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(54) PLANTES MODIFIEES GENETIQUEMENT AVEC L'INHIBITEUR DE LA PROTEINASE HETEROGENE SAPIN2A ET METHODES POUR L'UTILISER AFIN D'INHIBER LES ACTIVITES DE TYPE TRYPSINE ET CHYMOTRYPSINE ENDOGENES

(54) GENETICALLY MODIFIED PLANTS WITH HETEROGENEOUS PROTEINASE INHIBITOR SAPIN2A AND METHODS OF USE THEREOF FOR THE INHIBITION OF ENDOGENOUS TRYPSIN-AND CHYMOTRYPSIN-LIKE ACTIVITIES

(57)

The present invention relates to a proteinase inhibitor II cDNA, SaPIN2a from *Solanum americanum*, and its encoded protein product or a fragment thereof. The present invention also relates to genetically modified plants, and in particular genetically modified lettuce. The genetically modified plants have an inhibited endogenous protease activity, such as inhibited trypsin-like and chymotrypsin-like activities following transformation of the plant with a vector comprising a proteinase inhibitor II cDNA, SaPIN2a, using *Agrobacterium*-mediated transformation. The invention further relates to a recombinant vector that transforms lettuce so as to confer upon the lettuce inhibited endogenous trypsin-like and chymotrypsin-like activities. The vector comprises a proteinase inhibitor II cDNA, SaPIN2a with a CaMV 35S promoter. The invention further relates to a method of producing a plant with inhibited endogenous trypsin-like and chymotrypsin-like activities by expressing a proteinase inhibitor II cDNA. The invention also relates to methods of enhanced resistance of plants to pests or pathogens, including insects. In specific embodiments, the transgenic plants have enhanced resistance to cabbage looper (*Trichoplusia ni*). The invention further relates to a method of protection of heterogeneous protein production in transgenic plants.



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(54) **Titre :** PLANTES MODIFIEES GENETIQUEMENT AVEC L'INHIBITEUR DE LA PROTEINASE HETEROGENE  
SAPIN2A ET METHODES POUR L'UTILISER AFIN D'INHIBER LES ACTIVITES DE TYPE TRYPSINE ET  
CHYMOTRYPSINE ENDOGENES

(54) **Title:** GENETICALLY MODIFIED PLANTS WITH HETEROGENEOUS PROTEINASE INHIBITOR SAPIN2A AND  
METHODS OF USE THEREOF FOR THE INHIBITION OF ENDOGENOUS TRYPSIN-AND CHYMOTRYPSIN-LIKE  
ACTIVITIES

(57) **Abrégé/Abstract:**

The present invention relates to a proteinase inhibitor II cDNA, SaPIN2a from *Solanum americanum*, and its encoded protein product or a fragment thereof. The present invention also relates to genetically modified plants, and in particular genetically modified lettuce. The genetically modified plants have an inhibited endogenous protease activity, such as inhibited trypsin-like and chymotrypsin-like activities following transformation of the plant with a vector comprising a proteinase inhibitor II cDNA, SaPIN2a, using *Agrobacterium*-mediated transformation. The invention further relates to a recombinant vector that transforms lettuce so as to confer upon the lettuce inhibited endogenous trypsin-like and chymotrypsin-like activities. The vector comprises a proteinase inhibitor II cDNA, SaPIN2a with a CaMV 35S promoter. The invention further relates to a method of producing a plant with inhibited endogenous trypsin-like and chymotrypsin-like activities by expressing a proteinase inhibitor II cDNA. The invention also relates to methods of enhanced resistance of plants to pests or pathogens, including insects. In specific embodiments, the transgenic plants have enhanced resistance to cabbage looper (*Trichoplusia ni*). The invention further relates to a method of protection of heterogeneous protein production in transgenic plants.



**ABSTRACT**

The present invention relates to a proteinase inhibitor II cDNA, *SaPIN2a* from *Solanum americanum*, and its encoded protein product or a fragment thereof. The present invention also relates to genetically modified plants, and in particular genetically modified lettuce. The genetically modified plants have an inhibited endogenous protease activity, such as inhibited trypsin-like and chymotrypsin-like activities following transformation of the plant with a vector comprising a proteinase inhibitor II cDNA, *SaPIN2a*, using *Agrobacterium*-mediated transformation. The invention further relates to a recombinant vector that transforms lettuce so as to confer upon the lettuce inhibited endogenous trypsin-like and chymotrypsin-like activities. The vector comprises a proteinase inhibitor II cDNA, *SaPIN2a* with a CaMV 35S promoter. The invention further relates to a method of producing a plant with inhibited endogenous trypsin-like and chymotrypsin-like activities by expressing a proteinase inhibitor II cDNA. The invention also relates to methods of enhanced resistance of plants to pests or pathogens, including insects. In specific embodiments, the transgenic plants have enhanced resistance to cabbage looper (*Trichoplusia ni*). The invention further relates to a method of protection of heterogeneous protein production in transgenic plants.

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**GENETICALLY MODIFIED PLANTS WITH HETEROGENEOUS  
PROTEINASE INHIBITOR SAPIN2A AND METHODS OF USE THEREOF FOR  
THE INHIBITION OF ENDOGENOUS TRYPSIN-AND  
CHYMOTRYPSIN-LIKE ACTIVITIES**

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**1. FIELD OF INVENTION**

The present invention relates to a proteinase inhibitor II cDNA, *SaPIN2a* from *Solanum americanum*, and its encoded protein product or a fragment thereof. The present invention also relates to genetically modified plants, and in particular genetically modified lettuce. The genetically modified plants have inhibited endogenous trypsin-like and chymotrypsin-like activities following transformation of the plant with a vector comprising a proteinase inhibitor II cDNA, *SaPIN2a*, using *Agrobacterium*-mediated transformation. The invention further relates to a recombinant vector that transforms lettuce so as to confer upon the lettuce inhibited endogenous trypsin-like and chymotrypsin-like activities. The vector comprises a proteinase inhibitor II cDNA, *SaPIN2a* with a CaMV 35S promoter. The invention further relates to a method of producing a plant with inhibited endogenous trypsin-like and chymotrypsin-like activities by expressing a proteinase inhibitor II cDNA. The invention further relates to transgenic plants having enhanced resistance to insects. In specific embodiments, the transgenic plants have enhanced resistance to cabbage looper (*Trichoplusia ni*). The invention also relates to methods of enhanced resistance of plants to pests or pathogens, including insects. The invention further relates to a method of protection of heterogeneous protein production in transgenic plants by the co-expression of proteinase inhibitor *SaPIN2a* that has demonstrated trypsin-like and chymotrypsin-like inhibitor activities.

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**2. BACKGROUND**

A common opinion is that most known plant proteinase inhibitors (PIs) do not inhibit endogenous plant proteases but have specificities for animal or microbial enzymes (Laskowski and Sealock, 1971; Ryan, 1981; Sanchez-Serrano *et al.*, 1986). Therefore the function of plant proteinase inhibitors was thought to be in the prevention of invasion through inhibition of foreign proteolytic enzymes of pest or pathogen (Ryan, 1989; Brzin and Kidric, 1995). These observations may result from the fact that most studies use commercially available proteases, e.g. trypsin, chymotrypsin, elastase, and subtilisin from animal or microbial sources, as test enzymes in activity assays (Laskowski and Kato, 1980; Brzin and Kidric, 1995). However, none of these proteases are likely to be the true physiological target for these characterized plant PIs (Laskowski and Kato, 1980).

Reports on the developmental regulation and tissue-specific accumulation of plant PIs (Rosahl *et al.*, 1986; Sanchez-Serrano *et al.*, 1986; Margossian *et al.*, 1988; Hendriks *et al.*, 1991; Pena-Cortes *et al.*, 1991; Lorberth *et al.*, 1992) do suggest they have endogenous functions. A soybean cysteine proteinase inhibitor has been designated a novel  
5 role in modulating programmed cell death (Solomon *et al.*, 1999) and another, from cucumber leaves, which does not significantly inhibit commercial proteases of animal or microbial origin, inhibited cucumber glutamyl endopeptidase (CGEP) suggesting a role in the regulation of CGEP activity (Yamauchi *et al.*, 2001).

Proteinase inhibitor II from *Solanum americanum* (SaPIN2a) is highly  
10 expressed in the phloem and has possible involvement in regulating proteolysis in the sieve elements (Xu *et al.*, 2001). The localization of *SaPIN2a* mRNA and protein to the companion cells and sieve elements suggests regulation of proteolysis in phloem development/function.

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### 3. SUMMARY

The present invention is based, in part, on the isolation of cDNA encoding *SaPIN2a* (SEQ ID NO:1) from a *Solanum americanum* cDNA library using a tomato proteinase inhibitor II (PIN2) cDNA as hybridization probe (Xu *et al.*, 2001, *Plant Molecular Biology* 47:727-738, which is incorporated by reference in its entirety) by the  
20 present inventors. Accordingly, the present invention relates to a nucleotide sequence of SEQ ID NO:1 and a nucleotide sequence that encodes SaPIN2a having an amino acid sequence of SEQ ID NO:2. In another embodiment, the present invention relates to a nucleotide sequence that hybridizes under stringent conditions to the complement of a nucleotide sequence of SEQ ID NO:1. By way of example and not limitation, high  
25 stringency hybridization conditions can be defined as follows: The filter-bound DNA were hybridized in a solution containing 50% deionized formamide, 6xSSC, 5x Denhardt's, 1% SDS, 100 µg/ml denatured salmon sperm DNA at 42°C overnight (about 4-16 hours), and washing in 0.1xSSC, 0.1% SDS at 65°C (Ausubel F.M. *et al.*, eds., 1989, *Current Protocols in Molecular Biology*, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons,  
30 Inc., New York).

The present invention further provides an isolated SaPIN2a polypeptide (SEQ ID NO:2) or a fragment thereof. In another embodiment, the present invention provides isolated polypeptides that are encoded by a nucleic acid molecule comprising a nucleotide sequence of SEQ ID NO:1 or a fragment thereof, or a nucleotide sequence that  
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hybridizes under stringent conditions to the complement of a nucleotide sequence of SEQ ID NO:1 or a fragment thereof.

The present invention is further based on the observation of the present inventors that when SaPIN2a is expressed in transgenic lettuce, it results in the inhibition of plant endogenous protease activity. Lettuce was chosen for expression of SaPIN2a because it neither possesses detectable trypsin inhibitory activity in its leaves nor responds to any treatments by accumulating inhibitors (Walker-Simmons and Ryan, 1977). Further, lettuce is an economically important vegetable crop, grown globally (Ryder, 1999) and its transformation methods are well-established (Michelmore *et al.*, 1987; Curtis *et al.*, 1994). Previous reports have shown that the heterogeneous expression of PIN2 confers insect-resistance (Johnson *et al.*, 1989; Duan *et al.*, 1996; Klopfenstein *et al.*, 1997).

