Processing, Targeting, and Antifungal Activity of Stinging Nettle Agglutinin in Transgenic Tobacco

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The gene encoding the precursor to stinging nettle (*Urtica dioica* L.) isolectin I was introduced into tobacco (*Nicotiana tabacum*). In transgenic plants this precursor was processed to mature-sized lectin. The mature isolectin is deposited intracellularly, most likely in the vacuoles. A gene construct lacking the C-terminal 25 amino acids was also introduced in tobacco to study the role of the C terminus in subcellular trafficking. In tobacco plants that expressed this construct, the mutant precursor was correctly processed and the mature isolectin was targeted to the intercellular space. These results indicate the presence of a C-terminal signal for intracellular retention of stinging nettle lectin and most likely for sorting of the lectin to the vacuoles. In addition, correct processing of this lectin did not depend on vacuolar deposition. Isolectin I purified from tobacco displayed identical biological activities as isolectin I isolated from stinging nettle. In vitro antifungal assays on germinated spores of the fungi *Botrytis cinerea*, *Trichoderma viride*, and *Colletotrichum lindemuthianum* revealed that growth inhibition by stinging nettle isolectin I occurs at a specific phase of fungal growth and is temporal, suggesting that the fungi had an adaptation mechanism.

UDA is a single-chain peptide found in roots and rhizomes of stinging nettle (*Urtica dioica* L.) (Peumans et al., 1984; Van Damme and Peumans, 1987). In most ecotypes UDA is present as a mixture of isolectins with similar chitin-binding and agglutination activities (Van Damme and Peumans, 1987; Van Damme et al., 1988). Recently, one acidic and five basic UDA isolectins were identified in the Weerselo ecotype of the stinging nettle (Does et al., 1999). In in vitro assays, UDA showed antifungal activity toward various plant pathogenic fungi containing chitin in their cell walls (Broekaert et al., 1989).

UDA consists of two Cys-rich chitin-binding domains (Beintema and Peumans, 1992). These domains are homologous to hevein, a small chitin-binding peptide of 43 amino acids from the lutoid bodies of rubber tree latex (Archer, 1960; Walujono et al., 1975). Mature UDA is processed from a precursor that comprises an N-terminal signal peptide, the two chitin-binding or hevein domains, a small hinge region, and a C-terminal chitinase domain (Lerner and Raikhel, 1992; Does et al., 1999). Genes encoding precursors to UDA isolectins contain two introns in the chitinase-encoding region located at the same positions as those in class I, II, IV, and VI chitinase genes (Does et al., 1999). Because of their homology with other plant chitinases and the presence of two hevein domains, UDA precursors are classified as class V or Chia5 chitinases (Neuhaus et al., 1996).

Many hevein domain-containing proteins are localized in vacuoles. The precursors to these proteins are synthesized on the rough ER and translocated into its lumen (Blobel, 1980; Gomord and Faye, 1996). For targeting to the vacuoles, soluble proteins that pass the Golgi apparatus require additional information (Dorel et al., 1989). Such vacuole-sorting determinants reside within the C-terminal or N-terminal propeptides of the precursor proteins or within the mature protein sequence (Chrispeels and Raikhel, 1992; Neuhaus, 1996). In precursors to vacuolar hevein domain-containing proteins, these targeting sequences have been shown to be located on the C terminus. Mature vacuolar tobacco (*Nicotiana tabacum*) class I chitinase contains one hevein domain fused to the catalytic domain (Mauch and Staehelin, 1989; Shinshi et al., 1990).

The targeting determinant of prochitinase is contained within a C-terminal propeptide of seven amino acids and has been shown to be necessary and sufficient for vacuolar sorting (Neuhaus et al., 1991). Without this C-terminal extension the protein is secreted (Neuhaus et al., 1991; Melchers et al., 1993). The vacuolar lectins wheat germ agglutinin, rice lectin, and barley lectin are each composed of four hevein-like domains (Mishkind et al., 1983; for review, see Raikhel and Lerner, 1991) and are all processed from precursors that contain a small C-terminal propeptide (Stinissen et al., 1984; Mansfield et al., 1988; Lerner and Raikhel, 1989; Wilkins and Raikhel, 1989). Like the chitinase propeptide, the C-terminal propeptide of the barley prolectin is required for correct vacuolar deposition of the mature lectin and is able to redirect an extracellular cucumber chitinase to the vacuoles (Bednarek et al., 1990; Bednarek and Raikhel, 1991; Neuhaus et al., 1991).

Hevein is localized in vacuole-derived lutoid bodies and, similarly to UDA, is processed from a precursor with a large C-terminal domain (Archer, 1960; Lee et al., 1991). In contrast to the UDA precursor, this C-terminal domain

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Abbreviations: ESI, electrospray ionization; MALDI, matrix-assisted laser desorption ionization; UDA, *Urtica dioica* (stinging nettle) agglutinin.

does not show homology to chitinases but rather to tobacco PR4-type (pathogenesis-related) proteins (Broekaert et al., 1990; Linthorst et al., 1991). A putative vacuole-targeting signal has been shown to be cleaved off from the C terminus of the PR4-like domain of the hevein precursor (Soedjanaatmadja et al., 1995). Other known C-terminal propeptides containing essential vacuole-sorting determinants are present in precursors to tobacco class I β-1,3-glucanase (Shinshi et al., 1988; Sticher et al., 1992; Melchers et al., 1993), vacuolar PR5 proteins (Singh et al., 1989; Melchers et al., 1993), Brazil nut 2S albumin (Harris et al., 1993; Saalbach et al., 1996), and kiwifruit proactinidin (Paul et al., 1995).

In the present study we show that the precursor to UDA is processed to mature-sized isolectin in transgenic tobacco and that this isolectin is localized intracellularly, most likely in the vacuoles. Removal of the C-terminal 25 amino acids of the precursor results in the secretion of UDA. Extracellularly targeted UDA is correctly processed and displays similar agglutination, chitin-binding, and antifungal activities as the same isolectin from stinging nettle. The growth-inhibiting effect of UDA on *B. cinerea* and *T. viride* appears to be temporal, and adaptation of both fungi coincides with a phase of accelerated hyphal growth. *C. lindemuthianum* did not adapt to UDA within 2 d after addition and showed a severely stunted and inhibited growth.

