

Correct Processing of the Kiwifruit Protease Actinidin in Transgenic Tobacco Requires the Presence of the C-Terminal Propeptide¹

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A 35S cauliflower mosaic virus promoter and a tapetum-specific promoter were used to direct the synthesis in tobacco of preproactinidin and a derivative that lacked a C-terminal extension. Preproactinidin was processed into a form that migrated identically on protein gels with mature actinidin extracted from kiwifruit. This protein was proteolytically active in vitro, and high-level accumulation of this protein appeared to be detrimental to plant growth. Plants expressing an actinidin cDNA construct that lacked the sequence encoding the C-terminal propeptide were phenotypically normal but accumulated N-proactinidin, which was proteolytically active in vitro but did not self-cleave to mature actinidin. In transgenic tobacco, the C-terminal extension of actinidin is therefore required for correct processing.

Actinidin is a well-characterized Cys protease, with a wide pH activity range and wide substrate specificity (McDowall, 1970; Boland and Hardman, 1972) found in the Chinese gooseberry, or kiwifruit (*Actinidia chinensis*, and the cultivated hexaploid *Actinidia deliciosa*). Protein and DNA sequence data show that actinidin is a member of a group of closely related Cys proteases found in plants (e.g. papain from papaya, ficin from fig, aleurain, EP-A and EP-B from barley, and bromelain from pineapple), slime molds, insects, and mammals (e.g. cathepsin B and cathepsin L) (Praekelt et al., 1988). Several plants accumulate high concentrations of Cys proteases, either in specific organs or in specific cell types. *Carica papaya* (papaya) produces a range of Cys proteases contained within an extensive laticifer system, the most intensively studied being papain. Figs accumulate high levels of ficin in the fruit and pineapples produce bromelains in both the fruit and stem. *Actinidia* accumulates actinidin to very high concentrations in the fruit, where it constitutes up to 60% of soluble protein, but it is absent in other tissues (Praekelt et al., 1988). The function of proteases that are present in such high concentrations is uncertain (for speculations concerning functions, see Boller, 1986), but other plant Cys proteases, such as those found in the aleurone of barley, are thought to have important roles in the mobilization of seed storage reserves during germination (EP-A and EP-B) or in

cellular metabolism (aleurain) (Holwerda and Rogers, 1992).

It is not known where actinidin is located in the fruit cell or how such high accumulation of an active protease is possible without deleterious effects on cell metabolism. Possible solutions to the latter problem have emerged from the isolation and characterization of cDNAs encoding actinidin from kiwifruit (Praekelt et al., 1988; Podivinsky et al., 1989) and their comparison with cDNAs encoding other related Cys proteases. These Cys proteases were found to be encoded as preproproteins, with a putative ER-targeting signal and a large conserved NTPP. Some members of the family also possess a CTPP that varies in length between different proteases. Actinidin has a CTPP of 34 amino acids, one of the longest described to date. The papain-like proteases are therefore likely to be synthesized as zymogens and processed into mature active forms either on secretion or on sequestration to a location within the cell. In support of this view, Cys proteases have been found to be targeted to the lysosome (e.g. cathepsin B [Mort et al., 1981]) or the vacuole (e.g. aleurain [Holwerda et al., 1990] and bromelain [Boller and Kende, 1979]) or secreted (e.g. EP-A and EP-B [Koehler and Ho, 1990]). The proproteins either lack proteolytic activity in normal physiological conditions (e.g. proaleurain [Holwerda et al., 1990] and propapain [Vernet et al., 1990]) or are active only at the low pHs found in vacuoles and lysosomes (e.g. procathepsin B [Felleisen and Klinkert, 1990]).

The large cell size and highly vacuolate nature of the *Actinidia* fruit cells, coupled with the absence of an efficient transformation system for this species, make it technically difficult to determine the precise location of actinidin in kiwifruit and to determine the function of the actinidin propeptide sequences. Therefore, to address some of these problems, chimeric genes encoding preproactinidin or preproactinidin lacking a CTPP were expressed in tobacco. The results obtained provide evidence that the actinidin CTPP is required for actinidin processing. Additionally, to our knowledge this work is the first demonstration that a protease can be processed correctly into a proteolytically active form in a heterologous plant system.

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Abbreviations: CaMV, cauliflower mosaic virus; CTPP, C-terminal propeptide; NTPP, N-terminal propeptide; PG, polyacrylamide gelatin.