The present inventors successfully produced transgenic lettuce expressing SaPIN2a from the CaMV 35S promoter. Stable integration and inheritance of the *SaPIN2a* cDNA in the genome of transgenic lettuce were demonstrated by Southern blot analysis and segregation analysis of the R<sub>1</sub> progeny. Although *SaPIN2a* mRNA was detected in both the R<sub>0</sub> and R<sub>1</sub> transformants by northern blot analysis, western blot analysis using anti-peptide antibodies against SaPIN2a failed to detect the presence of SaPIN2a protein. Despite an absence of significant inhibitory activity against bovine trypsin and chymotrypsin in extracts from transgenic lettuce, the endogenous trypsin-like activity in all transgenic lines analyzed was almost completely inhibited, and the endogenous chymotrypsin-like activity, moderately inhibited. Preliminary insect bioassays with R<sub>0</sub> transgenic plants showed that some of them acquired enhanced resistance to cabbage looper (*Trichoplusia ni*). The present invention provides that heterogeneously expressed SaPIN2a in transgenic lettuce inhibited plant endogenous protease activity indicating that SaPIN2a regulates proteolysis. The present invention also provides that by inhibiting endogenous proteinases *in planta*, SaPIN2a can be used for the protection of heterogeneous protein production in transgenic plants.

The invention seeks to provide inhibition of endogenous protease activity in plants by transforming plants, such as crops, and specifically lettuce, with proteinase inhibitor II. In a specific embodiment, the invention provides transgenic lettuce with enhanced inhibition of endogenous trypsin-like activity by the expression of proteinase inhibitor II from *Solanum americanum* (SaPIN2a). In a specific embodiment, the inhibition is complete, *i.e.*, more than about 90% and less than or equal to 100%. In a specific embodiment, the inhibition is moderate, *i.e.*, more than about 50% and less than about 90%. In a specific embodiment, the inhibition is low, *i.e.*, more than 5% and less than about 50%.

The percentage inhibition may be determined by any assays well known to one skilled in the art.

The invention seeks to provide inhibition of endogenous trypsin-like activity in plants by transforming plants, such as crops, and specifically lettuce, with proteinase inhibitor II. In a specific embodiment, the invention provides transgenic lettuce with enhanced inhibition of endogenous trypsin-like activity by the expression of proteinase inhibitor II from *Solanum americanum* (SaPIN2a).

The invention seeks to provide inhibition of endogenous chymotrypsin-like activity in plants by transforming plants, such as crops, and specifically lettuce, with proteinase inhibitor II. In a specific embodiment, the invention provides transgenic lettuce with enhanced inhibition of endogenous chymotrypsin-like activity by the expression of proteinase inhibitor II from *Solanum americanum* (SaPIN2a).

The invention seeks to provide pests and pathogen protection in plants by transforming plants, such as crops, and specifically lettuce, with proteinase inhibitor II. In a specific embodiment, the invention provides transgenic lettuce with enhanced protection against cabbage looper by the expression of proteinase inhibitor II from *Solanum americanum* (SaPIN2a).

The present invention provides transgenic plant having therein a gene comprising a promoter, operably associated with a coding sequence for SaPIN2a and a terminator. Plant cells containing a gene comprising a nucleic acid sequence encoding proteinase inhibitor II are also an aspect of this invention, as are other plant parts, such as for example, seed of the transformed plant containing a gene according to the invention.

In a specific embodiment, the present invention provides transgenic lettuce having therein a gene comprising a promoter, operably associated with a coding sequence for SaPIN2a, and a terminator. Lettuce plant cells containing a gene comprising a nucleic acid sequence encoding SaPIN2a are also an aspect of this invention, as are other plant parts, such as for example, seed of the transformed plant containing a gene according to the invention.

In a specific embodiment, the genetically modified plants have inhibited endogenous protease activity following the introduction, by recombinant DNA techniques, of coding sequences for proteinase inhibitor II. In a specific embodiment, lettuce is transformed via *Agrobacterium*-mediated transformation.

In a specific embodiment, the genetically modified plants have inhibited trypsin-like activity following the introduction, by recombinant DNA techniques, of coding

sequences for proteinase inhibitor II. In a specific embodiment, lettuce is transformed via *Agrobacterium*-mediated transformation.

In a specific embodiment, the genetically modified plants have inhibited chymotrypsin-like activity following the introduction, by recombinant DNA techniques, of coding sequences for proteinase inhibitor II. In a specific embodiment, lettuce is transformed via *Agrobacterium*-mediated transformation.

In a specific embodiment, the genetically modified plants have enhanced resistance to cabbage looper, *Trichoplusia ni* following the introduction, by recombinant DNA techniques, of coding sequences for proteinase inhibitor II. In a specific embodiment, lettuce is transformed via *Agrobacterium*-mediated transformation.

In another embodiment, additional gene sequences coding for proteinase inhibitors may be introduced into the plant in addition to the coding enzyme for proteinase inhibitor II. Such proteins includes, but are not limited to, genes encoding ribosome-inactivating proteins, lectins, agglutinins, and other pathogenesis-related proteins. In a specific embodiment, serine proteinase inhibitor may be used.

In a specific embodiment, the present invention further provides a method of inhibiting endogenous protease activity in plant. The method comprising the steps of introducing into the plant genome a nucleic acid sequence encoding a proteinase inhibitor II and regenerating a plant having an altered genome. In a specific embodiment, the proteinase inhibitor II is from *Solanum americanum*. In another specific embodiment, the proteinase inhibitor II is SaPIN2a, under the direction of a suitable promoter and a suitable terminator.

In a specific embodiment, the present invention further provides a method of inhibiting endogenous trypsin-like activity in plant. The method comprising the steps of introducing into the plant genome a nucleic acid sequence encoding a proteinase inhibitor II and regenerating a plant having an altered genome. In a specific embodiment, the proteinase inhibitor II is from *Solanum americanum*. In another specific embodiment, the proteinase inhibitor II is SaPIN2a, under the direction of a suitable promoter and a suitable terminator.

In a specific embodiment, the present invention further provides a method of inhibiting endogenous chymotrypsin-like activity in plant. The method comprising the steps of introducing into the plant genome a nucleic acid sequence encoding a proteinase inhibitor II and regenerating a plant having an altered genome. In a specific embodiment, the proteinase inhibitor II is from *Solanum americanum*. In another specific embodiment, the



proteinase inhibitor II is SaPIN2a, under the direction of a suitable promoter and a suitable terminator.

In a specific embodiment, the present invention further provides a method of enhancing the resistance of plant to pests or pathogens. The method comprising the steps of  
 5 introducing into the plant genome a nucleic acid sequence encoding a proteinase inhibitor II and regenerating a plant having an altered genome. In a specific embodiment, the proteinase inhibitor II is from *Solanum americanum*. In another specific embodiment, the proteinase inhibitor II is SaPIN2a, under the direction of a suitable promoter and a suitable terminator. In specific embodiment, the pest is cabbage looper. In another specific  
 10 embodiment, the cabbage looper is *Trichoplusia ni*.

In a specific embodiment, the present invention further provides a method of protecting heterogeneous protein production in plant. The method comprising the steps of introducing into the plant genome a nucleic acid sequence encoding a proteinase inhibitor II and regenerating a plant having an altered genome. In a specific embodiment, the  
 15 proteinase inhibitor II is from *Solanum americanum*. In another specific embodiment, the proteinase inhibitor II is SaPIN2a, under the direction of a suitable promoter and a suitable terminator.

#### 4. DESCRIPTION OF THE FIGURES

20 **Figures 1a & b.** (a) Nucleotide sequence (SEQ ID NO:1) and (b) amino acid sequence (SEQ ID NO:2) of *SaPIN2a*.

**Figures 2a & b.** (a) Structure of T-DNA region of pSa7. RB, right border sequence of the transferred DNA (T-DNA); NOS-Pro, nopaline synthase (NOS) promoter; NPT II,  
 25 neomycin phosphotransferase II gene encoding resistance to kanamycin (Kan<sup>R</sup>); NOS-ter, NOS terminator; CaMV 35S-Pro, promoter of cauliflower mosaic virus (CaMV) 35S RNA; *SaPIN2a*, *Solanum americanum* proteinase inhibitor IIa; LB, left border sequence of the T-DNA. (b) DNA sequence of junction of CaMV 35S promoter and *SaPIN2a* cDNA in pSa7. The sequence in italics is from parent binary vector pBI121 and the remaining  
 30 sequence is from the plasmid pSa2, a pBluescript II SK-derivative containing the full-length *SaPIN2a* cDNA (Xu *et al.*, 2001). The transcription start site (Jefferson *et al.*, 1987) is underlined. The start codon (atg) of *SaPIN2a* is in boldface.

**Figures 3a & b.** Southern blot analysis of DNA from R<sub>0</sub> transgenic lettuce plants. Twenty  
 35 μg of genomic DNA was digested with *EcoRI* (a) or *BamHI* (b) and fractionated on a 0.8%

agarose gel. A  $^{32}\text{P}$ -labeled probe prepared by random-primed labeling of the 0.64 kb *SaPIN2a* cDNA was used. The identification number of the individual transgenic lettuce line is indicated on the top of each lane. WT, wild-type control plant. The DNA molecular size markers are shown on the sides. Arrows indicate the 0.58 kb hybridization band  
 5 corresponding to the *SaPIN2a* cDNA.

**Figures 4a & b.** Northern blot analysis of RNA from R0 transgenic lettuce plants. Total RNA (20 mg) was isolated from leaves of wild-type (WT) or transgenic lettuce (TL) and from stems of *S. americanum* (ST), as positive control. The blots were probed with a  
 10  $^{32}\text{P}$ -labeled *SaPIN2a* cDNA. The 25S rRNA bands stained with methylene blue are at the bottom panel. The hybridization band corresponding to the transcript of *SaPIN2a* cDNA is indicated by an arrowhead (in *S. americanum* stems) or an arrow (in transgenic lettuce leaves). The 25S rRNA bands stained with methylene blue are at the bottom panel. Blot shown in (b) is a repeat of RNA samples from TL10, 11 and 34 that were degraded in (a)  
 15 together with TL52 as an internal control.