MATERIALS AND METHODS

Cloning Procedures

A genomic sequence encoding the precursor to stinging nettle (*Urtica dioica* L.) UDA isolectin I was isolated previously (Does et al., 1999). The sequence was cloned into the vector pMOG181 (clone 3N11; Does et al., 1999), which contains an expression cassette consisting of the cauliflower mosaic virus 35S promoter with a double enhancer followed by a unique *Bam*HI site and the nopaline synthase transcription terminator on an *Eco*RI-*Hin*dIII fragment. The expression unit containing the wild-type UDA-precursor construct was cloned into the binary vector pMOG402 (Jongedijk et al., 1995) and is referred to as the wild-type construct.

We used site-directed mutagenesis by PCR to introduce a stop codon in the targeting mutant construct. PCR was performed on 0.1 ng of clone 3N11 plasmid DNA, as described previously (Does et al., 1999), using the 5′-terminal primer UD1 (Does et al., 1999) and the mutagenesis primer UD14 (5′-ACTTCAAGTCATGTCAATAGCTCAC-3′). The PCR fragment obtained was cloned, together with the *Sca*I-*Hin*dIII fragment from clone 3N11, into pMOG181, thereby restoring the *Sca*I restriction site. To exclude a PCR amplification misreading by the polymerase, PCR sequences were exchanged with clone 3N11 sequences or sequenced (Sanger et al., 1977). The presence of the introduced stop codon was verified by sequencing. The expression unit containing the targeting mutant construct was cloned into pMOG402.

Both binary constructs were transferred from *Escherichia coli* DH5α into *Agrobacterium tumefaciens* strain MOG101 (Hood et al., 1993) by triparental mating (Pen et al., 1992). Standard recombinant DNA procedures were performed as described previously (Sambrook et al., 1989).

Tobacco Transformation and Leaf Extraction

Transgenic tobacco (*Nicotiana tabacum* L. cv Samsun NN) plants were obtained by the standard leaf disc transformation method using kanamycin selection (100 mg L^{-1}) (Horsch et al., 1985). Leaf discs were prepared from the top leaves of axenically grown tobacco plants.

Leaf samples were ground in 0.1 N HCl to obtain the total extract. Extracellular washing fluid was isolated using 0.1 N HCl according to a procedure described previously (De Wit and Spikman, 1982). After the extracellular washing fluid was isolated, the remnant extract (the remains of the leaves) was obtained by grinding in 0.1 N HCl. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Sigma-Aldrich).

Protoplast and Vacuole Isolation

Protoplasts and vacuoles were purified from mature tobacco leaves as described previously (Dombrowski et al., 1994). The purity of protoplast and vacuole preparations was determined by light microscopy, because vacuoles could be visualized using neutral red dye (Dombrowski et al., 1994). Proteins in the vacuole preparations that were clear of other cell material were precipitated with acetone and subjected to western analysis.

Western Analysis

After 20% Tricine-SDS-PAGE (Schägger and Von Jagow, 1987), proteins were blotted onto nitrocellulose membranes by the semidry blotting method described previously (Does et al., 1999). For immunological detection of UDA, α-UDA antibodies that had been raised by Eurogentec (Seraing, Belgium) against a synthetic peptide of 15 amino acids (WSGERSDHRCGAAVG), corresponding to amino acids 40 to 54 of UDA isolectin I, were used. Blots were treated as described by Does et al. (1999). A goat-anti-rabbit peroxidase-linked antibody (rabbit IgG-horseradish peroxidase, Zymed Laboratories, South San Francisco, CA) was used for the secondary antibody. Detection was performed using an enhanced chemiluminescence western-blotting detection system (ECL, Amersham Life Science, 's-Hertogenbosch, The Netherlands) according to the manufacturer's protocol. Antibodies that had been raised against the bacteria-produced chitinase domain of the UDA precursor were obtained from Dr. Beatrice Iseli (Botanisches Institut, Basel, Switzerland). The gels were silver stained with a kit (Bio-Rad) according to the manufacturer's protocol.

UDA Isolation from Transgenic Tobacco

The purification of UDA isolectin I from tobacco leaves was based on a procedure described by Peumans et al.

(1984). Extracellular washing fluid was isolated using 0.1 N HCl as described above. Total extract was prepared with the following procedure. The main leaf veins were removed from mature leaves. After 1 teaspoon of charcoal was added to the sample, 100 g of leaf material was homogenized in 200 mL of ice-cold extraction buffer (0.1 N HCl and 0.1% [w/v] ascorbic acid) using a blender. The material was squeezed through three layers of gauze, and the extract was kept on ice. Pulp material was again homogenized with 100 mL of extraction buffer and squeezed through three layers of gauze. The extract was centrifuged at 3,200g for 30 min at 4°C. The supernatant was filtered through eight layers of gauze and centrifuged for 60 min at 20,000g at 4°C. The subsequent supernatant was again filtered through eight layers of gauze, and the extract obtained is referred to as the total extract. From this point on, the extracellular washing fluid and the total extract were treated equally.

The extract was brought to pH 3.8 with 2 N NaOH and kept on ice for at least 1 h. Subsequently, the extract was centrifuged at 20,000g for 60 min at 4°C. The resulting supernatant was applied at the rate of 3.5 mL min^{-1} to a chitin-affinity column (±100 mL) that was prepared as described previously (Peumans et al., 1984) and equilibrated with sodium acetate buffer (50 mM sodium acetate and 0.1 M NaCl, pH 3.8). The column was washed at the rate of 0.8 mL min^{-1} with 1 L of the same buffer. Bound protein was eluted with 0.5 N acetic acid at the rate of 2 mL min^{-1}. Fractions were tested for the presence of UDA by western analysis. UDA-containing fractions were pooled and brought to pH 3.8 with 2 N NaOH and 0.1 M NaCl was added.