MATERIALS AND METHODS

Construction of Chimeric Genes

Chimeric genes were constructed to encode the full-length preproprotein (NAC = NTPP-actinidin-CTPP) and the N-actinidin propeptide (NA = NTPP-actinidin) as shown in Figure 1. The actinidin gene from pKIWI450 (Podivinsky et al., 1989) was first transferred to pBluescript KS⁻ (Stratagene) as an *EcoRI*-*Bam*HI fragment, forming pWP100. The oligonucleotide primers 5'-GGGTCTAGAC-CATGGGTTTGCCCAAATCC-3' and 5'-GGGCCGCGGC-TAGTTGTTGTACTTGACGGG-3' were used in a PCR to generate an actinidin fragment (NA), which encodes a protein lacking the CTPP, from pWP100. These primers introduce an *Xba*I site immediately prior to the initiating ATG of the NA sequence, an *Nco*I site around this ATG and an *Sac*II site following the premature stop codon. The PCR product was cloned as an *Xba*I-*Sac*II fragment into pBlue-script KS⁻ (Stratagene), forming pNA. The C-terminal portion of actinidin was isolated as an *Mlu*I-*Sac*II fragment

from pWP100 and used to replace the *Mlu*I-*Sac*II fragment of pNA, forming pNAC. Therefore, the new plasmid encodes actinidin with the CTPP.

The actinidin derivatives were then cloned as *Xba*I-*Sac*II fragments between the double 35S CaMV promoter and polyadenylation sequence of pWP83, forming transcriptional promoter fusions (p35S-NA-int and p35S-NAC-int) (pWP83 is identical with pJIT60 [Guerineau et al., 1988] except that the *Pst*II-*Sma*I polylinker region has been replaced by the *Xba*I-*Sac*II polylinker fragment of pBlue-script). The 35S CaMV promoter-actinidin chimeric genes were then excised as *Sst*II-*Xho*I fragments and cloned into *Sst*II-*Sall*-cut pBin19 (Bevan, 1984), forming p35S-NA and p35S-NAC. Similarly, the actinidin derivatives were cloned into pWP112 as *Nco*I-*Sac*II fragments between the tapetum-specific A9 promoter (Paul et al., 1992) and CaMV polyadenylation sequence, forming pA9-NA-int and pA9-NAC-int (pWP112 is based on pWP83 except that the 35S CaMV promoter is replaced with a 950-bp A9 promoter fragment in which the sequence around the first ATG of A9 is mutated to an *Nco*I site). The chimeric genes were then transferred to pBin19 as *Hind*III-*Xho*I fragments, forming pA9-NA and pA9-NAC.

Transformation of Tobacco

Nicotiana tabacum was transformed using *Agrobacterium* pGV2260 as described by Draper et al. (1988).

Protein Blot Analysis

Protein blot analysis was performed as described by Worrall et al. (1992). The actinidin antibody (raised in a rabbit using actinidin purified from kiwifruit) was used at a dilution of 1:1000.

Protease Assays

A 1.5-mL microcentrifuge tube was filled one-quarter full with leaf pieces or kiwifruit slices, liquid nitrogen was added, and the plant tissue was ground to a powder with a microhomogenizer. Extraction buffer (150 μ L of 100 mM potassium phosphate buffer, pH 6.0, 10 mM sodium ascorbate, 5 mM EDTA, and 1% PVP) was added and the grinding continued until the extract was thawed and mixed. After centrifugation at 10,000 rpm for 5 min at 4°C, the supernatant was stored on ice until required. The assay mix consisted of 40 μ L of extract, 160 μ L of assay buffer (extraction buffer plus 25 mM β -mercaptoethanol), and 200 μ L of 10 mg/mL azocasein. At appropriate intervals after incubation at 37°C, 100- μ L aliquots were removed and precipitated by the addition of 175 μ L of 12% perchloric acid. The samples were then centrifuged at 10,000 rpm for 5 min and the release of soluble azo groups from azocasein measured spectrophotometrically at A_{340} . Protein concentrations were determined by the method of Bradford (1976).

The gel assay for protease activity was essentially as described by Mitsuhashi et al. (1984). Extracts from leaves and kiwifruit were prepared as described above and combined with 3 $\times$ native gel sample buffer. Protein extracts