**Figures 5a, b, c & d.** Northern blot and western blot analyses of R<sub>1</sub> transgenic lettuce plants. Total RNA (20  $\mu\text{g}$ ) and total protein (20  $\mu\text{g}$ ) were isolated from identical leaves and stems of 38-day R<sub>1</sub> transgenic (TL1, 11 & 33) and wild-type (WT) lettuce plants. Total  
 20 RNA (20  $\mu\text{g}$ ) and total protein (14  $\mu\text{g}$ ) isolated from *S. americanum* (Sa) stems were included as a positive control. (a): Northern blot analysis with a  $^{32}\text{P}$ -labeled *SaPIN2a* cDNA probe. The hybridization band corresponding to the transcript of *SaPIN2a* cDNA is indicated by an arrowhead (0.67 kb mRNA in *S. americanum* stems) or an arrow (0.93 kb mRNA in transgenic lettuce leaves). (b): The 25S rRNA bands stained with ethidium  
 25 bromide are shown to demonstrate amounts of total RNA loaded in (a). (c): western blot analysis with SaPIN2a-specific antibodies. Cross-reacting bands in lettuce leaves (18.1-kDa) and *S. americanum* stems (16.7-kDa) are indicated. (d): Coomassie blue staining of total protein from transgenic and wild-type plants separated on a 4-20% gradient SDS-PAGE to demonstrate amounts of total protein loaded in (c).  
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**Figures 6a & b.** Proteinase inhibitory activity assay of transgenic lettuce leaf extracts. Leaf extracts were prepared from 54-day old wild-type (WT) or transgenic (TL1, 11 & 33) R<sub>1</sub> plants. Each value represents the mean  $\pm$  SE of three replicates. (a) trypsin inhibitory activity assay. One  $\mu\text{g}$  of bovine trypsin (Calbiochem) was incubated with 150  $\mu\text{l}$  of assay  
 35 buffer (standard) or leaf extracts and the residual trypsin activity was determined by

measuring the increase of absorbance at 247 nm during the hydrolysis of substrate. (b) chymotrypsin inhibitory activity assay. Two  $\mu\text{g}$  of bovine  $\alpha$ -chymotrypsin (Calbiochem) was incubated with 50  $\mu\text{l}$  of assay buffer (standard) or leaf extract and the residual chymotrypsin activity was determined by measuring the increase of absorbance at 256 nm during the hydrolysis of substrate.

**Figures 7a & b.** Trypsin- and chymotrypsin-like activity assay of transgenic lettuce leaf extracts. Leaf extracts were prepared from 54-day old wild-type (WT) or transgenic (TL1, 11 & 33)  $R_1$  plants. Each value represents the mean  $\pm$  SE of three replicates. (a) trypsin-like activity assay. Leaf extract (150  $\mu\text{l}$ ) was directly incubated with substrate in the absence of bovine trypsin and the trypsin-like activity was determined by measuring the increase in absorbance at 247 nm during the hydrolysis of substrate. (b) chymotrypsin-like activity assay. Leaf extract (50  $\mu\text{l}$ ) was directly incubated with substrate in the absence of bovine  $\alpha$ -chymotrypsin and the chymotrypsin-like activity was determined by measuring the increase in absorbance at 256 nm during the hydrolysis of substrate.

**Figures 8a, b, c & d.** Growth of cabbage loopers (*T. ni*) fed on leaves of  $R_0$  transgenic and wild-type lettuce. Newly hatched first-instar larvae were fed with daily fresh leaves from  $R_0$  transgenic plants TL1(a), TL7(b), TL11(c) and TL15(d), each with leaves from wild-type control plants (WT). The points shown represent the total weight of ten larvae.

**Figures 9a, b, c & d.** Cabbage loopers (*T. ni*) fed on leaves of transgenic (TL1) and wild-type lettuce. (a) *T. ni* larvae fed 8 days on leaves of transgenic lettuce (TL1); (b) *T. ni* larvae fed 8 days on leaves of wild-type lettuce; (c) resultant leaves of TL1  $R_0$  plants on day 8 of feeding trials; (d) resultant leaves of wild-type lettuce on day 8 of feeding trials.

## 5. DETAILED DESCRIPTION OF THE INVENTION

### 5.1 SAPIN2A cDNA

The cloning of *SaPIN2a* cDNA is disclosed in Xu *et al.* (2001), which is incorporated by reference in its entirety. Other proteinase inhibitor II DNA related to *SaPIN2a* DNA may be isolated and characterized using techniques known in the art. A cDNA or genomic DNA specific for proteinase inhibitor II protein or nucleic acid may be cloned and sequenced in a variety of ways, *e.g.*, dideoxy chain termination sequencing, *see, e.g., Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*

The polynucleotides that may be used in the present invention include polynucleotides having the DNA sequences presented herein, and additionally include any nucleotide sequence encoding a contiguous and functional proteinase inhibitor II encoding open reading frame (ORF) that hybridizes to a complement of the DNA sequences presented herein under highly stringent conditions. By way of example and not limitation, high stringency hybridization conditions can be defined as follows: The filter-bound DNA were hybridized in a solution containing 50% deionized formamide, 6xSSC, 5x Denhardt's, 1% SDS, 100 µg/ml denatured salmon sperm DNA at 42°C overnight (about 4-16 hours), and washing in 0.1xSSC, 0.1% SDS at 65°C (Ausubel F.M. *et al.*, eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York) and encodes a functionally equivalent gene product.

For oligonucleotide probes, by way of example and not limitation, highly stringent conditions may refer, *e.g.*, to washing in 6XSSC/0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base oligos).

Additionally contemplated polynucleotides that may be used in the present invention include any nucleotide sequences that hybridize under moderately stringent conditions to the complement of the DNA sequences that encodes a proteinase inhibitor II. By way of example but not limitation, such moderately stringent conditions may include, *e.g.*, washing in 0.2XSSC/0.1% SDS at 42°C (Ausubel *et al.*, 1989, *supra*).

Additionally contemplated polynucleotides that may be used in the present invention include any nucleotide sequences that hybridize under low stringency conditions to the complement of the DNA sequences that encode a proteinase inhibitor II. By way of example and not limitation, procedures using such conditions of low stringency are described in Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792.

Moreover, a variant of proteinase inhibitor II can also be used in the present invention. A variant may comprise one or more changes in the amino acid sequence of the proteinase inhibitor, *e.g.*, by way of addition, substitution, or deletion of one or more amino acids, compared with the wild type proteinase inhibitor. Any change should not abolish the ability of the proteinase inhibitor to perform its function, though it may increase or decrease this ability depending on the nature of the changes. Preferably, the amino acid changes are conservative.

In various embodiments, the proteinase inhibitor II, fragment, variant, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the proteinase inhibitor, fragment, analog, or derivative joined via a peptide bond to a

heterologous protein sequence (of a different protein)). Such a chimeric gene product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. Preferably, the fragment, analog, and derivative of the proteinase inhibitor in the fusion protein retains the ability to perform its function. Alternatively, DNA for the expression of the heterogeneous desired protein (*e.g.*, edible vaccines, antibodies, *etc.*) intended for protection against endogenous plant proteases could be introduced on a separate plasmid, using a different selectable marker, into transgenic lettuce (or any plant species) that is already expressing SaPIN2a. The resultant transgenic plant will co-express the heterogeneous protein and SaPIN2a and thus the heterogeneous protein would gain protection against endogenous plant proteases.

A cDNA or genomic DNA specific for a plant may be cloned through screening a cDNA or genomic DNA library. Such a library may be prepared, for example, from messenger RNA or genomic DNA from the plant. For general background on molecular biology techniques and on how to prepare a cDNA library and a genomic library, *see, e.g.*, Ausubel F.M. *et al.*, *supra*; Sambrook *et al.*, 1989, *supra*; and U.S. Patent No. 5,650,148.

The library may be screened with a nucleotide fragment specific for a part of the proteinase inhibitor II. For example, the protein sequence of a proteinase inhibitor II may be determined using techniques well known to those of skill in the art, such as via the Edman degradation technique. (*See, e.g.*, Creighton, 1983, "Proteins: Structures and Molecular Principles", W.H. Freeman & Co., New York, pp. 34-49). The amino acid sequence obtained may be used as a guide for the generation of oligonucleotide mixtures that can be used to screen a cDNA library for the cDNA sequence encoding the proteinase inhibitor II.

Or, for example, two stretches of protein sequences specific for the proteinase inhibitor II may be determined. A set of degenerate oligonucleotides specific for each stretch is prepared and the oligonucleotides are used in a polymerase chain reaction ("PCR") amplification. Oligonucleotides are at least about 6 nucleotides long, more preferably at least about 10, more preferably at least about 15, more preferably at least about 20, more preferably at least about 30, more preferably at least about 40 nucleotides. The template in the PCR reaction would be, for example, a mixture of cDNA or genomic DNA that is known to contain or suspected to contain a DNA polynucleotide specific for the

proteinase inhibitor II of interest. A cDNA template may be obtained in a variety of ways, for example, by isolating a mixture of different cDNA species from a cDNA library or, for example, by reverse transcribing total mRNA from a cell or organism known to (or suspected to) express the proteinase inhibitor II. For background on PCR, *see, e.g.*,  
 5 Ausubel, *supra*, and PCR Protocols: A Guide to Methods and Applications, 1990, Innis, M. *et al.*, eds. Academic Press, Inc., New York.

In order to clone a full length cDNA or genomic DNA sequence from any species or to clone variant or heterologous forms of the proteinase inhibitor II, labeled DNA probes made from nucleic acid fragments corresponding to any of the polynucleotides  
 10 discussed herein or made using the methods of the invention may be used to screen a cDNA library or a genomic DNA library (for example, a phage library) as described in, *e.g.*, Ausubel F.M. *et al.*, *supra*; Sambrook *et al.*, 1989, *supra*.

## 5.2 PRODUCTION OF ANTIBODIES

15 For the production of antibodies, various host animals may be immunized by injection with the proteinase inhibitor II (*e.g.*, one corresponding to functional domain of the proteinase inhibitor II), truncated proteinase inhibitor II (a proteinase inhibitor II in which one or more domains have been deleted), functional equivalents of the proteinase inhibitor II, mutants of the proteinase inhibitor II, or short peptides (or fragments) of  
 20 proteinase inhibitor II. Such host animals may include but are not limited to rabbits, mice, goats, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions,  
 25 keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Antibodies useful for the present invention include monoclonal antibodies  
 30 (*see, e.g.*, Kohler *et al.*, 1975, Nature 256:495-497; and U.S. Patent No. 4,376,110), chimeric antibodies (*see, e.g.*, Morrison *et al.*, 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger *et al.*, 1984, Nature, 312:604-608; Takeda *et al.*, 1985, Nature, 314:452-454), single chain antibodies (*see, e.g.*, U.S. Patent 4,946,778; Bird, 1988, Science 242:423-426; Huston *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward *et al.*, 1989,  
 35 Nature 334:544-546), antibody fragments (*see, e.g.*, Huse *et al.*, 1989, Science, 246:1275-

1281), anti-idiotypic antibodies or Fab fragments of such anti-idiotypes (*see, e.g.*, Greenspan & Bona, 1993, FASEB J 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438).