The solution was kept on ice for at least 12 h and then centrifuged at 20,000g for 60 min at 4°C. The volume of the supernatant was reduced to approximately 30 mL through an ultrafiltration membrane (YM1 Diaflo, Amicon, Beverly, MA) at 4°C using a 400-mL stirred cell (Amicon). To this concentrate was added 250 mL of 50 mM sodium acetate buffer. The solution was subjected to cation-exchange chromatography using a column (HiLoad SP Sepharose Fast-Flow column 26/10, Pharmacia) equilibrated with sodium acetate buffer. The column was washed with the same buffer and protein was eluted using a 1000-mL gradient of 0.1 to 0.5 M NaCl in buffer. Fractions containing UDA were pooled, concentrated over an ultrafiltration membrane, dialyzed against PBS, and concentrated to 2.5 mL by ultrafiltration.

The concentrate was applied to a gel-filtration column (Biogel P10, 16/90, Bio-Rad) at the rate of 0.2 mL min^{-1}. The pure UDA peak eluted after 24 h. Fractions were concentrated, dialyzed thoroughly against water, and concentrated by ultrafiltration, and then a final concentration was obtained with a concentrator (Centricon-3, Amicon).

For antifungal assays the UDA solution was filter-sterilized over a 0.22-μm filter. The concentration of the purified UDA was calculated from A_{280} using the calculated molar-extinction coefficient of UDA isolectin I (ϵ_{280} = 29 mM^{-1} cm^{-1}).

UDA Isolation

UDA isolectin I was further purified from a fraction containing UDA isolectin I and II that was obtained earlier (Does et al., 1999). The fraction was 50-fold diluted with sodium acetate buffer and subjected to cation-exchange chromatography. Isolectins were separated using a 700-mL gradient of 0.1 to 0.3 M NaCl. Only the fractions containing isolectin I were pooled, concentrated, thoroughly dialyzed against water, and concentrated as described for the tobacco isolectin. Uniformity of the isolectin was examined by ESI-MS. For antifungal assays, the UDA isolectin preparation was filter-sterilized. MALDI-MS and ESI-MS were performed as described by Does et al. (1999).

Agglutination Assays

Agglutination by crude leaf extracts was performed on microscope glasses with squashed leaves to which trypsin-treated rabbit erythrocytes (Peumans et al., 1984) and PBS were added. After agglutination had occurred the soluble contents were transferred to a 96-well microtiter dish and photographed.

To test for agglutination activity, different concentrations of purified UDA in PBS were added to 30 μL of trypsin-treated rabbit erythrocytes in small glass test tubes. PBS was added to a final volume of 60 μL. Agglutination was scored after 30 min of incubation at room temperature.

In the agglutination-inhibition assay, 0.3 μg of UDA or 0.5 μg of concanavalin A was added to 30 μL of trypsin-treated rabbit erythrocytes to which 5 μL of a chitin suspension of 1 mg mL^{-1} was added. PBS was added to a final volume of 60 μL. After agglutination had occurred the mixtures were transferred to a 96-well microtiter dish and photographed.

In Vitro Antifungal Assay

Botrytis cinerea and *Trichoderma viride* strains were obtained from Zeneca Mogen (Leiden, The Netherlands). The *Colletotrichum lindemuthianum* UPS9 strain was obtained from Dr. M. Dufresne (Laboratoire de Phytopathologie Moléculaire, Université Paris Sud). *B. cinerea* and *T. viride* were kept on potato dextrose agar (Difco Laboratories, Detroit, MI) and cultured in the dark at 20°C and 23°C, respectively. To obtain fresh spores of *C. lindemuthianum*, 2 × 10^5 spores were plated onto potato dextrose agar and cultured at 23°C in the dark.

Spores were harvested after 5 weeks (*B. cinerea*), 2 weeks (*T. viride*), or 3 weeks (*C. lindemuthianum*) by flooding the agar plates with sterile water and filtering through three layers of Miracloth (Calbiochem). Spores were stored at −80°C at a concentration of 1.8 × 10^5 spores mL^{-1} in 15% glycerol. In germination tests, 99% of the *B. cinerea* spores, 25% of the *T. viride* spores, and 50% of the *C. lindemuthianum* spores appeared to germinate. Before antifungal assays, spores were diluted to a concentration of 1.2 × 10^4 spores mL^{-1} (*B. cinerea* and *C. lindemuthianum*) or 4.8 × 10^4 spores mL^{-1} (*T. viride*).

Assays were performed in 48-well microtiter dishes as described previously (Woloshuk et al., 1991). Wells were filled with 125 μL of potato dextrose agar, on top of which 25 μL of the spore suspension was layered. For pregermination of the *B. cinerea* spores, the microtiter dish was kept in the dark on ice in a 4°C cold room for 12 h and then incubated at 23°C for 3.5 to 4 h in the dark. UDA was added when ≥95% of the spores were germinated and the length of the germ tubes was 1 to 4 times the size of the spores. Pregermination of *T. viride* spores occurred at 23°C in the dark after 16 h. UDA was added when germ tube emergence was observed. Pregermination of *C. lindemuthianum* spores occurred after 12 to 14 h overnight at 14°C. For further germination, the microtiter dish was kept at 23°C in the dark for another 2.5 h. UDA was added when 10% of the spores showed germ tube emergence.

UDA (2.5, 5.0, and 22.5 μg) and BSA (22.5 μg) were added to the germinated spores as 50-μL solutions in water to obtain the final UDA concentrations of 33, 67, and 300 μg mL^{-1}, respectively, and 300 μg BSA mL^{-1}. Images of fungal growth at 0, 2, 4, 6, 24, 48, and 72 h were acquired with an inverted light microscope (model IMT-2, Olympus) equipped with a cooled CCD (charge-coupled device) camera (model DDE/3200, Astromed, Cambridge, UK). The image-processing package ScilImage (Ten Kate et al., 1990) was used to measure hyphal length. Overall fungal-growth inhibition was estimated by two persons independently.