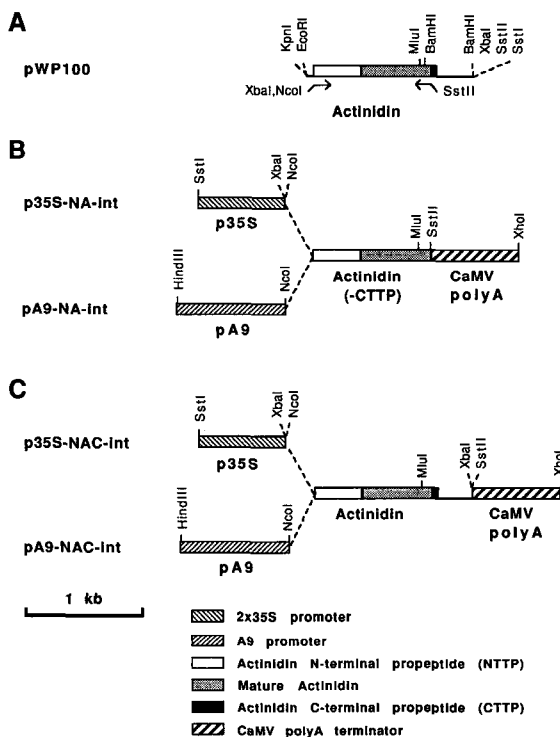


Figure 1. Construction of the chimeric actinidin genes. **A**, The actinidin gene of pWP100 showing relevant restriction enzyme sites. The positions where the primers bind to actinidin are indicated by arrows, together with the restriction sites introduced by PCR. **B**, Chimeric genes containing the actinidin gene lacking the CTPP. These were made by cloning the actinidin PCR fragment between the double 35S CaMV promoter and terminator of pWP83 and the A9 promoter and CaMV terminator of pWP112 (see "Materials and Methods"). **C**, Chimeric genes containing the complete actinidin gene. These are identical with p35S-NA-int and pA9-NA-int except that the *Mlu*I-*Sst*II fragments were replaced with the *Mlu*I-*Sst*II fragment of pWP100.

were loaded onto a 10% anodic native gel and electrophoresed at 4°C. The gel was then immersed in 100 mM phosphate buffer, pH 6 to 8 (or 100 mM sodium acetate buffer, pH 3–5), 5 mM EDTA, and 10 mM β -mercaptoethanol for 10 min. The gel was then laid onto a 0.3-mm-thick, 7.5% polyacrylamide overlay gel containing 1% gelatin, and both gels were incubated at 30°C for 2 h. The overlay gel was then stained with Coomassie blue and destained to reveal clear areas where the gelatin had been degraded.

RESULTS

Construction of Chimeric Actinidin Genes

Figure 1 shows the chimeric genes that were constructed and transformed into tobacco. The actinidin derivatives were produced, as described in "Materials and Methods," from the plasmid KIWI450 (Podivinsky et al., 1989), which contains a full-length actinidin cDNA. Two oligonucleotides, including one designed against the sequence around the junction of the CTPP with mature actinidin, were used in PCRs to generate two actinidin sequences. The first was a full-length preproactinidin sequence retaining the signal and both the NTPP and CTPP sequences (NAC), and the second was actinidin lacking the CTPP sequence (NA). Transcriptional fusions of these sequences were then made to a double 35S CaMV promoter (Guerineau et al., 1988), forming the constructs p35S-NAC and p35S-NA. Because this promoter is active in vegetative tissues, it was possible that actinidin produced from these chimeric genes would kill plant cells, thereby preventing the recovery of transgenic plants. Therefore, the actinidin sequences were also linked translationally to the tapetum-specific A9 promoter (Paul et al., 1992), forming pA9-NAC and pA9-NA. The tapetum is essential for the formation of pollen, which has been demonstrated by the expression of an RNase specifically within tapetal cells (Mariani et al., 1990). A male-sterile phenotype from plants containing the A9-actinidin genes and an inability to recover plants expressing 35S-actinidin genes would therefore suggest that proteolytically active actinidin was being synthesized, leading to cell death.

The CTPP Is Required for Efficient Processing of Preproactinidin to Mature Actinidin in Tobacco

Figure 2 shows immunoblots of protein extracts of the leaves of plants transformed with the 35S-NAC and 35S-NA constructs (lanes 3 and 4) and anthers of plants transformed with the A9-NAC and A9-NA constructs (lanes 6 and 7). Tobacco leaves apparently contain a protein migrating at approximately 43 kD that binds the anti-actinidin antibody nonspecifically, because this band is seen in nontransformed leaf extracts (lane 2) but not in extracts of nontransformed anthers (lane 5). Transformation with the full-length NAC constructs led to the accumulation of a protein that co-migrated with authentic actinidin (lane 1) and bound the anti-actinidin antibody strongly (lanes 3 and 6). The "mature" tobacco actinidin protein was excised from an SDS polyacrylamide gel and the N-terminal sequence Val-Leu-Pro-X-Tyr (where X is an

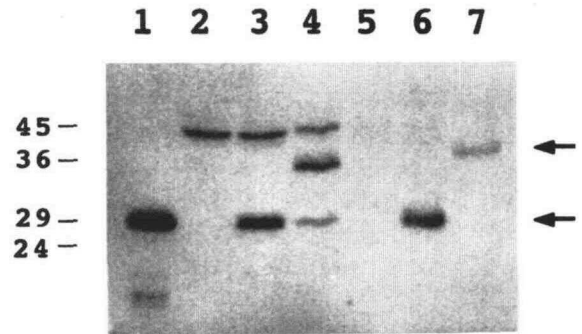


Figure 2. Expression of chimeric actinidin genes in tobacco. Gel blot of protein samples probed with an actinidin antibody. Lane 1, Kiwi-fruit; lanes 2 to 4, tobacco leaf protein; and lanes 5 to 7, tobacco anther protein. Lanes 2 and 5, Wild type; lane 3, 35S-NAC; lane 4, 35S-NA; lane 6, A9-NAC; and lane 7, A9-NA tobacco plant. Arrows indicate the size of mature actinidin and N-proactinidin. The distances migrated by molecular mass markers are shown to the left of the blot in kD.

