUS activity in most tissues of the plant, including all leaf tissues, all stem tissues (except cambium), root tips, receptacles, carpel, stamens (particularly anthers), and ovules (data not shown). In mature seed pods, the mas promoter produced strong expression in placental tissues, where expression was preferentially in the vascular tissue (Fig. 4K). The promoterless GUS gene showed no activity in any tissues, above the background level of wild-type (non-transgenic) plants, which exhibit light blue staining in the style, stigma, and pollen (data not shown).

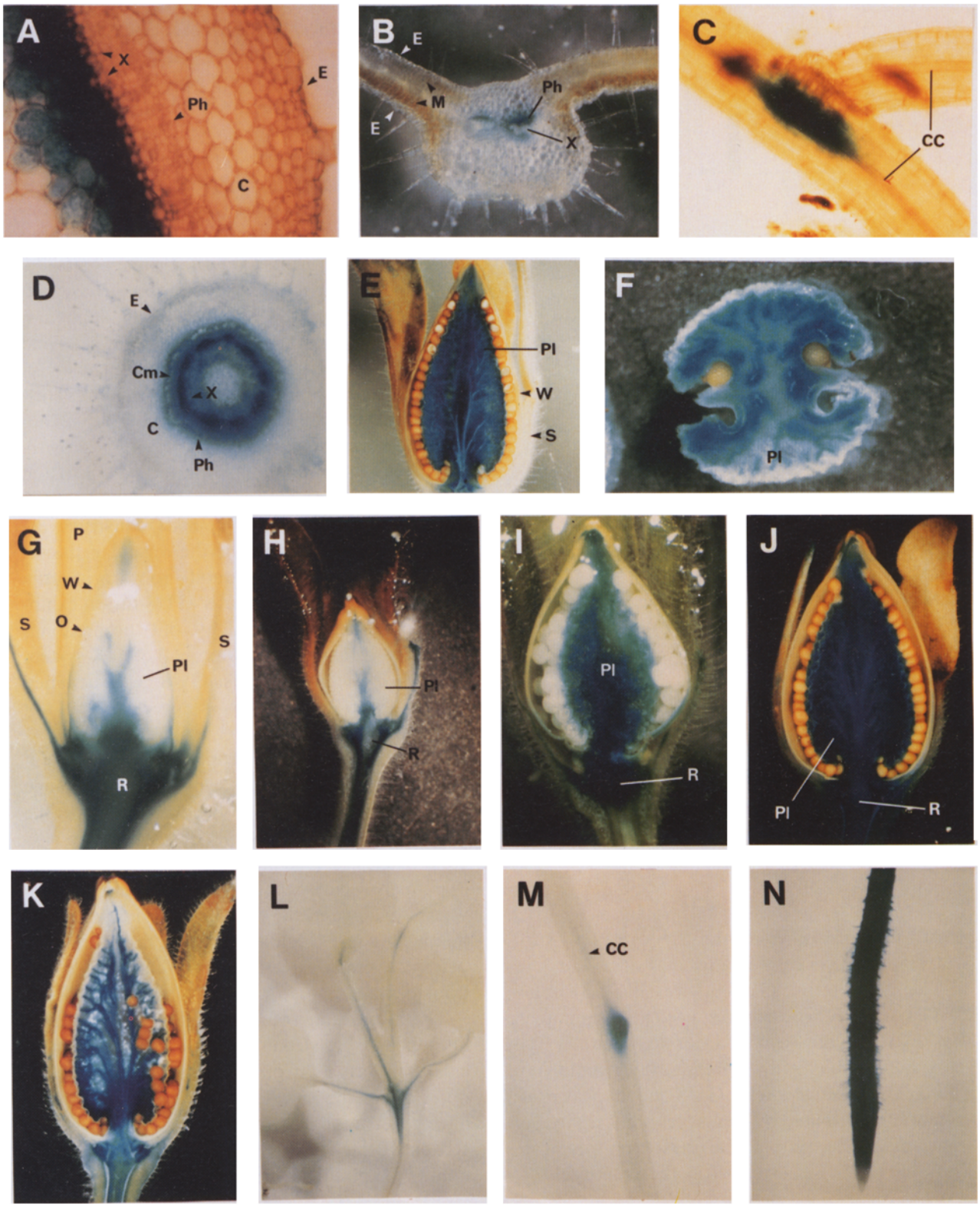
We also carried out quantitative assays of the GUS activity in cell extracts of the respective organs of transgenic plants (shown in Fig. 5A). In petunia, the actinidin promoter directed high-level expression in receptacles and placenta of mature seed pods and weak expression in leaves and petals, whereas the mas promoter was predominantly active in leaves and placental tissues. The low-level expression driven by the actinidin promoter in leaves and petals is consistent with limited GUS staining seen in the major veins.