RESULTS

Processing and Localization of UDA in Tobacco

We previously isolated a gene encoding the precursor to UDA isolectin I from the stinging nettle ecotype Weerselo (Does et al., 1999). Mature UDA was processed from the precursor by cleavage between the signal peptide and the first chitin-binding domain and by an unknown processing event located C-terminally of the second chitin-binding domain (Fig. 1A). To study the subcellular localization of mature UDA, the gene was placed behind the cauliflower mosaic virus 35S promoter (the wild-type construct) and transformed into tobacco.

For detection of UDA in transgenic tobacco, α-UDA antibodies were raised against a synthetic peptide corresponding to 15 amino acids of mature isolectin I (Fig. 1B). These antibodies were used to detect UDA migrating at the predicted size in total leaf extracts from different tobacco lines by western analysis (Fig. 2A). Some cross-reactivity with larger proteins was found. In nontransformed control plants, α-UDA antibodies cross-reacted with the larger proteins only, and proteins with a size corresponding to that of UDA were not detected. Crude extracts from several transgenic lines showed agglutination activity on trypsin-treated rabbit erythrocytes (Fig. 3A). Agglutination was not observed in the control extract.

Intracellular localization of UDA was examined by comparison of the total extract, the extracellular washing fluid, and the remnant extract of leaves of different tobacco lines expressing UDA. Equal amounts of proteins from the total extract, the extracellular washing fluid, and the remnant
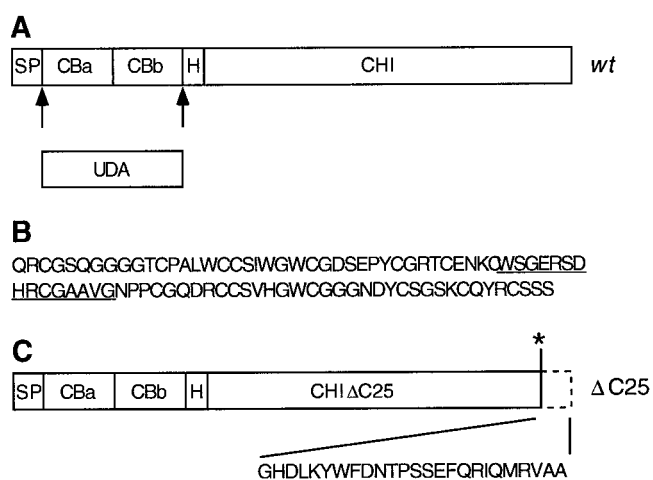

Figure 1. A, Model for processing of the wild-type (*wt*) UDA precursor to mature UDA. SP, Signal peptide; CBa and CBb, chitin-binding domains a and b; H, hinge region; CHI, chitinase domain. B, Amino acid sequence of UDA isolectin I from stinging nettle rhizomes. The sequence of the synthetic peptide used for antibody production is underlined. C, The C-terminal 25 amino acids are deleted from the targeting mutant precursor ΔC25 by introduction of a stop codon (*) in the encoding DNA sequence.

extract were used in SDS-PAGE. The presence of UDA was determined immunologically. The results obtained with a high expressor are shown in Figure 4A. In the extracellular washing fluid of this plant, a very low signal for UDA was detected compared with the total extract and the remnant extract. Similar results were obtained for other plants. These results indicate that plants transformed with the wild-type construct retain most of the produced UDA intracellularly.

The subcellular localization of UDA in mature tobacco leaves was further assessed by the isolation of protoplasts and vacuoles. Similar amounts of UDA were detected in an equal number of purified leaf mesophyll protoplasts and vacuoles (Fig. 4A). These results suggest a vacuolar localization of UDA in plants producing the wild-type precursor.

Extracellular Targeting of UDA

To study the presence of a vacuole-targeting determinant at the C terminus of the UDA precursor, targeting mutant construct ΔC25 was transformed into tobacco. This mutant construct, which encodes a precursor lacking the C-terminal 25 amino acids, was created by introduction of a stop codon in the encoding DNA (Fig. 1C). Extracts of tobacco leaves expressing the mutant construct revealed the presence of mature-sized UDA (Fig. 2B) and agglutination activity took place on the rabbit erythrocytes (Fig. 3A). The total extract, extracellular washing fluid, and remnant extract were also examined from a high-expressor ΔC25 line (Fig. 4B). Compared with the total extract, the extracellular washing fluid fraction was enriched in isolectin. In contrast, only a small amount of UDA was detected in the remnant extract. Evidently the removal of the C-terminal
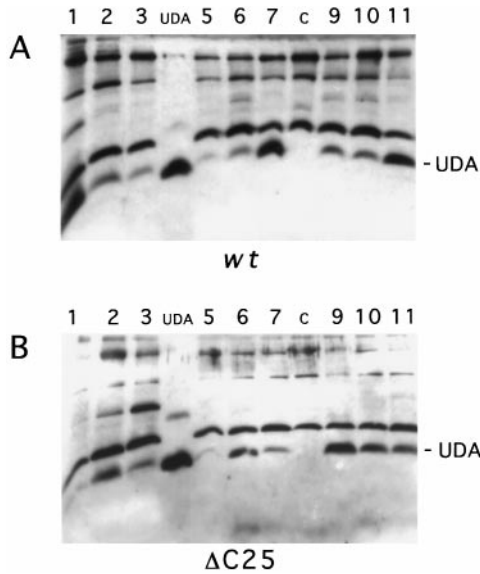
Figure 2. Detection of mature-sized UDA by western analysis of 2.55 μg of protein from the total extracts of leaves from different tobacco lines expressing the wild-type (*wt*) (A) or the ΔC25 mutant precursor construct (B) using α-UDA antibodies. Lanes 1 to 3, 5 to 7, and 9 to 11, Transgenic lines; lanes c, control total extract of nontransformed tobacco; and lanes UDA, 50 ng of UDA-isolectin mixture from stinging nettle rhizomes.