undefined residue) was determined. However, the N-terminal sequence of mature actinidin purified from *Actinidia* has been reported as Leu-Pro-Ser-Tyr (Carne and Moore, 1978; Baker, 1980), which corresponds to a cleavage of the preproprotein between residues Val¹²⁶ and Leu¹²⁷. To check that the reported N-terminal sequence of kiwifruit actinidin was correct, actinidin was extracted from immature fruit, excised from an SDS polyacrylamide gel, and directly sequenced. Results obtained suggested that actinidin was a mixture of two proteins, one with an N-terminal sequence of Val-Leu-Pro-Ser and the other with sequence Leu-Pro-Ser-Tyr. The Val-Leu-Pro-Ser form was more abundant than the Leu-Pro-Ser-Tyr form. Thus, the tobacco actinidin precursor is cleaved at a site used in kiwifruit.

Plants transformed with the construct lacking the coding sequence for the CTPP (i.e. NA constructs) accumulated a protein with an apparent molecular mass of approximately 35 kD (Fig. 2, lanes 4 and 7). Plants expressing the 35S-NA constructs also accumulated small amounts of mature actinidin (lane 4), but the anthers of plants expressing the A9-NA construct lacked this band. The size of the major cross-reacting product in these plants is close to that of 36 kD predicted for the unprocessed preprotein lacking the CTPP (i.e. the N-proprotein). There was no evidence of a significant accumulation of preproactinidin (i.e. NAC) in any of the transformed plants.

Therefore, the results indicate that preproactinidin can be expressed in tobacco and processed to a form that is similar in size to that of mature actinidin. Deletion of the CTPP results in the accumulation of a protein that apparently retains the NTPP. Thus, the CTPP is required for efficient processing of actinidin into a mature form in tobacco.

Both N-Proactinidin and Mature Actinidin from Tobacco Are Proteolytically Active in Vitro

The substrate azocasein was used to determine whether whole cell protein extracts from the 35S-NA and 35S-NAC

plants contained elevated protease activity (see "Materials and Methods"). Figure 3A shows that both plant lines had protease activities that were considerably above that of the untransformed control. The addition of the Ser and Cys protease inhibitor, leupeptin, at a final concentration of 100 μM to the assay abolished protease activity in kiwifruit extracts and also the elevated protease activity in transgenic tobacco extracts (data not shown). The specific activity of mature actinidin present in the 35S-NAC extract was similar to that found in kiwifruit extracts (data not shown).