5                   **5.3    EXPRESSION OF PROTEINASE INHIBITOR II  
                  USING RECOMBINANT DNA TECHNOLOGY**

Proteinase inhibitor II, fragments thereof or fusion proteins thereof, are advantageously produced by recombinant DNA technology using techniques well known in the art. Such methods can be used to construct chimeric gene or expression vectors  
10 containing a proteinase inhibitor II nucleotide sequence and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. *See*, for example, the techniques described in Sambrook *et al.*, 1989, *supra*, and Ausubel *et al.*, 1989, *supra*. As used herein, the term chimeric gene refers to a combination of nucleic acid  
15 sequences for each part of the chimeric gene, which sequences have been engineered into relationship by recombinant DNA techniques, which sequences may also be in their separate parts endogenous or exogenous to the plant into which the chimeric gene is to be introduced.

Alternatively, RNA corresponding to all or a portion of a transcript encoded  
20 by a proteinase inhibitor II nucleotide sequence may be chemically synthesized using, for example, synthesizers. *See*, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety.

Any of host-expression vector system known in the art of biotechnology may  
25 be utilized to express the proteinase inhibitor II nucleotide sequence including, but not limited to, expression in bacteria, yeast, insect cells, mammalian cells, eukaryotic cells and plant cells. In these expression systems, any selection system may be used. Such selection may comprise growth on a selective medium (*e.g.*, antibiotics, minimal media, etc.) or the  
30 use of an indicator (*e.g.*, a dye, a fluorescent reagent, etc.).

In cases where plant expression vectors are used, the expression of the proteinase inhibitor II coding sequence may be driven by any of a number of regulatory elements. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson *et al.*, 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu *et al.*, 1987, EMBO J. 6:307-311) may be used; alternatively, plant promoters  
35 such as the small subunit of RUBISCO (Coruzzi *et al.*, 1984, EMBO J. 3:1671-1680;

Brogie *et al.*, 1984, *Science* 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley *et al.*, 1986, *Mol. Cell. Biol.* 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, biolistics/particle bombardment, microinjection, electroporation, *etc.* For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, *Methods for Plant Molecular Biology*, Academic Press, New York, Section VIII, pp. 421-463; and Grierson & Corey, 1988, *Plant Molecular Biology*, 2d Ed., Blackie, London, Ch. 7-9. As used herein, regulatory elements include but are not limited to inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression.

Preferably the promoter is capable of directing expression in a particular tissue of the plant and/or at particular stages of development of the plant. The promoter may be heterologous or homologous to the plant. Preferably the promoter directs expression to the fruit or leaves (as in the case of lettuce) for easy delivery of edible plant vaccines. Fruits or leaves eaten raw would be ideal for this purpose (e.g., tomatoes, lettuce, apples, bananas). Preferably the promoter directs expression to the endosperm of the plant seed or to the roots or tuber of the plant. A preferred promoter is the high molecular weight glutenin (HMWG) gene of wheat. Other suitable promoters will be known to the skilled in the art, such as the promoters of gliadin, branching enzyme, ADPG pyrophosphorylase, starch synthase and actin, for example.

#### 5.4 **TRANSGENIC PLANTS EXPRESSING PROTEINASE INHIBITOR II**

A transgenic plant with the ability to express a plant proteinase inhibitor II polypeptide may be engineered by transforming a plant cell with a gene construct comprising a sequence encoding a plant proteinase inhibitor II protein or polypeptide. In one embodiment, a plant promoter is operably associated with a sequence encoding the desired plant proteinase inhibitor II protein or polypeptide. As used herein, the term "Operably associated" or "operably linked" refers to an association in which the regulatory regions (e.g., promoter, enhancer) and the nucleic acid sequence to be expressed are covalently joined and positioned in such a way as to permit transcription, and under the appropriate condition, translation. In a preferred embodiment of the present invention, the associated promoter is a strong and non tissue- or developmental-specific plant promoter (e.g., a promoter that strongly expresses in many or all plant tissue types). Examples of such strong, "constitutive" promoters include, but are not limited to, the CaMV 35S



promoter (Odell *et al.*, 1985, Nature 313:810-812), the T-DNA mannopine synthetase promoter, and their various derivatives. In another preferred embodiment, an inducible or repressible promoter is used to express the proteinase inhibitor II of interest in a plant, for example, a tet operator promoter as described in Weinmann *et al.*, 1994, The Plant Journal 5:559-569; or a glucocorticoid-inducible promoter as described in McNellis *et al.*, 1998, The Plant Journal 14:247-257; or an ethanol inducible promoter as described in Caddick *et al.*, 1998, Nature Biotechnology 16:177-180. See, also, Gatz, 1995, Methods In Cell Biology 50:411-424, which describes inducible and repressible gene expression systems for plants.

10 In one embodiment of the invention, a proteinase inhibitor II is expressed in a plant so that the proteinase inhibitor II polypeptide will be localized in the apoplastic space. The proteinase inhibitor II may be directed to the apoplastic space, when expressed in a plant, by expressing the proteinase inhibitor II polypeptide as a fusion protein together with a peptide that acts as a signal or transporter so that proteinase inhibitor II is localized in  
15 the apoplastic space of the transgenic plant. A variety of signal or transporter peptides can be used, for example, the PR1b signal sequence as described in Lund *et al.*, 1992, Plant Molecular Biology 18:47-53; or the PR-1a, b and c signal sequences as described in Pfitzner *et al.*, 1987, Nucleic Acids Research 15:4449-4465. A fusion protein comprising a signal or transporter peptide and a proteinase inhibitor II polypeptide may be constructed by linking  
20 polynucleotides specific for each component to each other (*e.g.*, the polynucleotides are linked in frame) so that the desired fusion protein is made when the fusion polynucleotide is expressed in a transgenic plant. A skilled artisan would know how to construct a polynucleotide useful for expressing a proteinase inhibitor II in the apoplastic space of a transgenic plant.

25 In another embodiment of the present invention, it may be advantageous to engineer a plant with a gene construct comprising a sequence encoding a plant proteinase inhibitor II protein or polypeptide operably associated with a tissue- or developmental-specific promoter, such as, but not limited to, the CHS promoter, the PATATIN promoter, etc.

30 In yet another embodiment of the present invention, it may be advantageous to transform a plant with a gene construct comprising a sequence encoding a plant proteinase inhibitor II protein or polypeptide operably linked to a modified or artificial promoter. Typically, such promoters, constructed by recombining structural elements of different promoters, have unique expression patterns and/or levels not found in natural  
35

promoters. See, e.g., Salina *et al.*, 1992, *Plant Cell* 4:1485-1493, for examples of artificial promoters constructed from combining cis-regulatory elements with a promoter core.

In yet an additional embodiment of the present invention, the expression of a proteinase inhibitor II polynucleotide may be engineered by increasing the copy number of the gene encoding the desired protein or polypeptide using techniques known in the art.

### 5.5 TRANSFORMATION OF PLANTS AND PLANT CELLS

Plants and plant cells may be transformed using any method known in the art. In an embodiment of the present invention, *Agrobacterium* is employed to introduce the gene construct into plants. Such transformation preferably uses binary *Agrobacterium* T-DNA vectors (Bevan, 1984, *Nuc. Acid Res.* 12:8711-8721), and the co-cultivation procedure (Horsch *et al.*, 1985, *Science* 227:1229-1231). Generally, the *Agrobacterium* transformation system is used to engineer dicotyledonous plants (Bevan *et al.*, 1982, *Ann. Rev. Genet* 16:357-384; Rogers *et al.*, 1986, *Methods Enzymol.* 118:627-641). The *Agrobacterium* transformation system may also be used to transform, as well as transfer, DNA to monocotyledonous plants and plant cells. (see Hernalsteen *et al.*, 1984, *EMBO J* 3:3039-3041 ; Hooykass-Van Slogteren *et al.*, 1984, *Nature* 311:763-764; Grimsley *et al.*, 1987, *Nature* 325:1677-179; Boulton *et al.*, 1989, *Plant Mol. Biol.* 12:31-40.; and Gould *et al.*, 1991, *Plant Physiol.* 95:426-434).

In other embodiments, various alternative methods for introducing recombinant nucleic acid constructs into plants and plant cells may also be utilized. These other methods are particularly useful where the target is a monocotyledonous plant or plant cell. Alternative gene transfer and transformation methods include, but are not limited to, particle gun bombardment (biolistics), protoplast transformation through calcium-, polyethylene glycol (PEG)- or electroporation-mediated uptake of naked DNA (see Paszkowski *et al.*, 1984, *EMBO J* 3:2717-2722, Potrykus *et al.* 1985, *Molec. Gen. Genet.* 199:169-177; Fromm *et al.*, 1985, *Proc. Nat. Acad. Sci. USA* 82:5824-5828; and Shimamoto, 1989, *Nature* 338:274-276) and electroporation of plant tissues (D'Halluin *et al.*, 1992, *Plant Cell* 4:1495-1505). Additional methods for plant cell transformation include microinjection, silicon carbide mediated DNA uptake (Kaepler *et al.*, 1990, *Plant Cell Reporter* 9:415-418), and microprojectile bombardment (see Klein *et al.*, 1988, *Proc. Nat. Acad. Sci. USA* 85:4305-4309; and Gordon-Kamm *et al.*, 1990, *Plant Cell* 2:603-618). In any methods, selectable markers may be used, at least initially, in order to determine whether transformation has actually occurred. Useful selectable markers include enzymes which confer resistance to an antibiotic, such as gentamycin, hygromycin, kanamycin and

the like. Alternatively, markers which provide a compound identifiable by a color change, such as GUS, or luminescence, such as luciferase, may be used.

The chimeric gene may also comprise a gene switch mechanism which determines under what conditions or when the coding sequence is to be expressed. The gene switch may be a chemically induced promoter or a temperature controlled promoter, for example.

According to the present invention, a wide variety of plants and plant cell systems may be engineered for the desired physiological and agronomic characteristics described herein using the nucleic acid constructs of the present invention and the various transformation methods mentioned above. In preferred embodiments, target plants and plant cells for engineering include, but are not limited to, those monocotyledonous and dicotyledonous plants, such as crops including grain crops (*e.g.*, wheat, maize, rice, millet, barley), fruit crops (*e.g.*, tomato, apple, pear, strawberry, orange), forage crops (*e.g.*, alfalfa), root vegetable crops (*e.g.*, carrot, potato, sugar beets, yam), leafy vegetable crops (*e.g.*, lettuce, spinach); flowering plants (*e.g.*, petunia, rose, chrysanthamum), conifers and pine trees (*e.g.*, pine fir, spruce); plants used in phytoremediation (*e.g.*, heavy metal accumulating plants); oil crops (*e.g.*, sunflower, rape seed) and plants used for experimental purposes (*e.g.*, *Arabidopsis*, tobacco).