25 amino acids of the UDA precursor caused the secretion of UDA into the intercellular space. The mutant precursor appeared to be processed to the mature UDA size.

## Correct Processing of Extracellularly Targeted UDA in Tobacco

Closely related UDA isolectins can be identified accurately by mass determination using MALDI-MS or ESI-MS as described previously (Does et al., 1999). This technique was therefore used to investigate the processing of UDA isolectin I in tobacco. The isolectin was purified from the extracellular washing fluid of leaves of the homozygous high-expressor line ΔC25–33.14. Mass determination by MALDI-MS showed that the molecular mass of the tobacco extracellular washing fluid isolectin was identical to the theoretical mass (Table I). Since the isolation of UDA from extracellular washing fluid was inefficient, the isolectin was purified from a total extract of the ΔC25–33.14 line in subsequent experiments. At low temperature (4°C), purified UDA appeared to precipitate at concentrations higher than 0.7 μg μL^{-1}. We also observed a decrease in the agglutination activity of the resolved purified isolectin after lyophilization. Therefore, unlyophilized UDA was used and stored at concentrations of ≤0.6 μg μL^{-1} water.

Purity of the isolectin was examined by western analysis and SDS-PAGE, followed by silver staining (not shown). Mass determination again showed a pure, homogeneous, and correctly processed isolectin (Table I). The same isolectin was purified from stinging nettle and homogeneity was verified using ESI-MS (Table I). The results showed the presence of a single molecule in samples of isolectin I purified from stinging nettle and tobacco (data not shown). The absence of molecules with "ragged ends" indicates that extracellularly targeted UDA was correctly processed at its C terminus in transgenic tobacco. UDA contains 16 Cys residues, which are involved in eight disulfide bridges (Beintema, 1994). Formation of one disulfide bridge causes a 2-D decrease in mass because of the loss of one hydrogen atom for each Cys residue involved. The determined mass differed from the theoretical mass by only 0.1 D (Table I), which suggests that all of the disulfide bridges were present in the tobacco-derived UDA.
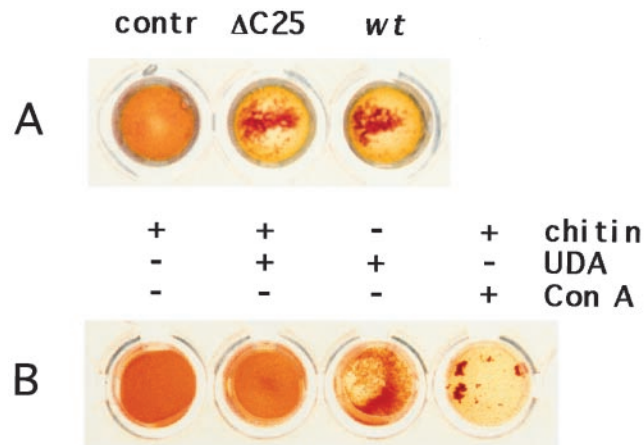
Figure 3. Tobacco-produced UDA agglutinates erythrocytes and binds to chitin. A, Agglutination of trypsin-treated rabbit erythrocytes by crude leaf extracts of tobacco expressing a wild-type (*wt*) or a ΔC25 mutant precursor construct but not by a crude extract of nontransformed tobacco (contr). B, Agglutination of trypsin-treated rabbit erythrocytes by 0.3 μg of UDA purified from tobacco was inhibited by the addition of chitin; agglutination by 0.5 μg of concanavalin A (Con A) was not inhibited.
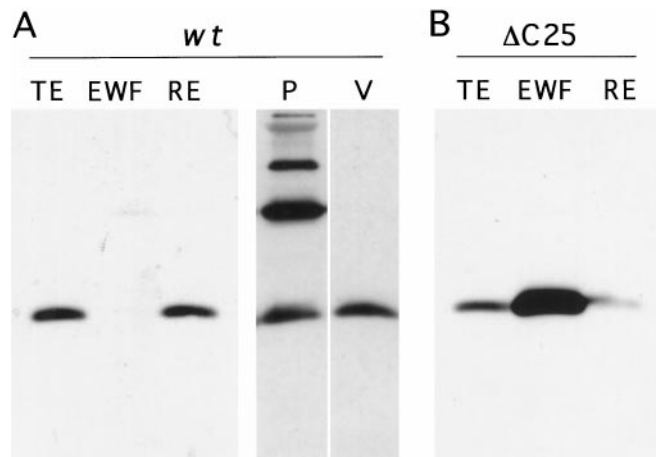
Figure 4. Subcellular localization of UDA in tobacco expressing the wild-type (*wt*) (A) or the ΔC25 mutant precursor construct (B) by western analysis using α-UDA antibodies. A, Analysis of 9 μg of protein from the total extract (TE), extracellular washing fluid (EWF), and remnant extract (RE): 1.8×10^3 protoplasts (P) and proteins of 1.8×10^3 vacuoles (V) of a wild-type precursor tobacco line. B, Analysis of 9 μg of protein from the total extract, extracellular washing fluid, and remnant extract of a ΔC25 mutant precursor line.

Table I. *Molecular mass determination of UDA isolectin I purified from stinging nettle and transgenic tobacco*

UDA Isolectin I Source	Molecular Mass		
	Theoretical[a]	MALDI[b]	ESI[c]
			D
Stinging nettle	9382.5	ND[d]	9382.1 ± 1.3
			9380.7 ± 0.4
			9382.3 ± 1.4
Tobacco			
Extracellular washing fluid	9382.5	9382.6	ND
Total extract	9382.5	ND	9382.1 ± 0.8
			9382.0 ± 0.7

[a] Theoretical average mass of UDA isolectin I has been corrected for the N-terminal pyroglutamate (Chapot et al., 1986) and for the presence of disulfide bridges. [b] MALDI masses were corrected for protonization of the proteins during the experiment (–1 D). MALDI was internally calibrated. [c] Results of individual experiments. [d] ND, Not determined.