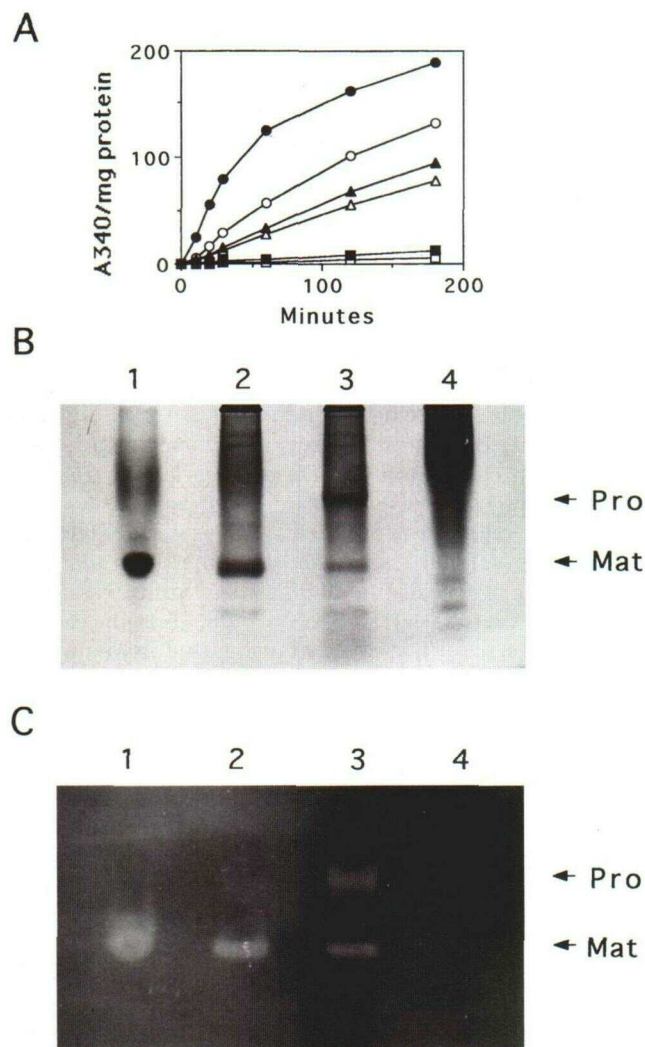


Figure 3. Proteolytic activity of the mature and proactinidin proteins. A, Crude extracts from 35S-NA (Δ , $\blacktriangle$), 35S-NAC ($\circ$, $\bullet$), and wild-type tobacco ($\square$, $\blacksquare$) assayed for the ability to degrade azocasein at pH 5 (filled symbols) or pH 6 (open symbols). B and C, Gel assay. Crude extracts from kiwifruit and for wild-type and transgenic tobacco were separated by native PAGE. One gel was Coomassie blue stained (gel B) and a duplicate was equilibrated in assay buffer for 10 min before being laid onto a PG gel. After incubation at 30°C for 2 h, the PG gel was Coomassie blue stained (gel C). Clear areas are due to the degradation of gelatin by proteases. Lane 1, Kiwifruit; lane 2, 35S-NAC; lane 3, 35S-NA; lane 4, wild-type tobacco. Pro, Proactinidin; Mat, mature actinidin.

This estimate was made by calculating, from denaturing polyacrylamide gels, the percentage of total cell protein that was actinidin in these extracts.

A PG gel assay confirmed that the increased protease activity in the 35S-NA and 35S-NAC extracts was due to the presence of actinidin. In this assay, protein samples were separated on a nondenaturing gel, and this gel was subsequently incubated in contact with a PG gel. After Coomassie staining, clear areas in the PG gel indicate the position of protease activity. Duplicate nondenaturing gels were prepared of extracts of kiwifruit, 35S-NAC- and 35S-NA-transformed tobacco, and wild-type tobacco; one of the duplicates was stained with Coomassie (Fig. 3B) and the other was incubated in contact with a PG gel (Fig. 3C). These data show that untransformed tobacco has no protease activity detectable by this method (lane 4). However both 35S-NAC (lane 2) and 35S-NA (lane 3) extracts contained a protease activity co-migrating with that found in kiwifruit (lane 1). In addition, the 35S-NA extract contains a slower migrating band of protease activity not present in the other extracts (Fig. 3C, lane 3). This band was electroeluted from the gel and the N-terminal sequence X-Thr-Asn-Asp-Glu obtained, where X represents an undefined amino acid. This sequence matches that found between amino acids 33 and 37 of the actinidin precursor sequence, i.e. Arg-Thr-Asn-Asp-Glu. Therefore, this protein represents the actinidin N-proprotein with the signal sequence, presumably extending from amino acids 1 to 32, removed. However, the method of von Heijne (1983) predicts that the most likely processing position for removing the signal peptide is between residues 24 and 25. Thus, it cannot be discounted that the creation of the N terminus of the actinidin N-proprotein is the result of more than one processing event.

These results demonstrate that both the mature actinidin and the N-proprotein are proteolytically active when extracted from transgenic tobacco. However, comparison of the relative amounts of N-proprotein and mature protein in a 35S-NA transformant, revealed by Coomassie staining, with the PG protease activity gel suggests that the N-proprotein may be less active than the mature protein (Fig. 3, B and C).

The N-Proprotein Does Not Self-Cleave to Mature Actinidin in Vitro

To determine whether the proteolytic activity of the N-proprotein was due to cleavage to the mature form and whether the N-proprotein is capable of self-cleavage, the following experiment was conducted. First, crude protein extracts from a 35S-NA plant were separated by nondenaturing PAGE. The gel was then equilibrated in buffer at various pH values (pH 3.0, 4.0, and 8.0) for 10 min before being laid onto a PG gel and incubated for 2 h at 30°C as described in "Materials and Methods." The PG gel was then stained to reveal protease activity (Fig. 4A). No differences were observed in the relative activities of the N-proprotein and the mature protein in the assay conditions used (lane 1, pH 3.0; lane 3, pH 4.0; lane 4, pH 8.0).