## 5.6 SCREENING OF TRANSFORMED PLANTS AND PLANT CELLS

According to the present invention, desired plants may be obtained by engineering one or more of the gene constructs expressing a proteinase inhibitor II as described herein into a variety of plant cell types, including but not limited to, protoplasts, tissue culture cells, tissue and organ explants, pollens, embryos, as well as whole plants. In an embodiment of the present invention, the engineered plant material is selected or screened for transformants (those that have incorporated or integrated the introduced gene construct(s)) following the approaches and methods described below. An isolated transformant may then be regenerated into a plant. Alternatively, the engineered plant material may be regenerated into a plant before subjecting the derived plant to selection or screening for the marker gene traits. Procedures for regenerating plants from plant cells, tissues or organs, either before or after selecting or screening for marker gene(s), are well known to those skilled in the art.

A transformed plant cell, callus, tissue or plant may be identified and isolated by selecting or screening the engineered plant material for traits encoded by the marker genes present on the transforming DNA. For instance, selection may be performed by

growing the engineered plant material on media containing inhibitory amount of the antibiotic or herbicide to which the transforming gene construct confers resistance. Further, transformed plants and plant cells may also be identified by screening for the activities of any visible marker genes (e.g., the  $\beta$ -glucuronidase, luciferase, B or C1 genes) that may be present on the recombinant nucleic acid constructs of the present invention. Such selection and screening methodologies are well known to those skilled in the art.

Physical and biochemical methods may also be used to identify plant or plant cell transformants containing the gene constructs of the present invention. These methods include but are not limited to: 1) Southern analysis or PCR amplification for detecting and determining the structure of the recombinant DNA insert; 2) Northern blot, S1 RNase protection, primer-extension or reverse transcriptase-PCR amplification for detecting and examining RNA transcripts of the gene constructs; 3) enzymatic assays for detecting enzyme activity, where such gene products are encoded by the gene construct; 4) protein gel electrophoresis (PAGE), Western blot techniques, immunoprecipitation, or enzyme-linked immunoassays, where the gene construct products are proteins. Additional techniques, such as *in situ* hybridization, enzyme staining, and immunostaining, also may be used to detect the presence or expression of the recombinant construct in specific plant organs and tissues. The methods for doing all these assays are well known to those skilled in the art.

## 20           5.7    **TRANSGENIC PLANTS EXPRESSING AN ENGINEERED PROTEINASE INHIBITOR II POLYNUCLEOTIDES**

Transgenic plants are generated that express an engineered proteinase inhibitor II gene. A transgenic plant expressing a proteinase inhibitor II has inhibited endogenous protease activity. In particular, a transgenic plant expressing a proteinase inhibitor II has inhibited endogenous trypsin-like activity or inhibited endogenous chymotrypsin-like activity. A transgenic plant expressing a proteinase inhibitor II is less susceptible to the pathogenic effects of the pathogen of interest. Transgenic plants may be made using any of the techniques known in the art as described for plant proteinase inhibitor II expressing transgenic plants.

30           Transgenic plants expressing one or more proteinase inhibitor II gene polynucleotides capable of rendering said plants more resistant to a pathogen of interest may be from any plant species, plant genus, plant family, plant order, plant class, plant division of the kingdom of plants. See, e.g., U.S. Patent Nos. 5,889,189; 5,869,720; 5,850,015; 5,824,842; PP10,742; PP10,704; PP10,682, which recite plant species, genera, families, orders, classes and divisions in which the proteinase inhibitor II genes may be used.

Examples of plants are monocots, dicots, crop plants (*i.e.*, any plant species grown for purposes of agriculture, food production for animals including humans, plants that are typically grown in groups of more than about 10 plants in order to harvest for any reason the entire plant or a part of the plant, *e.g.*, a fruit, a flower or a crop, *e.g.*, grain, that the plants bear, etc.), trees (*i.e.*, fruit trees, trees grown for wood production, trees grown for decoration, etc.), flowers of any kind (*i.e.*, plants grown for purposes of decoration, for example, following their harvest), cactuses, etc.

Further examples of plants in which the proteinase inhibitor II genes may be expressed include *Viridiplantae*, *Streptophyta*, *Embryophyta*, *Tracheophyta*,  
 10 *Euphyllophytes*, *Spermatophyta*, *Magnoliophyta*, *Liliopsida*, *Commelinidae*, *Poales*,  
*Poaceae*, *Oryza*, *Oryza sativa*, *Zea*, *Zea mays*, *Hordeum*, *Hordeum vulgare*, *Triticum*,  
*Triticum aestivum*, *Eudicotyledons*, *Core eudicots*, *Asteridae*, *Euasterids*, *Rosidae*, *Eurosids*  
 II, *Brassicales*, *Brassicaceae*, *Arabidopsis*, *Magnoliopsida*, *Solananae*, *Solanales*,  
*Solanaceae*, *Solanum*, *Nicotiana*.

15 Also included are, for example, crops of particular interest including  
 Solanaceae, including processing and fresh market tomatoes, pepper and eggplant; leafy  
 plants, including lettuce and spinach; *Brassicaceae*, including broccoli, brussels sprouts,  
 calabrese, kale, cauliflower, red cabbage and white cabbage; cucurbits, including cucumber,  
 melon, watermelon, zucchini and squash; large seeded plants, including peas, beans and  
 20 sweetcorn; rooted plants, including carrots and onions; vegetatively propagated plants,  
 including berries, grapes, banana, pineapple and rosaceous fruit and nut crops; and tropical  
 crops, including tobacco, mango and papaya.

Thus, the invention has use over a broad range of plants including, but not  
 limited to, species from the genera *Anacardium*, *Arachis*, *Asparagus*, *Atropa*, *Avena*,  
 25 *Brassica*, *Citrus*, *Citrullus*, *Capsicum*, *Carthamus*, *Cocos*, *Coffea*, *Cucumis*, *Cucurbita*,  
*Daucus*, *Elaeis*, *Fragaria*, *Glycine*, *Gossypium*, *Helianthus*, *Heterocallis*, *Hordeum*,  
*Hyoscyamus*, *Lactuca*, *Linum*, *Lolium*, *Lupinus*, *Lycopersicon*, *Malus*, *Manihot*, *Majorana*,  
*Medicago*, *Nicotiana*, *Olea*, *Oryza*, *Panicum*, *Pannaserum*, *Persea*, *Phaseolus*, *Pistachia*,  
*Pisum*, *Pyrus*, *Prunus*, *Raphanus*, *Ricinus*, *Secale*, *Senecio*, *Sinapis*, *Solanum*, *Sorghum*,  
 30 *Theobromus*, *Trigonella*, *Triticum*, tobacco, *Vicia*, *Vitis*, *Vigna*, and *Zea*.

## 5.11 POLYNUCLEOTIDE CONSTRUCTS FOR EXPRESSION OF ENGINEERED GENE IN TRANSGENIC PLANTS

35 A polynucleotide construct capable of directing the expression of an  
 engineered proteinase inhibitor II gene product in a transgenic plant of interest is

constructed using general recombinant DNA and cloning techniques known in the art of biotechnology, *see, e.g., Sambrook et al., supra; Ausubel et al., supra*. Such a polynucleotide construct typically comprises a polynucleotide sequence that encodes an engineered proteinase inhibitor II gene product and one or more regulatory polynucleotide  
5 sequence. Regulatory sequences useful for the polynucleotide construct of the invention include, but are not limited to, a promoter, an enhancer, an intron, a splice donor, a splice acceptor, a polyadenylation sequence, a RNA stability regulating sequence, or an element of any one of the above (*e.g., promoter elements including, but not limited to, a TATA box*).

The polynucleotide construct comprises one or more regulatory elements  
10 capable of directing the expression of the engineered proteinase inhibitor II gene product of the invention. In a preferred aspect, the regulatory elements are capable of directing expression in a plant species in which expression of the engineered proteinase inhibitor II gene product is desired. In another preferred aspect, the regulatory elements are capable of directing expression in a cell type in which expression of the engineered proteinase inhibitor  
15 II gene product is desired in the plant species of interest.

Regulatory elements useful for the polynucleotide construct of the present invention are known to those of skill in the art, for example, promoter and enhancer elements of genes known to be expressed in the cell type and plant species of interest. A promoter useful for expression of the engineered proteinase inhibitor II gene product in a  
20 cell type of a plant species of interest may also be isolated using routine experimentation, for example, by isolating a promoter region of a gene known to be expressed in the desired fashion. For example, one may screen a genomic library with a cDNA probe specific for the 5' end of a messenger RNA known to be expressed in the cell type of interest of the plant species of interest. Such a 5' end cDNA probe should preferably be only about 100  
25 base pairs to about 300 base pairs so that the clones identified in the genomic library are likely to include the 5' end of the gene possibly including the promoter region of the gene for which the probe is specific. The promoter region typically includes about 1,000 to about 2,000 base pairs upstream of the transcription initiation site. Thus, a promoter useful for the expression of the engineered proteinase inhibitor II genes of the present invention is a  
30 polynucleotide from about 2,000 base pairs upstream to about 50 base pairs downstream of the transcription initiation site of a gene known to be expressed in the cell type of interest in the plant species of interest, or is a portion of the polynucleotide.

In order to facilitate the proper processing of the engineered proteinase inhibitor II gene product, it may be necessary to include a nucleotide stretch that encodes a  
35 peptide sequence necessary for such processing. For example, a peptide sequence which is

recognized by and functional in the transgenic host plant, for example, to facilitate the entry of the proteinase inhibitor II gene product into the endoplasmic reticulum may be necessary, *i.e.*, signal sequence.

5           **5.12    ASSAYS FOR TESTING AN ENGINEERED  
RESISTANT PLANT LINE**

Plant lines generated using methods of the present invention that express an engineered proteinase inhibitor II gene product are more resistant to the pathogenic effects of a pathogen of interest when compared to a plant line of the same species that does not  
10 express the engineered proteinase inhibitor II gene product (*i.e.*, a wild-type plant). The increased resistance of a plant line generated using methods of the invention may be assayed for by any technique known to the skilled artisan. For example, one may infect a plant of the generated plant line and a plant of a wild-type plant line with a pathogen of interest. After such infection, the plant of the generated plant line will have at least an approximately  
15 20% higher probability of surviving infection than the wild-type plant, more preferably at least about 40%, more preferably at least about 60% and most preferably at least about 80%.

Another way of testing a transgenic plant made using the methods of the invention is by testing for necrosis inducing activity, for example, as described in Mahe *et al.*, 1998, *J. Peptide Res.* 52:482-494. Thus, one can express an engineered proteinase  
20 inhibitor II gene in a transgenic plant and infect the transgenic plant with the pathogen of interest. For example, when applying a pathogen to the transgenic plant expressing the engineered proteinase inhibitor II gene, one would observe clear necrosis or severe spreading necrosis in the wild-type plant but not in a transgenic plant of the plant line from which the transgenic plant was derived.

25           Necrotic cell death can also be observed using histochemical staining reactions in addition to visual inspection.