Agglutination and Chitin-Binding Activity of Purified UDA

Agglutination activity of the purified tobacco isolectin was similar to that of the genuine isolectin I. Trypsin-treated rabbit erythrocytes agglutinated best with isolectin concentrations of 5 μg mL^{-1} or more. Little agglutination was observed at a concentration of 2.5 μg mL^{-1}. The UDA

purified from tobacco was still bound to chitin, since the addition of chitin inhibited agglutination (Fig. 3B). Chitin did not directly inhibit the ability of the erythrocytes to agglutinate, since agglutination by the nonchitin-binding concanavalin A was still visible in the presence of chitin (Fig. 3B).

Antifungal Activity of UDA Isolectin I

UDA displays antifungal activity on several plant pathogenic fungi that contain chitin in their cell walls (Broekaert et al., 1989). We compared the antifungal activity of different amounts of tobacco UDA with the same isolectin from stinging nettle in assays on germinated spores of *B. cinerea*, *T. viride*, and *C. lindemuthianum*. Growth of the fungi was examined by inverted light microscopy, and images were obtained using a CCD camera at several times after the addition of the isolectin. BSA was used as a control. No differences in growth inhibition were detected between the tobacco-produced and the authentic UDA isolectin I from stinging nettle. The distinct sensitivity of the three fungi to UDA appeared to be time and concentration dependent, as shown in Table II.

Antifungal Activity on *B. cinerea*

Upon germination, *B. cinerea* grew fast and reached a length of 8 to 15 times that of the spores within 4 h and 20

Table II. *In vitro antifungal activity of UDA on different plant-pathogenic fungi containing chitin in their cell walls*

Different amounts of UDA isolectin I purified from the total extract of a ΔC25 tobacco line or from stinging nettle were added to pregerminated spores of *B. cinerea*, *T. viride*, and *C. lindemuthianum*. Fungal growth was monitored by inverted light microscopy. BSA was used as a control. The final concentrations of UDA and BSA in water are shown. Growth inhibition is the percentage of growth compared with the water control (no UDA or BSA added).

UDA Isolectin I	BSA	Time after Addition	Growth Inhibition		
			B. cinerea	*T. viride*	*C. lindemuthianum*
μg mL^{-1}		h		%	
0	0		0	0	0
33	0	2	0	0	0
		4	0	0	0
		6	0	10–20	0
		24	0	0	0
		48	ND[a]	ND	0
67	0	2	20	0	0
		4	5–10	0	0
		6	0	20–30	0
		24	0	0	10–30
		48	ND	ND	30
300	0	2	50–60	0	0
		4	50	10–20	0
		6	30–40	40–50	0
		24	0	30–40	50–60
		48	ND	0	80–90
0	300	2	0	0	0
		4	0	0	0
		6	0	0	0
		24	0	0	20
		48	ND	0	30

[a] ND, Not determined.

to 30 times greater within 6 h. Two hours after the addition of UDA, growth was inhibited approximately 20% by a concentration of 67 μg mL^{-1}. Compared with the control, growth inhibition of 50% to 60% was observed upon the addition of 300 μg mL^{-1} isolectin. However, inhibition declined after 4 h; after 6 h, some growth inhibition was still detectable only at the highest concentration. No differences from the control could be observed after 24 h. BSA did not visibly affect fungal growth.

Antifungal Activity on *T. viride*

Hyphae of *T. viride* grew to 5 to 8 times the size of the spores within 4 h and 20 to 30 times greater within 6 h. Compared with *B. cinerea*, *T. viride* appeared to be more sensitive to UDA but had a later response time. After 6 h, a growth inhibition of 10% to 20% and 20% to 30% was observed for 33 and 67 μg mL^{-1} isolectin, respectively. A concentration of 300 μg mL^{-1} isolectin inhibited growth within 4 h. In contrast to *B. cinerea*, growth inhibition increased after 2 h to between 40% and 50%. The effect of 300 μg mL^{-1} isolectin I was still visible after 24 h, because the surface of the agar was less covered with fungi than that in the control well. BSA did not affect the growth of *T. viride*.

Antifungal Activity on *C. lindemuthianum*

The most dramatic effects of UDA were observed for *C. lindemuthianum*. In contrast to *B. cinerea* and *T. viride*, *C. lindemuthianum* grew slowly and its hyphae reached a length of only 5 to 7 times the size of the spores within 6 h. No growth inhibition at all was detected before this time. By 24 h, 67 and 300 μg mL^{-1} isolectin had caused inhibited, altered hyphal growth. The addition of 300 μg mL^{-1} UDA caused severely stunted fungal growth. Compared with the control, a similar number of branches had been created but at much shorter distances from each other. Within 48 h, growth inhibition increased. However, in contrast to the other two fungi, *C. lindemuthianum* also showed some inhibited growth in the presence of 300 μg mL^{-1} BSA, with phenotypic differences similar to those shown for 67 μg mL^{-1} isolectin I. This indicates that the fungus might be sensitive to proteinous agents. However, it is clear that most (approximately 75%) of the observed growth inhibition of *C. lindemuthianum* was caused by UDA. Because of aerial growth and spore formation, we were not able to detect differences in growth after 72 h.

DISCUSSION

Correct Processing of UDA in Transgenic Tobacco

Heterologous plant systems have been used successfully in the past for protein production and vacuole-targeting studies. Several seed-specific storage proteins from different plant species are correctly processed, assembled, and transported to protein storage vacuoles or deposited invacuole-derived protein bodies in tobacco seed (Greenwood and Chrispeels, 1985; Bäumlein et al., 1987; Sturm et al., 1988; Altenbach et al., 1989; De Clercq et al., 1990; Saalbach et al., 1991; Holwerda et al., 1992). In tobacco leaves, certain heterologous seed-specific proteins have been shown to be targeted to vacuoles but are rapidly degraded or incorrectly processed (Beachy et al., 1986; Saalbach et al., 1996). Barley lectin, patatin, and sporamin and kiwifruit actinidin are specifically produced in roots, tubers, tuberous roots, and fruit, respectively (Racusen and Foote, 1980; Mishkind et al., 1983; Hattori et al., 1985; Preakelt et al., 1988). These proteins are targeted to the vacuoles in tobacco leaves as well and, with the exception of sporamin, processed to their mature sizes (Sonnewald et al., 1989; Matsuoka et al., 1990; Wilkins et al., 1990; Paul et al., 1995). Sporamin is processed to a protein that is three amino acids longer at its N terminus than authentic sporamin (Matsuoka et al., 1990).