The actinidin promoter is activated in the later stages of seed pod development

We next investigated the temporal regulation of GUS expression during seed pod development. Transgenic plants with strong GUS activity were selected for histochemical and quantitative analysis at different developmental stages.

Histochemical GUS staining of developing seed pods from one of the highly expressing transgenic plants, 503B, is shown in Figs 4G–4J The 1.36 kb construct produced only limited GUS activity in the ovary prior to pollination, even in strongly expressing plants, despite the presence of strong GUS expression in the neighbouring receptacle tissues. However, significant GUS activity was induced in the placenta of seed pods approximately two weeks after pollination, and GUS staining reached a high level four weeks after pollination, when the seed pods were start-

Fig. 4. Histochemical assay of GUS gene expression in transgenic petunia plants. E, epidermis; C, cortex; Ph, phloem, X, xylem, M, mesophyll tissue; CC, central cylinder; Cm, cambium; R, receptacle; O, ovule; W, ovary wall; P, petal; S, sepal. Magnification numbers below refer to the original image on 35 mm film. A. Cross-section of the stem from a pKIWI503 transformant (33 ×). B. Cross-section of the leaf from a pKIWI503 transformant (9 ×). C. Root branching point of a pKIWI503 transformant (33 ×). D. Cross-section of flower receptacle from a pKIWI503 transformant (7.5 ×). E. Longitudinally dissected seed pod at the matured stage from a pKIWI503 transformant (1.8 ×). F. Cross-section of the placenta of a mature seed pod from a pKIWI503 transformant (7.5 ×). G. The flower ovary from transgenic plant 503B dissected longitudinally prior to pollination (4.5 ×). H. The seed pod from 503B dissected longitudinally 1 week after pollination (2 ×). I. The seed pod from 503B dissected longitudinally 2 weeks after pollination (2 ×). J. The seed pod from 503B dissected longitudinally 4 weeks after pollination (2 ×). K. Longitudinal section of the mature seed pod from a pKIWI 103 transformant (1.8 ×). L. Vascular system of a T2 seedling from a 503 transgenic petunia line (4.5 ×). M. The root from a T2 503 petunia line, showing the initiation site of the lateral root (12 ×). N. The root of a pKIWI103-transgenic T2 petunia line (12 ×).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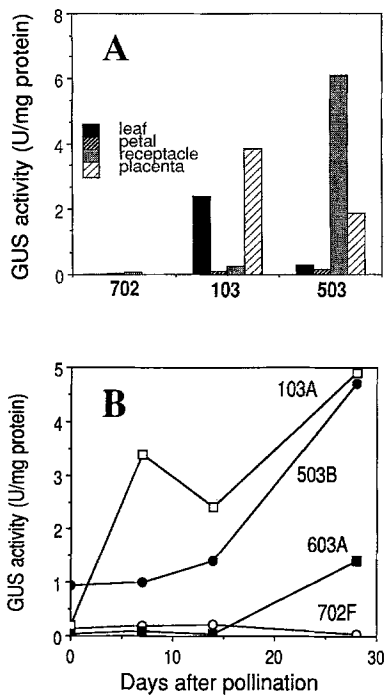