**5.13.   PATHOGENS**

Pathogen resistance is an important property in plants and a useful tool for  
30 the protection of plants, especially crop plants. The term "plant", as used herein, includes whole plants, plant parts, individual plant cells, groups of plants cells (*e.g.*, cultured plant cells) and progeny thereof. The term "enhance" when used to describe an increase of resistance of a plant to a pathogen, as used herein, includes the increase of the resistance of a plant that may have no resistance, or some resistance or substantial resistance to the  
35 pathogen to effecting the increase in resistance.

Plant pathogens include, but are not limited to, bacteria, viruses, fungi, nematodes and insects. A pathogen may infect a plant and cause severe damage to that plant, including death. Upon infection, a plant may initiate a protective reaction to the pathogen, *e.g.*, a hypersensitive response, depending on whether the plant can recognize the  
 5 pathogen.

Pathogens of the various classes may change, for example, through mutagenesis. Also, new pathogens may arise that were not previously encountered by a plant species. For example, when a plant (*e.g.*, a crop, a fruit, a vegetable, etc.) is introduced into a continent (for example, through importation), a plant species is likely  
 10 exposed to pathogens it has not encountered before.

The following examples are provided to further illustrate the current invention but are not provided to in any way limit the scope of the current invention.

15

## 6. EXAMPLES

### 6.1 MATERIALS AND METHODS

#### *Plant material and growth conditions*

Seeds of lettuce (*Lactuca sativa L.* var. Great Lakes No.118) were obtained from Northrup King Co., Mpls., MN 55440, USA. Plant tissue cultures were maintained and propagated *in vitro* in a growth chamber at 22-24°C under a 12 h light/12 h dark regime. Plants in soil  
 20 were grown under natural conditions in a greenhouse.

#### *Generation of transgenic lettuce plants expressing SaPIN2a*

The *Agrobacterium tumefaciens*-mediated transformation vector pSa7 containing the  
 25 *SaPIN2a* cDNA (Figure 1a; SEQ ID NO:1) was constructed by replacing the GUS gene (Jefferson *et al.* 1987) in pBI121 (Clontech) with the *SaPIN2a* cDNA fragment (Xu *et al.*, 2001). The junction region of the CaMV 35S promoter and the *SaPIN2a* cDNA in pSa7 was sequenced with a primer 5'-CAA TCC CAC TAT CCT TCG CAA GAC C-3' (SEQ ID NO:3) (corresponding to 7372-7396 of CaMV genome; Franck *et al.*, 1980) to confirm for  
 30 the absence of any spurious ATG codon between transcription start site (Jefferson *et al.*, 1987) and the SaPIN2a initiator codon. The binary vector pSa7 was then transferred into *A. tumefaciens* LBA4404 by direct transformation (Holsters *et al.* 1978). Lettuce transformation was performed as described by Curtis *et al.* (1994), with modifications, in that petunia nurse cell cultures were omitted in callus-inducing medium and 100 µg/ml of  
 35



kanamycin sulphate was added to the media for callus induction, shoot regeneration and rooting.

*Segregation analysis of the progeny (R<sub>1</sub>) plants of transgenic lettuce*

- 5 The segregation ratios of kanamycin-resistant (Km<sup>R</sup>) to kanamycin-sensitive (Km<sup>S</sup>) plants in the progeny (R<sub>1</sub>) of the self-fertilization of primary (R<sub>0</sub>) transgenic lettuce plants were determined by germinating surface-sterilized seeds from each of R<sub>0</sub> plants on Murashige and Skoog medium (MS, Murashige and Skoog, 1962) containing 100 µg/ml of kanamycin sulphate. After incubation for 2 to 3 weeks in a tissue culture chamber (22-24°C, 12 h  
10 light/12 h dark), the seedlings were scored for the kanamycin resistance. The segregation ratios were assessed by Chi-square analysis.

*Southern blot analysis*

- Twenty µg DNA, isolated (Dellaporta *et al.*, 1983) from lettuce leaves, were digested with  
15 restriction endonucleases, separated by electrophoresis in 0.8% agarose gel and blotted onto Hybond-N membrane (Amersham) according to Sambrook *et al.* (1989). The blot was pre-hybridized in 30% deionized formamide, 6 × SSC, 5 × Denhardt's, 1% SDS, 50 µg/ml denatured, sonicated salmon sperm DNA at 42°C for 4 h. The random-primed <sup>32</sup>P-labeled *SaPIN2a* cDNA probe was added and hybridized at 42°C overnight. The blot was washed  
20 in 0.1 × SSC, 0.1% SDS at room temperature.

*Northern blot analysis*

- Total RNA was extracted from nightshade plants, wild-type lettuce or transgenic lettuce (Nagy *et al.*, 1988) and analyzed in northern blot analysis as previously described by Xu *et al.* (2001).  
25

*Western blot analysis*

- Total plant protein was extracted according to the procedure of Wu *et al.* (1997). Protein concentration was determined following Bradford (1976). Total protein was separated by  
30 4-20% gradient SDS-PAGE (Gallagher, 1995) for western blot analysis (Sambrook *et al.*, 1989) using polyclonal antibodies raised in rabbit against a synthetic peptide (GESDPRNPKDC) (SEQ ID NO:4) corresponding to amino acids 77 to 87 of SaPIN2a (Figure 1b; SEQ ID NO:2) (Xu *et al.*, 2001). The Amplified Alkaline Phosphatase Immun-Blot Assay Kit (Bio-Rad) was used to detect cross-reacting bands.  
35

*Trypsin and chymotrypsin inhibitory activity and endogenous trypsin- and chymotrypsin-like activity assays*

Total plant proteins extracted with 50 mM Tris, pH 8.1, 20 mM CaCl<sub>2</sub> were used for spectrophotometric assays of trypsin or chymotrypsin inhibitor activity as described by  
 5 Kollipara and Hymowitz (1992).

For the trypsin inhibitor activity assay, 150 µl of leaf extract was pre-incubated for 3 min at room temperature (RT) in a quartz cuvette (10-mm path length, 3.5-ml capacity) with 100 µl of bovine trypsin (20 µg/ml in 1 mM HCl, Calbiochem Cat. No. 6502) and assay buffer (46 mM Tris-HCl, pH 8.1, 11.5 mM CaCl<sub>2</sub>), to give a final volume of 1.5 ml. To  
 10 start the reaction, 1.5 ml of substrate [2 mM p-toluenesulfonyl-L- argininc methyl ester (TAME, Sigma Cat. No. T4626) in assay buffer] were added and mixed with the pre-incubation mixture. The recording of absorbance at 247 nm (A<sub>247</sub>) was immediately initiated. The spectrophotometer was set to auto-zero just before the start of recording and the absorbance was measured at 30-sec intervals for 3 min. In the control, 100 µl of bovine  
 15 trypsin (20 µg/ml in 1 mM HCl) was pre-incubated with 1.4 ml of assay buffer without inhibitor. For the chymotrypsin inhibitor activity assay, 50 µl of leaf extract was pre-incubated for 3 min at RT with 100 µl of bovine α-chymotrypsin (20 µg/ml in 1 mM HCl, Calbiochem Cat. No. 230832) and assay buffer (0.1 M Tris-HCl, pH 7.8, 0.1 M CaCl<sub>2</sub>), to give a final volume of 1.5 ml. To start the reaction, 1.5 ml of substrate [1 mM  
 20 N-benzoyl-L-tyrosine ethyl ester (BTEE, Sigma Cat. No. B6125) in 50% (w/w) methanol] were added and mixed with the pre-incubation mixture. The absorbance at 256 nm (A<sub>256</sub>) was monitored like that of trypsin described above. For control, 100 µl of chymotrypsin (20 µg/ml in 1 mM HCl) was pre-incubated with 1.4 ml of assay buffer without inhibitor.

Endogenous trypsin- and chymotrypsin-like activities in leaves of wild-type and  
 25 transgenic R<sub>1</sub> lettuce plants were determined using the same procedures as in proteinase inhibitory activity assays except that no bovine trypsin or chymotrypsin was added to the reaction.

*Insect feeding experiments*

30 Insect feeding trials were carried out as described by Johnson *et al.* (1989). The larvae of cabbage looper (*Trichoplusia ni*) were reared on lettuce grown in growth chamber (16 h light, 20±2°C) until pupation. Their pupae were collected and put into a box with a net to give rise to adults. The moths were fed with 5% honey obtained from the local market. The eggs were collected and hatched in Petri dishes. The first and second instar larvae were  
 35 used for the experiments. Detached lettuce leaves from wild-type and transgenic plants

were placed on top of 3 sheets of Whatman No.1 papers (125 mm diameter) wetted with 6 ml of distilled water in sterile Petri dishes (145 mm diameter). Ten first or second instar larvae of *T. ni* were put into each dish and incubated in plant growth chamber (20±2°C). Each day, the larvae were weighed and transferred with a brush to new dishes containing  
 5 fresh leaves.

## 6.2. RESULTS

### Transformation of lettuce with pSa7 containing SaPIN2a cDNA

Figure 2a shows the binary vector pSa7 used in lettuce transformation. The expression of  
 10 *SaPIN2a* cDNA is driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter. DNA sequence analysis at the junction of the CaMV 35S promoter and the *SaPIN2a* cDNA in pSa7 confirmed the absence of any spurious ATG codon between the transcription start site (Jefferson *et al.*, 1987) and the SaPIN2a initiator codon (Figure 2b). *A. tumefaciens* LBA4404 harboring pSa7 was used to transform lettuce and transformants were selected on  
 15 kanamycin-containing media.

### Southern blot analysis of transgenic lettuce

To confirm the integration of the *SaPIN2a* cDNA into the lettuce genome and to estimate the copy number of the transgene in different transgenic lines, Southern blot analyses were  
 20 performed with a <sup>32</sup>P-labeled *SaPIN2a* cDNA probe.

Results of Southern blot analysis with *EcoRI*-digested DNA extracted from putative transgenic and regenerated (R<sub>0</sub>) lettuce plants is shown in Figure 3a. The presence of an expected 0.58 kb *EcoRI* fragment (indicated by arrows in Figure 3a) in all transgenic lines (Figure 3a, TL1, 7, 11, 15 and 33) suggests integration of the *SaPIN2a* cDNA, lacking in  
 25 wild-type (Figure 3a, lane WT). Additional strong hybridization bands in TL7 and 33 suggest the presence of rearranged copies of the *SaPIN2a* cDNA in the genome of these transgenic lettuce lines. Rearrangements of the transgene have been observed previously in many other transgenic plants (Jorgensen *et al.*, 1987; Deroles and Gardner, 1988; Radke *et al.*, 1988; Lee *et al.*, 1999).