We used tobacco plants to study the targeting of UDA, which is normally present in the roots and rhizomes of stinging nettle. Constructs containing the gene encoding the UDA-isolectin I precursor were expressed in tobacco plants under the control of the cauliflower mosaic virus 35S promoter. The results show that the UDA precursor is processed to its mature size. The determined molecular mass of UDA isolectin I isolated from transgenic ΔC25 tobacco leaves matches the theoretical mass and the mass of authentic isolectin I from stinging nettle. Therefore, we conclude that UDA is processed correctly in transgenic tobacco.

It is unknown whether cleavage of the UDA precursor occurs with a single cleavage near the hinge region or in a stepwise fashion, removing parts from the C-terminal end of the precursor. In contrast to mature UDA, the UDA precursor and the C-terminal chitinase domain could not be detected on immunoblots of extracts from tobacco leaves using α-UDA antibodies or antibodies that had been raised against the bacteria-produced chitinase domain of the UDA precursor (not shown). This could be explained by a rapid processing of the UDA precursor and subsequent degradation of the chitinase domain. Processing of the UDA precursor might be similar to processing of the hevein precursor. Soedjanaatmadja and coworkers (1995) showed that the hevein precursor is processed to mature hevein by the removal of a C-terminal propeptide followed by the removal of the 14-kD PR4-like domain. In the lutoid-body fraction of rubber latex, both the hevein precursor and the PR4-like domain have been found in a molar ratio of 1:30 relative to hevein, which indicates rapid degradation of the PR4-like domain (Soedjanaatmadja et al., 1995). However, it is possible that the extraction buffer used for leaf extraction was unsuitable for isolation of the UDA precursor and the chitinase domain.

Vacuolar and Extracellular Targeting of UDA

In tobacco transformed with the wild-type construct, UDA is detected intracellularly, most likely in the vacuoles, suggesting a vacuolar localization of this isolectin in stinging nettle roots and rhizomes. Unlike the N-terminal part of the chitinase domains of the UDA precursor and plant chitinases, the sequence of the C-terminal 25 amino acids of

the UDA precursor is not similar to the C termini of any known plant chitinase (Fig. 5A). We imagined that this C-terminal region of the UDA precursor might be involved in the vacuolar targeting of UDA. To examine the presence of a vacuole-targeting signal in the C terminus of the UDA precursor, ΔC25, a mutant encoding a precursor lacking the C-terminal 25 amino acids, was constructed.

In plants transformed with the ΔC25 construct, UDA was present in the extracellular washing fluid. In contrast, in the extracellular washing fluid of tobacco plants producing the wild-type precursor, UDA was not observed. Thus, within the C-terminal segment of 25 amino acids of the UDA precursor, a signal is present that retains UDA intracellularly, presumably in the vacuoles. However, in both protoplasts and vacuoles isolated from leaves of a ΔC25 line, low amounts of UDA were reproducibly detected (not shown). It is possible that these protoplast and vacuole fractions were contaminated with extracellular UDA. Another possibility is that the deletion of the C-terminal sorting signal causes only partial secretion of the isolectin, which would suggest the presence of additional vacuole-targeting determinants within the precursor. A possible candidate harboring such a determinant is the hinge region, which is located C-terminally of the mature UDA sequence.

This hinge region shows similarity to the vacuole-targeting propeptides located C-terminally of mature wheat germ agglutinin (Fig. 5B) and mature barley lectin. The homologous amino acid sequences might cause partial sorting of UDA to the vacuoles. It is possible that the hinge region of the UDA precursor is evolutionarily related to the C-terminal vacuole-targeting propeptides of the Gramineae prolectins. UDA and the four hevein-domain lectins have been hypothesized to be products of duplications of an ancestral single-hevein domain (Wright et al., 1991).

Shinshi et al. (1990) postulated that a hevein domain containing chitinases and prohevein-like proteins arose by transposition of the hevein domain in front of an ancestral chitinase sequence. In accordance with these hypotheses, the hinge region might have been introduced by translocation together with UDA into the UDA precursor genes during evolution.

In contrast to N-terminal propeptides, C-terminal propeptides do not share homology, a common motif, or a specific conformation (Dombrowski et al., 1993; Nakamura and Matsuoka, 1993). The exact sequence harboring the C-terminal vacuole-targeting determinant of the UDA precursor and the question of whether this sequence is sufficient for proper vacuolar targeting of UDA remains to be elucidated.