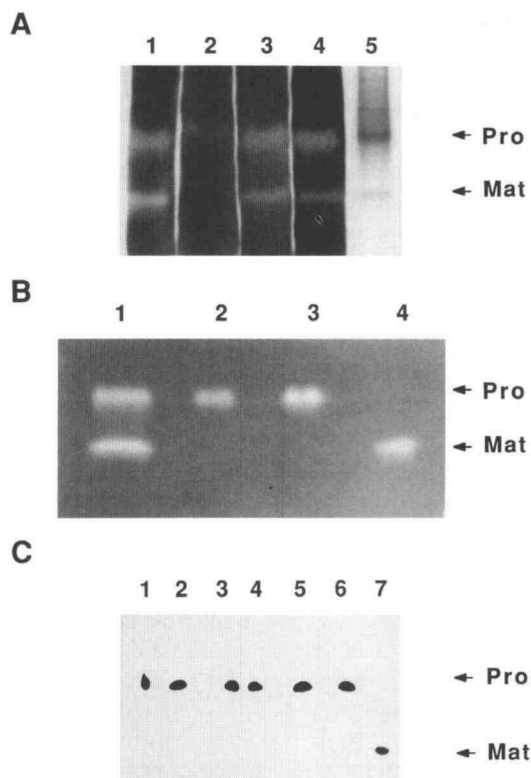


Figure 4. Proteolytic activity and processing of N-proactinidin *in vitro*. A, 35S-NA crude extracts were separated by native PAGE and checked for activity using the PG gel assay. Lanes 1 to 4, Coomassie blue-stained PG gel; lane 5, Coomassie blue-stained native gel. Duplicate native gel slices were incubated in buffers ranging from pH 3 to 8 in the presence or absence of 100 μ M leupeptin. Lane 1, pH 3; lane 2, pH 4 plus 100 μ M leupeptin; lane 3, pH 4; lane 4, pH 8. B, PG gel assay (performed at pH 5) of mature actinidin and N-proactinidin samples excised from native gel slices. Lane 1, Original p35S-NA extract; lane 2, N-proactinidin excised from a gel slice previously incubated at pH 4; lane 3, N-proactinidin from a gel slice incubated at pH 7; lane 4, mature protein from a gel slice incubated at pH 4. C, Protein gel blot of mature actinidin and N-proactinidin samples excised from the native gel of Figure 4A and probed with an actinidin antibody. Lanes 1 to 6, N-proactinidin; lane 7, mature actinidin. Samples were previously incubated in the following conditions: Lane 1, pH 3; lane 2, pH 4; lane 3, pH 5; lane 4, pH 6; lane 5, pH 7; lane 6, pH 8; lane 7, pH 4. Pro, N-proactinidin; Mat, mature actinidin.

The proteolytic activity of both proteins was inhibited by leupeptin (lane 2). Bands corresponding to the positions of the pro and mature actinidin proteins were then excised from the original nondenaturing gel, the slices were placed into the wells of another nondenaturing gel, and the proteins were separated out as before. This second gel was laid onto a PG gel, incubated as described above, and the PG gel was stained (Fig. 4B). The isolated N-proprotein exhibited proteolytic activity at both pH 4 (lane 2) and pH 7 (lane 3) but in neither case was activity observed co-migrating with that of the mature actinidin (lane 4). Immunoblots of a replicate gel indicated that no mature actinidin was produced under any conditions in which isolated N-proactini-

din was incubated (Fig. 4C). Thus, *in vitro*, the N-proprotein does not self-cleave at an appreciable rate to mature actinidin.

Transgenic Tobacco Plants Accumulate High Levels of Mature Actinidin with Phenotypic Effects Apparent in Only the Highest Expressers

35S-NA and A9-NA plants that accumulated actinidin N-proprotein were phenotypically normal, whereas 35S-NAC and A9-NAC plants that accumulated high levels of mature actinidin exhibited growth or fertility abnormalities. High-expressing A9-NAC plants showed reduced male fertility (data not shown), and one of the 35S-NAC plants (designated 35S-NAC-6) was stunted and had thick leathery leaves and recessed stamens and stigma and was self-sterile, since no pods formed. The 35S-NAC-6 plant was the highest actinidin expresser with up to 8% of the total soluble leaf protein being actinidin, as judged by density scanning of Coomassie blue-stained gels. Lower-level expressers (approximately 3%) (e.g. 35S-NAC-1 and 35S-NAC-2) appeared normal throughout vegetative growth, although recessed stamens, small stigmas, and markedly reduced seed set were observed. To determine whether the phenotype of the 35S-NAC-6 plant was associated with the accumulation of mature actinidin, the 35S-NAC-6 plant was recalled, and 24 new plants were analyzed. Three of these plants, termed "revertants" and designated 6A, 6P, and 6X, had normal morphology and seed set; the remaining plants were phenotypically identical with 35S-NAC-6, although a few small pods containing viable seed were formed on most plants. Revertant plants were shown not to produce actinidin. For example, Figure 5A shows a gel of leaf extracts of plants regenerated by recalling 35S-NAC-6; lanes 1 to 5, 9 to 12, 14, and 15 are from plants that produced actinidin that was detectable by Coomassie staining, whereas lane 6 is from revertant plant 6A that was phenotypically normal and lacked detectable levels of actinidin. Immunoblot analysis of the recalled plants derived from 35S-NAC-6 are shown in Figure 5B. Lanes 1 and 7 are from actinidin-producing plants and lanes 2, 4, and 5 are from nonproducing plants 6X, 6P, and 6A, respectively. Additional revertant plants were generated by recalling the actinidin-producing plants, 6H and 6I.