Fig. 5. Quantitative assay of GUS activity in transgenic petunia plants. The indicated tissues of transgenic plants were used to prepare cell extracts and assayed for GUS activity by fluorimetry (see Materials and methods). The units of GUS activity are nmols of substrate converted to product per minute, normalized for soluble protein content of the extract. A. GUS activity in transgenic petunia plant tissues. Tissue samples from all the T1 transformants of pKIWI503, pKIWI702, and pKIWI103 were pooled and measured for their GUS activity. The respective vectors and tissue types are indicated. B. GUS activity during seed pod development. Time refers to weeks after pollination. The curves show results for individual, highly expressing plants for each construct. Samples consisted of XX seed pods pooled from each plant, and the points are the average of three measurements for each sample. Standard deviations were less than 0.5 units of GUS activity for all the samples.

ing to turn brown. Placental tissues rapidly underwent senescence thereafter and dried out.

The quantitative GUS assay (Fig. 5B) confirmed that GUS induction started approximately half way through seed pod development, as exemplified by the highly expressing transgenic line 503B. The initial low-level GUS activity was presumably contributed by the tissues adjacent to the receptacle. In contrast, the mas promoter was induced to its highest level in the first week of pod development, consistent with histochemical results (not shown).

Taken together, these results suggest that the kiwifruit actinidin promoter is able to direct expression of a heterologous gene during the late stages of seed pod development in petunias.

Segregation and expression of the GUS gene in the T2 progeny

To confirm the results from the primary transformants (the T1 generation), seeds obtained by selfing were germinated on minimal medium and seedlings from germination to three weeks old were stained for GUS activity. Before the emergence of cotyledons from the seed, GUS expression was generally found in all tissues of the plant (data not shown). After one week of growth, seedlings of the actinidin promoter-GUS fusion displayed the typical tissue-specific pattern of the parental T1 plant. The 1.36 kb construct expressed the GUS gene in the vascular tissues (Fig. 4L), and near lateral root primordia (Fig. 4M). The mas promoter-GUS fusion exhibited ubiquitous GUS activity, but the predominant site of expression was in the root system (Fig. 4N). The promoterless GUS gene remained silent in the T2 generation as expected (data not shown).

T2 plants showing strong GUS expression were monitored to the flowering stage. No change of tissue specificity was noticed compared to the T1 generation, although GUS activity in the vascular tissues of vegetative organs appeared to be marginally weaker than in the T1 plants. There was no change in the GUS expression in reproductive organs. Qualitatively then, the expression patterns were in agreement with those of primary transformants.

The T2 seedlings of four pKIWI503 transgenic lines expressing strong GUS activity in the vasculature were stained to determine the segregation ratio of the reporter gene (Table 1). All four lines gave ratios close to 3:1, consistent with insertion of the T-DNA at a single site in the genome of each T1 transformant.

Table 1. Segregation ratios of T2 generation pKIWI503 transgenic petunia plants.

Transgenic plant	Number of GUS +	Number of GUS −	Ratio
503A	87	27	3.22:1
503B	104	41	2.54:1
503I	77	25	3.08:1
503M	67	22	3.05:1

GUS expression by the short actinidin promoter

In most of the transgenic petunia plants containing the shorter 173 bp actinidin promoter-GUS fusion, GUS expression was at background levels. However, in a few plants GUS expression was detectable. In all of these cases, expression occurred in the same tissues in which the 1.36 kb construct showed strong activity. For example, one transgenic petunia plant, out of eleven examined for the short promoter, showed expression in the seed pod. (This compares with twelve of 14 plants for the longer promoter.) This plant, designated 603A, showed GUS induction late in pod development (see Fig. 5B), in the same way as plants containing the 1.36 kb construct. In addition, five of the eleven 603 plants showed expression in pollen, two showed expression in placental tissues, and one in the stem xylem. In no case did the shorter construct express GUS in the tissues where the long construct showed no activity (data not shown).