30 Since only one *Bam*HI site is present between the 35S promoter and the *SaPIN2a* cDNA within the T-DNA region of pSa7 (Figure 2a), the number of bands observed in the Southern blot of *Bam*HI-digested genomic DNA probed with a <sup>32</sup>P-labeled *SaPIN2a* cDNA fragment should give a good estimation of the transgene copies integrated into lettuce genomic DNA. The different hybridization patterns of *Bam*HI-digested DNA shown in  
 35 Figure 3b suggest these transgenic plants resulted from independent transformation events,

and also revealed that the number of strong bands which hybridized to *SaPIN2a* cDNA probe ranged from one (TL1, 2, 10, 11, 12, 14, 20, 43) to four (TL34 and 52) corresponding to single or multiple copies of the transgene in these lines. Single copies of the transgene in TL1 and 11 and multiple copies of the transgene in TL33 were further confirmed by  
 5 segregation analysis of their progenies (Table 1).

#### Expression of SaPIN2a mRNA in transgenic lettuce

The transcription of *SaPIN2a* in transgenic lettuce plants was examined by northern blot analysis. Total RNA, isolated from leaves of R<sub>0</sub> transgenic lettuce identified in Southern  
 10 blot analysis (Figure 3) and from wild-type plants, was hybridized to the *SaPIN2a* cDNA probe. As shown in Figure 4, *SaPIN2a* mRNA was detected in most transgenic lines, with the exception of TL14, and no signal was present in the RNA from leaves of wild-type plants. The size of most *SaPIN2a* transcripts in transgenic lettuce (0.93 kb, indicated by an arrow in Figure 4) is slightly larger than that of endogenous transcript of 0.67 kb in *S.*  
 15 *americanum* (indicated by an arrowhead in Figure 4 lane Sa) due to ca. 200 additional nucleotides from the NOS-terminator (Figure 2a).

#### Non-detection of SaPIN2a protein in transgenic lettuce by western blot analysis

Total leaf proteins were extracted from leaves of R<sub>0</sub> transgenic plants that showed *SaPIN2a*  
 20 mRNA accumulation in northern blot analysis (Figure 4) and wild-type plants for western blot analysis with affinity purified SaPIN2a-specific antibodies. No SaPIN2a protein could be detected in the leaves of these R<sub>0</sub> transgenic plants in western blot analysis despite detection of *SaPIN2a* mRNA in northern blot analyses (data not shown). Western blot analysis was also carried out with total proteins extracted from the progeny (R<sub>1</sub>) plants of  
 25 three self-pollinated R<sub>0</sub> transgenic plants (TL1, 11 and 33) as well as the progeny of self-pollinated regenerated wild-type plants. Figure 5 shows the results of western blot analysis of total proteins prepared from R<sub>1</sub> transgenic plants, together with the corresponding results of northern blot analysis of total RNA prepared from identical tissue samples. Although very high levels of *SaPIN2a* mRNA accumulated in the leaves of these  
 30 transgenic plants (Figure 5a), again no protein band corresponding to SaPIN2a in *S. americanum* stem was detected in transgenic lettuce leaves (Figure 5c). Some non-specific cross-reacting bands were found and one of them (18.1 kDa), present in lettuce leaves but not in stem, is very close to the size of native SaPIN2a (16.7 kDa) (Figure 5c). Since native SaPIN2a in *S. americanum* accumulates in stems (Xu *et al.*, 2001), the cellular transport of  
 35 SaPIN2a might account for its apparent absence in transgenic lettuce leaves. To investigate

this possibility, northern blot and western bolt analyses were carried out with samples from stems of transgenic lettuce plants. Although *SaPIN2a* mRNA was detected in their stems (Figure 5a) at lesser amounts than leaves, SaPIN2a protein remained undetected in both, on western blot with SaPIN2a-specific antibodies (Figure 5c).

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#### **Trypsin and chymotrypsin inhibitory activities and endogenous trypsin- and chymotrypsin-like activity assays**

Although the SaPIN2a coding sequence in pSa7 was confirmed by DNA sequencing (Figure 2b), the integration of *SaPIN2a* cDNA in transgenic lines, by Southern blot analysis (Figure 3) and *SaPIN2a* mRNA by northern blot analysis (Figure 4), the SaPIN2a protein was undetectable upon western blot analysis. The relatively low sensitivity of western blot analysis may result in the failure to detect SaPIN2a protein in transgenic lettuce. Based on the quantitative assays of *Bacillus thuringiensis* (*B.t.*) protein expressed in transgenic plants using three assay methods (bioassay, ELISA and western blot analysis), it has been shown that the western blot analysis is the least sensitive assay, although it is the most frequently used (Fuchs *et al.*, 1990). Hence we further examined the presence of SaPIN2a in transgenic lettuce by using proteinase inhibitory activity assays, which may be more sensitive than western blot analysis.

Crude leaf extracts prepared from leaves of R<sub>1</sub> transgenic and wild-type plants were tested for inhibitory activity against bovine trypsin and chymotrypsin. Results of trypsin inhibitory activity assays (Figure 6a) showed no significant inhibitory activity against bovine trypsin in transgenic lettuce. Leaf extracts from all three transgenic lines analyzed (TL1, 11 & 33) showed trypsin activity similar to the standard reaction. Surprisingly, leaf extracts of wild-type plants had much higher trypsin activity than the standard reaction containing bovine trypsin (Figure 6a). In the case of chymotrypsin inhibitory activity assays (Figure 6b), all reactions containing leaf extracts from both transgenic and wild-type plants showed higher chymotrypsin activity than the standard, while the transgenic plants had slightly decreased activity compared with wild-type plants. These results of the inhibitory activity assays suggest that lettuce leaves might possess considerable endogenous trypsin- and chymotrypsin- like activities.

Accordingly, the endogenous trypsin- and chymotrypsin-like activity in leaves of wild-type and transgenic R<sub>1</sub> lettuce plants were determined using the same procedures for above proteinase inhibitory activity assay except that no bovine trypsin or chymotrypsin was added to the reaction. The results of these experiments are shown in Figure 7. As expected, both endogenous trypsin- and chymotrypsin-like activities were detected in leaf extracts

from wild-type lettuce plants. The results of assays on leaves from transgenic plants, however, are unexpected and interesting. The endogenous trypsin-like activity in all three transgenic plants was almost completely inhibited (Figure 7a), while the endogenous chymotrypsin-like activities in these transgenic plants moderately decreased (Figure 7b).

5 The inhibition of endogenous trypsin- and chymotrypsin-like activities in transgenic lettuce leaves suggests that SaPIN2a protein accumulates in transgenic lettuce at amounts that are undetectable by western blot analysis but that could inhibit the endogenous trypsin- and chymotrypsin-like activities.

#### 10 **Preliminary insect feeding assay with primary transgenic lettuce plants**

Since transgenic plants expressing potato or tomato PIN2 show the enhanced resistance to herbivorous insects (Johnson *et al.*, 1989; Duan *et al.*, 1996; Klopfenstein *et al.*, 1997), transgenic lettuce plants expressing SaPIN2a were tested for their protection against the cabbage looper (*Trichoplusia ni*), a very destructive lettuce pest (Barbour, 1999).

15 Due to limited availability of the larvae at the same developmental stage, all of the feeding trials could not be carried out concurrently. The experiments shown in Figure 8 were performed separately, each trial with its own set of control. Larvae fed on leaves from TL1 and TL11 plants (Figures 8a & c) grew significantly slower than those fed on control leaves, with a 49.8% and 37.7% reduction in larval weight respectively after feeding for 8

20 days. A moderate reduction (25.5% on day 9) in larval growth was observed with TL15 (Figure 8d), while growth was not retarded when larvae were fed on TL7 leaves (Figure 8b). Figure 9 shows the growth of larvae fed on leaves of TL1 was arrested and the larvae consumed much less leaf tissue compared to the larvae fed on leaves of wildtype lettuce. Since considerable variability of the growth of larvae fed even on leaves from wild-type

25 plants was observed in four independent trials presented in Figure 8, the insect resistance observed in this study with primary ( $R_0$ ) transgenic plants needs to be further confirmed with homogenous progeny transgenic plants and a larger population of larvae to confirm the observations in Figure 8 & 9.

30

### **6.3. Discussion**

Here, we demonstrate that the heterologous expression of a plant PI, SaPIN2a, not only confers insect-resistance in transgenic lettuce but also inhibits the endogenous trypsin- and chymotrypsin-like activities. Since it has been recently shown that the yield and quality of antibodies produced in transgenic plants are significantly affected by endogenous

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proteolytic degradation (Stevens *et al.*, 2000), heterologous expression of PIs could be exploited in the protection of heterogeneous protein production in transgenic plants.

The significant inhibition of trypsin-like activity and the moderate inhibition of chymotrypsin-like activity in transgenic lettuce, resulting from the expression of SaPIN2a, could not be due to a mutation caused by the T-DNA insertion because all three independent lines (TL1, 11 and 33) tested show similar inhibition. This finding further supports our previous hypothesis that other than a possible role in plant defense, SaPIN2a could have an endogenous role in regulating the activity of endogenous proteases in the phloem (Xu *et al.*, 2001). The identification of the target endogenous proteinases for SaPIN2a would be our future goal. So far, only a trypsin-like enzyme and its endogenous inhibitor have been identified in lettuce seeds (Shain and Mayer, 1965, 1968). In barley, an endogenous proteinase, a carboxypeptidase, has been shown to hydrolyse a number of ester substrates of trypsin and chymotrypsin (Mikola and Pietila, 1972).

The striking difference in levels of *SaPIN2a* mRNA between leaves and stems in transgenic lettuce plants was unexpected, because the CaMV 35S promoter is generally regarded as a strong constitutive promoter and directs high level transcription in nearly all plant organs (Nagy *et al.*, 1985; Odell *et al.*, 1985). This result, together with other studies (Jefferson *et al.*, 1987; Benfey and Chua, 1989; Williamson *et al.*, 1989; Yang and Christou, 1990; Sunilkumar *et al.*, 2002) suggest CaMV 35S promoter is not constitutive.

Despite a high expression of *SaPIN2a* mRNA in transgenic lettuce plants, SaPIN2a protein was not detected on western blot analysis. This failure could be attributed to the instability of SaPIN2a protein in transgenic lettuce. It has been shown that some transgene proteins accumulate to very low levels in transgenic plants because of degradation (Lawton *et al.*, 1987; Higgins and Spencer, 1991). Alternatively, the relatively low sensitivity of western blot analysis may also result in the failure to detect SaPIN2a protein in transgenic lettuce. Based on the quantitative assays of *Bacillus thuringiensis* (*B.t.*) protein expressed in transgenic plants using three assay methods (bioassay, ELISA and western blot analysis), it has been shown that bioassays are the most sensitive assay ( $\geq 0.5$  ng *B.t.* protein/mg plant protein) and western blot analysis the least ( $\geq 10$  ng *B.t.* protein/mg plant protein), although the latter is most frequently used (Fuchs *et al.*, 1990). It has been shown that presence of PIN2 protein at levels  $>50$   $\mu\text{g/g}$  of tissue in transgenic tobacco (Johnson *et al.*, 1989) and 0.5-2.0% of total soluble proteins in transgenic rice (Duan *et al.*, 1996) may provide insect resistance. The efficacy of a particular insecticide protein, however, is dependent on the target insect. An approximately 2000-fold range in levels of *B.t.* protein CryIA(b) for 50% mortality was observed across the 7 agronomically important insects (MacIntosh *et al.*,

1990). To undoubtedly detect and quantify the SaPIN2a protein in transgenic lettuce, the protein will be purified from transgenic plants.