The three revertant plants from 35S-NAC-6 (i.e. 6A, 6P, and 6X) had no detectable levels of actinidin by Coomassie staining or by probing protein blots with actinidin antibody. These plants were analyzed by PCR and shown to contain the chimeric actinidin gene (data not shown). Selfed seed from 6A was planted, and of the 20 kanamycin-resistant plants tested, 17 expressed high levels of actinidin and possessed a phenotype similar to the original 35S-NAC-6 plant. The three phenotypically normal plants from this selection did not accumulate immunodetectable actinidin protein, and RNA gel blots demonstrated that the revertant plants did not express significant levels of actinidin mRNA (Fig. 5C).

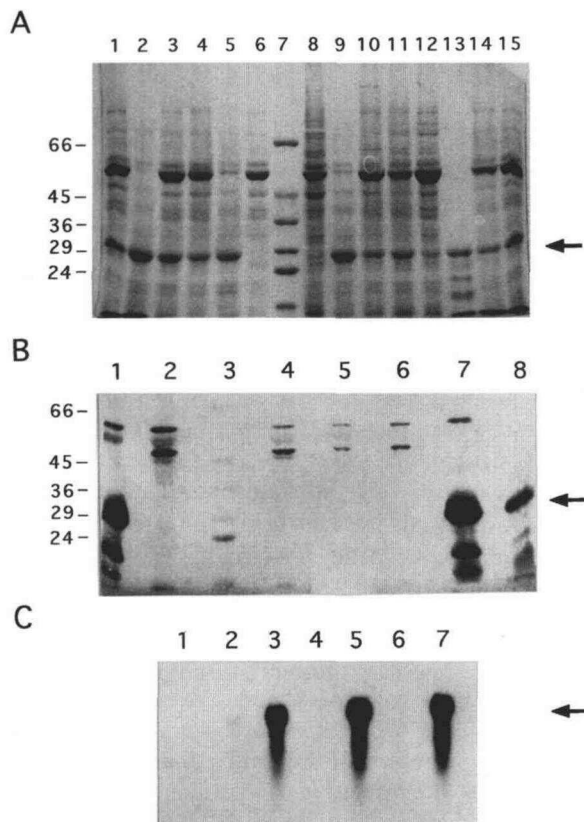


Figure 5. Actinidin expression in 35S-NAC-6-derived plants. A, Coomassie blue-stained protein gel of extracts of a representative group of plants derived by recalling the sterile, high-level actinidin-expressing plant, 35S-NAC-6. Of 24 plants analyzed (plants 6A–6X), all expressed actinidin and were phenotypically similar to the original 35S-NAC-6 plant, except for plants 6A (lane 6) and 6P and 6X (not shown), which lacked actinidin and were phenotypically normal. Lane 7, Molecular mass markers; lane 8, wild-type tobacco; lane 13, kiwifruit. Arrow indicates actinidin. The distances migrated by molecular mass markers are indicated in kD. B, Protein blot of plant extracts described in Figure 5A, probed with actinidin antibody. Lanes 1 and 7, Extracts of 35S-NAC-6-derived plants that express actinidin; lane 2, plant 6X; lane 3, molecular mass markers; lane 4, plant 6P; lane 5, plant 6A; lane 6, wild-type tobacco; lane 8, kiwifruit. C, Actinidin transcript levels in 35S-NAC-6 derived plants. RNA was extracted from anthers and probed with ^{32}P -labeled actinidin DNA. Lane 1, Wild-type tobacco; lanes 2 and 3, T_2 progeny of plant 6A; lanes 4 and 5, plants derived from recalling the actinidin expresser 6H; lanes 6 and 7, plants derived from recalling the actinidin expresser 6I. Only plants expressing actinidin transcript accumulate immunodetectable levels of actinidin and are phenotypically abnormal.

DISCUSSION

These data demonstrate that the preproprotein of the kiwifruit protease actinidin can be successfully expressed and correctly processed in transgenic tobacco. Plants in which the transgene is driven by the CaMV 35S promoter can accumulate up to 8% of their total extractable leaf protein as the mature form of actinidin. But this level achieved falls far short of that reported for kiwifruit fruit

cells (up to 60% of total protein). Even at these lower levels, deleterious phenotypic effects were observed in tobacco. High-level 35S-NAC expressers were stunted in growth, with a high frequency of silencing of actinidin expression, and a few A9-NAC plants exhibited reduced male fertility. This suggests that kiwifruit may have additional mechanisms to allow such high accumulation. On the other hand, the phenotypic effects in tobacco may be due to an interference of normal cellular function, either due to the proteolytic activity of the transgene product or because the synthesis of large amounts of foreign protein is energetically disadvantageous. These factors may not be a problem in kiwifruit, since the fruit cells have no vital function for plant growth. Overall, it is somewhat surprising that a heterologous host can survive the synthesis and accumulation of relatively large quantities of a broad-specificity thiol protease. This may indicate that the actinidin accumulates in a compartment, such as the vacuole, that is isolated from the general metabolic machinery of the cell.