These results suggest that the 1.36 kb promoter is necessary for consistent high level expression, but that the 173 bp promoter may contain information that is sufficient to confer spatial and temporal regulation on genes located downstream.

Discussion

The protease activity measurements reported here show that actinidin is developmentally regulated in fruit. Activity was first seen when fruit were about half their final size, and steadily increased until the time of normal harvest. There was no further increase in protease activity when fruit were allowed to ripen on the vine. These results are consistent with northern hybridizations which suggested that levels of actinidin mRNA increase during the later stages of fruit ripening [18, 17; see Introduction].

In transgenic petunia, the major site of GUS expression driven by a kiwifruit actinidin promoter was the seed pod, tissue which is analogous to the fruit of kiwifruit. Moreover, GUS expression increased during the latter half of petunia seed pod development, again consistent with the induction pattern of actinidin in kiwifruit. Detailed study of actinidin promoter activity during 'ripening' was not possible with petunia, because the tissues dehydrate rapidly as they change colour and senesce. We attempted to overcome this difficulty by introducing the actinidin-GUS constructs into transgenic tomato plants (data not shown). GUS expression in these plants was qualitatively similar to the patterns found in petunia, but we observed suppression of GUS expression in some T1 plants, and also in their progeny. This instability of expression has so far prevented us from obtaining accurate quantitative data for GUS expression during tomato fruit ripening.

While GUS expression in seed pods of transgenic petunias was as predicted by the pattern of actinidin protease activity in kiwifruit, some sites of expression were also noted in vegetative tissues of the transgenic petunia plants. For example, the actinidin promoter was activated in vascular tissues, particularly at the branching points, and also in cells of the receptacle. At present it is not clear whether a similar pattern of vegetative expression is directed by the actinidin promoter in kiwifruit. In order to address this issue, we have generated transgenic kiwifruit plantlets containing the fusion constructs described here. These plants so far show no GUS activity in vascular bundles of kiwifruit leaves (unpublished data). Further analysis of GUS expression in these plants as they develop will enable a direct comparison between the species.

Recently De Block and Debrouwer [5] have shown that localization of sites of GUS expression by histochemical staining with X-gluc can be

confused by staining artefacts, particularly near veins. To exclude the possibility that the GUS staining we observed near petunia veins was artefactual, we stained several T2 plants in the presence of the oxidizing agent ferricyanide (2 mM), which had been found to limit formation of the insoluble blue precipitate to the site of GUS gene expression [5]. Under these conditions, we observed staining that was fainter than that shown in Fig. 4, and was more localised to cells adjacent to xylem elements (data not shown). These results confirmed that the actinidin promoter does direct significant levels of GUS expression in the vascular system of petunia. We have also eliminated the possibility that GUS expression in petunia leaves was induced by wounding (sectioning), because GUS staining was not affected by cycloheximide treatment, in marked contrast to the results for the mas-GUS fusion (data not shown).

The region containing 173 bp of DNA immediately upstream of the ATG directed GUS expression in similar tissues (pollen, xylem, and seed pod) to those seen for the larger 1.36 kb promoter fragment. However, the expression patterns for the smaller actinidin promoter were much less consistent than those of the larger fragment. One interpretation of these results is that the 173 bp promoter fragment contains sequences which are sufficient for the tissue specificity of GUS expression, and that the adjacent upstream regions present in the larger 1.36 kb promoter contain sequences which modulate the level of expression. The reduced consistency of expression of the shorter promoter presumably results from 'position effects', since integration in the vicinity of a suitable enhancer might be required to obtain expression of GUS at detectable levels.

The central conclusion drawn from this work is that the pattern of late fruit expression observed for actinidin in kiwifruit was conserved when promoter sequences were introduced into petunia. These results add further experimental support to observations with other plant genes, which indicate that the genetic mechanisms effecting developmental and tissue-specific control are evolutionarily conserved between many dicotyledon species.

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