Both vacuolar and extracellular targeted UDA were correctly processed, indicating that the C-terminal vacuole-sorting information on the UDA precursor is not required for correct processing to mature UDA. In plant cells, proteins that are destined for secretion and soluble vacuolar proteins that harbor positive targeting information are transported from the ER to the Golgi apparatus (for review, see Neuhaus and Rogers, 1998). In the Golgi, vacuolar proteins are sorted from the secretory proteins by means of their targeting information. Subsequently, secretory proteins and vacuolar proteins are further transported in specific vesicles (for review, see Neuhaus and Rogers, 1998). In general, processing of vacuolar proteins occurs after their arrival in vacuoles or prevacuolar compartments other than the Golgi or ER (for review, see Müntz, 1998). Both the wild type and the mutant UDA precursor might be processed in the Golgi apparatus after they have been sorted into the different transport vesicles. If so, both the vesicles carrying the secretory UDA and the vesicles carrying the vacuolar UDA should also contain active proteases.
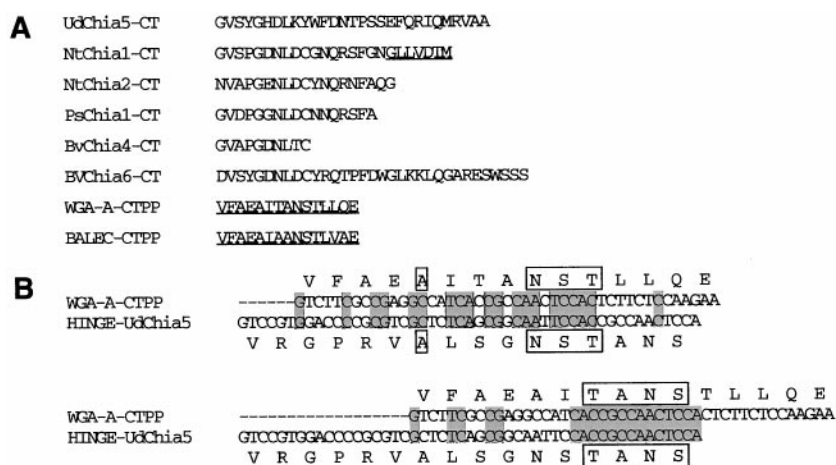
Figure 5. Alignments of C-terminal (CT) protein sequences of the UDA precursor (UdChia5) with C-terminal sequences of several (pro)chitinases and prolectins (A), and homology of the hinge region of the UDA-precursor with the C-terminal propeptide (CTPP) of wheat germ agglutinin A (WGA-A) at the DNA level and protein level (B). Two possible alignments are given. Sequences derived from published data are: UdChia5, Does et al. (1999); NtChia1, Shinshi et al. (1990); wheat germ agglutinin A, Smith and Raikhel (1989); barley lectin (BALEC), Lerner and Raikhel (1989). Other sequences were derived from the EMBL nucleotide database under the following accession numbers: NtChia2 from tobacco, M29869; PsChia1 from pea, X63899; and BvChia4 and BVChia6 from beet, L25826 and X79301, respectively. Known propeptide sequences are underlined, homologous nucleotide sequences are shaded, and homologous amino acid sequences are boxed.

Some vacuolar storage proteins have been reported to be transported with their specific maturation proteases in the same vesicle (for review, see Robinson et al., 1998). However, these proteases appeared to be in an inactive form until they had been deposited in the (pre)vacuoles (for review, see Robinson et al., 1998). Alternatively, the wild-type precursor and mutant precursor may be processed by proteases in the (pre)vacuoles and in extracellular space, respectively. In both cellular compartments the same type of proteases that are involved in the processing of vacuolar storage proteins or the degradation of pathogenesis-related proteins may be present, as has been shown for aspartic proteinases (Rodrigo et al., 1991; Runeberg-Roos et al., 1994; Hiraiwa et al., 1997). It is even possible that processing of the UDA precursors occurs by aspecific proteases, since the tertiary structure of the UDA molecule may protect mature UDA from further degradation. Additional experiments may elucidate the subcellular trafficking route and the processing of the UDA precursor.

Both Antifungal Activity of UDA and Adaptation of the Fungus Depend on the Phase of Fungal Growth

Purified UDA isolectin I from leaves of a ΔC25 tobacco line agglutinated red blood cells as effectively as purified UDA isolectin I from stinging nettle and bound to chitin in an agglutination-inhibition assay. In in vitro antifungal assays, UDA from transgenic tobacco and from stinging nettle displayed identical antifungal activities on germlings of the chitin-containing plant pathogenic fungi *B. cinerea*, *T. viride*, and *C. lindemuthianum*.

Little is known about the mechanism of fungus-growth inhibition by UDA. Unlike plant chitinases, mature UDA does not display hydrolase activity and is not able to lyse the cell walls of fungi that contain chitin (Schlumbaum et al., 1986; Mauch et al., 1988; Broekaert et al., 1989; Sela-Buurlage et al., 1993). It has been suggested that instead, because of its small size, UDA penetrates the cell wall and affects cell wall synthesis (Broekaert et al., 1989; Van Parijs et al., 1992).

We have presented evidence that inhibition of hyphal growth by UDA is only temporal and depends on the phase of accelerated growth. *B. cinerea* showed fast hyphal growth within the first 2 h after germination, and maximal growth inhibition by UDA had probably occurred within this period, since it declined within the next few hours. Similarly, *T. viride* showed accelerated growth and maximal growth inhibition by UDA between 4 and 6 h after germination. *C. lindemuthianum* grew the most slowly. No effect of the addition of UDA was detected in the first few hours, whereas maximal growth inhibition occurred at approximately 48 h. After 24 h and after 48 h, hyphal growth of *C. lindemuthianum* showed a phenotype of a severely stunted hyphae. Because of the time that is available to penetrate the cell wall and bind to chitin, a slow grower such as *C. lindemuthianum* is probably more affected by UDA. However, the growth inhibition of *C. lindemuthianum* is not only because of the biological action of UDA; it also appears to be sensitive to the addition of large amounts of protein; a concentration of 300 μg BSA mL^{-1} caused phenotypic differences similar to those caused by 67 μg UDA mL^{-1}.

It is not clear why the fungi showed increased sensitivity to UDA only at a specific phase of fast growth. The temporality of the inhibition might be explained by the adaptation of the fungal cell wall. Fungal adaptation to a chitin-binding protein has been demonstrated previously (Sela-Buurlage, 1996). Lysis of *Fusarium solani* by tobacco chitinase I appears to depend on the phase of germination of the macroconidia. Neither the first added chitinase, which remained active during the assays, nor a second boost of chitinase has been shown to be able to lyse germ tubes if the first amount was added to the macroconidia at an early stage of pregermination. These results indicate that the macroconidia were able to adapt to chitinase (Sela-Buurlage, 1996). It is possible that UDA alters the hyphal cell wall architecture of *B. cinerea*, *T. viride*, and *C. lindemuthianum*, as exemplified in *Phycomyces blakesleeanus* germlings treated with UDA (Van Parijs et al., 1992). A change in cell wall composition might result in the resistance of fungi to UDA.

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