In contrast to results with preproactinidin, in tobacco the CTPP-minus protein was not efficiently processed to mature actinidin, with most protein instead accumulating as an N-proprotein. This could be due to a direct requirement for the CTPP in the processing events. Alternatively, the lack of the CTPP could lead indirectly to the failure of processing through incorrect targeting of the actinidin precursor. Preliminary results have shown that mature actinidin accumulates in the vacuoles of transgenic tobacco plants, whereas the N-proactinidin is apparently secreted (J. Amiss and H. Smith, unpublished data). This indicates that the CTPP may contain signaling information for vacuolar sorting, which has been observed for other CTPPs such as that of a vacuolar chitinase (Neuhaus et al., 1991). Thus, if the proteolytic cleavage of the NTPP occurs en route to or in the destination vacuole, the N-proprotein would be secreted in an unprocessed form. Support for this view comes from studies of cell lines that secrete procathepsin B or L. The secretion of these lysosomal proproteins appears to be due to either saturation of the lysosomal targeting machinery (Moin et al., 1989) or a defect in the targeting process (Achkar et al., 1990).

The actual mechanism of processing appears to vary depending on the Cys protease. Procathepsin L (Mason et al., 1987), procathepsin B (Felleisen and Klinkert, 1990), and procruzain (Eakin et al., 1992) autoprocess at low pH to the mature enzyme but remain stable at neutral pH. Such a processing mechanism appears attractive, since the proprotein is inactive until it reaches the acidic environment of the lysosome. In contrast, proaleurain, secreted from a *Xenopus* oocyte expression system, has no proteolytic activity and is sequentially processed in a post-Golgi compartment to the mature enzyme by two proteases, one of which is a Cys protease (Holwerda et al., 1990). Proaleurain is not cleaved by aleurain. Propapain has been secreted from insect cells using recombinant baculovirus (Vernet et al., 1990). Propapain autocleaves to mature papain when incubated at 60°C in a reducing, low pH buffer; however, only 10% of the processing occurs at the site cleaved in the plant. Therefore, it seems likely that other proteases process propapain in

papaya. Even in the case of procathepsin L, autocleavage in vitro results in the formation of products that differ from those formed in vivo (Smith and Gottesman, 1989).

Thus, although some proproteins can in certain conditions autocleave to mature proteins, in vivo processing may require specific proteases. These studies, however, indicate that the NTPP acts as inhibitor of protease activity. Vernet et al. (1991) speculated that the papain NTPP may function in a similar way to that of procarboxypeptidase B (Coll et al., 1991) in forming a domain that lies over the active site of the mature portion of the enzyme. Temperature or pH changes might allow access to the active site and initiate autocleavage to the mature protein. Such structural transitions may be easier in some proproteins, perhaps explaining why some, such as proaleurain, have no autocatalytic activity.

N-proactinidin is markedly different from the other proproteins studied in that it has significant protease activity over a wide pH range in vitro (at least pH 3–8). This suggests that N-proactinidin is proteolytically active in the ER and in intercellular fluid. However, plants secreting the N-proprotein are phenotypically normal, which may conflict with the proposed role of the NTPP in preventing proteolysis during secretion or intracellular targeting. It is possible that other mechanisms exist to prevent proteolysis; perhaps specific inhibitors are present in the ER or conditions are unfavorable for protease activity. Alternatively, it is possible that a second N-terminal processing event, after the removal of the signal peptide (predicted to be between residues 24 and 25), creates the observed N-terminal of N-proactinidin (residue 33), and activates the protein. If this occurs on secretion or in the vacuole, it may explain why actinidin proproteins are not active in the ER.

N-proactinidin does not autocleave to the mature protein in vitro. This suggests that specific, probably vacuolar, proteases are required to form mature actinidin. However, the small amount of mature actinidin found in plants expressing the CTPP-minus construct under the 35S promoter might indicate processing by other proteases secreted from tobacco cells. It should be emphasized that N-proactinidin secreted by tobacco may have characteristics different from those of a proactinidin, which retains the CTPP. For example, it is possible that the CTPP may stabilize the precursor so that the NTPP is more effective in preventing protease activity. Synthesis of the full-length precursor in vitro or in appropriate expression systems should determine whether this is the case.

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