It has been observed that expression of transgenes in lettuce is not as efficient as in other transgenic plants and many transgenes are unstable in transgenic lettuce plants (McCabe, *et al.*, 1999; Ryder, 1999). The mechanism of low transgene expression in lettuce is not well understood and may be associated with DNA methylation (McCabe, *et al.*, 1999). The observations on lack of accumulation of transgene protein despite over-expression of corresponding mRNA have also been reported in transgenic petunia (Jones *et al.*, 1985), tobacco (Jones *et al.*, 1985; Florack *et al.*, 1994), tomato (Seymour *et al.*, 1993), cauliflower (Passelegue and Kerlan 1996) and potato (Gatehouse *et al.*, 1997). The transgenic lettuce generated in this study should provide good material for further exploring the mechanisms responsible for low protein accumulation in transgenic lettuce and other species.

Whether SaPIN2a possesses the putative inhibitory activity towards bovine trypsin and chymotrypsin, based on its sequence homology to known PIN2s, is still an outstanding problem. One possibility is that the amount of SaPIN2a protein accumulated in leaves of transgenic lettuce is not sufficient, as indicated by western blot analysis, for *in vitro* inhibitory activity assay using bovine trypsin or chymotrypsin, although this amount of SaPIN2a protein is sufficient for inhibiting endogenous trypsin-like activity in lettuce leaves. An alternative explanation is that SaPIN2a is specific to certain plant endogenous proteases and/or the midgut proteases from cabbage looper larvae, but not to bovine trypsin and chymotrypsin. It was found that the *Tribolium*-protease inhibitors from soybean (Birk *et al.*, 1963) and wheat (Applebaum and Konijn, 1966) could markedly inhibit larval gut proteolysis of *Tribolium castaneum* but were inactive towards either mammalian trypsin or chymotrypsin. The maize proteinase inhibitor (MPI), belonging to the potato proteinase inhibitor I (PIN1) family based on its sequence homology to known PIN1s (Cordero *et al.*, 1994), has been shown to effectively inhibit midgut chymotrypsin from *S. littoralis* larvae, but it only weakly inhibits bovine chymotrypsin, unlike most members of PIN1 family which are potent inhibitors of mammalian chymotrypsin (Tamayo *et al.*, 2000). To undoubtedly establish the inhibitory activities of SaPIN2a, purified SaPIN2a protein from *S. americanum* stems could be employed for *in vitro* activity assays.

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of



the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

5                    Various references are cited herein, the disclosure of which are incorporated by reference in their entirety.

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Blake, Cassels & Graydon LLP  
Box 25, Commerce Court West  
199 Bay Street  
Toronto, Ontario M5L 1A9

December 5, 2002

IN THE CANADIAN PATENT OFFICE  
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Canada Canada  
OPIC CIPO

The Commissioner of Patents  
Ottawa-Hull, Canada  
K1A 0C9

DEC 05 2002 340

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Application No.: Un-assigné  
 Applicants/Inventors: CHYE, Mee-Len; and XU, Zeng-Fu  
 Filed: 2002/11/29  
 Title: GENETICALLY MODIFIED PLANTS WITH  
 HETEROGENEOUS PROTEINASE INHIBITOR SAPIN2A  
 AND METHODS OF USE THEREOF FOR THE  
 INHIBITION OF ENDOGENOUS TRYPSIN-AND  
 CHYMOTRYPSIN-LIKE ACTIVITIES  
 Our Reference: 63373/1

Dear Sir:

A Petition for Grant of a Patent was submitted to the Canadian Intellectual Property Office on November 29, 2002.

We enclose herewith the following:

1. Preliminary Amendment with sequence listing on sheets numbered 1/3 to 3/3.
2. Sequence Listing in computer readable form.
3. Statement Pursuant to Rules 111(c) & 112(c).

We also enclose an amended Petition to insert the addresses of the applicants/inventors of this application.

The Office is authorized to charge all fees in connection with this submission to Deposit Account No. 600000021.

Should any Patent Office Official want to contact us by telephone with respect to the above, the call should be made to John C. Hunt at (416) 863-4344.

Yours very truly,

Enclosure

21090159.1

**BLAKE, CASSELS & GRAYDON LLP**  
Box 25, Commerce Court West  
199 Bay Street  
Toronto, Ontario M5L 1A9

December 5, 2002

**IN THE CANADIAN PATENT OFFICE**

The Commissioner of Patents  
Ottawa-Hull, Canada  
K1A 0C9

Canadian Patent Application

Inventors: CHYE, Mee-Len; XU, Zeng-Fu  
Title: GENETICALLY MODIFIED PLANTS WITH HETEROGENEOUS  
PROTEINASE INHIBITOR SAPIN2A AND METHODS OF USE  
THEREOF FOR THE INHIBITION OF ENDOGENOUS  
TRYPSIN-AND CHYMOTRYPSIN-LIKE ACTIVITIES

Filing Date: November 29, 2002

Classification:

Examiner:

Our Reference: 63373/00001

4 Industrie Industry 4  
Canada Canada  
OPIC CIPO

DEC 05 2002 340

Dear Sir:

This is a preliminary amendment.

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**IN THE SEQUENCE LISTING**

We enclose herewith a Sequence Listing, on sheets numbered 1/3 to 3/3, for insertion into the application.

**REMARKS**

The enclosed Sequence Listing has been prepared in accordance with Sections 113 to 130 of the Patent Rules.

No data content of the Sequence Listing goes beyond that contained the application as filed and no new matter has been added.

Any telephone call regarding this matter should be directed to John Hunt at (416) 863-4344.

Yours very truly,

*Blak, Cas & Gr*

|                                     |
|-------------------------------------|
| SEQUENCE LISTING FORMAT<br>ACCEPTED |
| FEB 04 2003                         |
| INIT.: <u>DL</u>                    |

**BLAKE, CASSELS & GRAYDON LLP**  
Box 25, Commerce Court West  
199 Bay Street  
Toronto, Ontario M5L 1A9

December 5, 2002

**IN THE CANADIAN PATENT OFFICE**



**DEC 05 2002 340**

The Commissioner of Patents  
Ottawa-Hull, Canada  
K1A 0C9

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Canadian Patent Application

Inventors: CHYE, Mee-Len; XU, Zeng-Fu

Title: GENETICALLY MODIFIED PLANTS WITH  
HETEROGENEOUS PROTEINASE INHIBITOR SAPIN2A  
AND METHODS OF USE THEREOF FOR THE INHIBITION  
OF ENDOGENOUS TRYPSIN-AND CHYMOTRYPSIN-LIKE  
ACTIVITIES

Filing Date: November 29, 2002

Classification:

Examiner:

Our Reference: 63373/00001

**STATEMENT PURSUANT TO RULES 111(c) & 112(c)**

Dear Sir:

A computer-readable Sequence Listing in the form of a floppy diskette is enclosed. Only sequences disclosed in the application as filed are contained in the enclosed Listing. No new matter has been added.

The content of the copy of the Sequence Listing in computer-readable form is the same as the content of the Sequence Listing contained in the description.

Yours very truly,

- 1/3 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANTS: CHYE, Mee Len;  
XU, Zeng-Fu
- (ii) TITLE OF INVENTION: GENETICALLY MODIFIED PLANTS WITH  
HETEROGENEOUS PROTEINASE INHIBITOR SAPIN2A  
AND METHODS OF USE THEREOF FOR THE  
INHIBITION OF ENDOGENOUS TRYPSIN- AND  
CHYMOTRYPSIN-LIKE ACTIVITIES
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Blake, Cassels & Graydon LLP
  - (B) STREET: Box 25, Commerce Court West
  - (C) CITY: Toronto
  - (D) PROVINCE: Ontario
  - (E) COUNTRY: Canada
  - (F) POSTAL CODE: M5L 1A9
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: Windows NT
  - (D) SOFTWARE: FastSEQ for Windows Version 4.0;  
WordPerfect 9.0
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE: 29-NOV-2002
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Blake, Cassels & Graydon LLP
  - (C) REFERENCE NUMBER: 63373/00001
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 416-863-4344
  - (B) TELEFAX: 416-863-2653

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 529 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY:
- (ii) MOLECULE TYPE: Genomic DNA
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Solanum americanum
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

cataatggct gttcacaaag ttagcttctc tgcttgcta cttgttcttg gatggatggt 60
tctacttgcg aaacatggtg atgccaaggc ttgtactaga gaatgtggtc attttagcta 120
tggcatatgc ccacgttcag aaggaagtcc ccaaaaacct atatgcacca attggtgctc 180
aggctataag ggttgcaact attacagtgc taaggagat ttgatttggtg aaggagaatc 240

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- 2/3 -

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tgaccctaga aacccaaaag attgtacctt cgaatgtgat acacagattg cttattcaaa 300
atgtcctcgt tcagaaggaa agatgataat taaaccctact ggatgcacca cttgttgac 360
gggctatcag ggttgctact atttcgatca agatgggtgat tttgtctgtg aaggagagag 420
tcctgaacce aagaccactg cttatttcta atcaatcata tgttgttatc tatcaaaaaa 480
aaatatgtat gcatgatata tgctggttac tgtaatgtgg actttattg 529

```

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 148 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Solanum americanum

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Ala Val His Lys Val Ser Phe Leu Ala Cys Leu Leu Val Leu Gly
 1           5           10           15
Trp Met Phe Leu Leu Ala Lys His Val Asp Ala Lys Ala Cys Thr Arg
 20           25           30
Glu Cys Gly His Phe Ser Tyr Gly Ile Cys Pro Arg Ser Glu Gly Ser
 35           40           45
Pro Gln Lys Pro Ile Cys Thr Asn Cys Cys Ser Gly Tyr Lys Gly Cys
 50           55           60
Asn Tyr Tyr Ser Ala Lys Gly Asp Leu Ile Cys Glu Gly Glu Ser Asp
 65           70           75           80
Pro Arg Asn Pro Lys Asp Cys Thr Phe Glu Cys Asp Thr Gln Ile Ala
 85           90           95
Tyr Ser Lys Cys Pro Arg Ser Glu Gly Lys Met Ile Ile Lys Pro Thr
 100          105          110
Gly Cys Thr Thr Cys Cys Thr Gly Tyr Gln Gly Cys Tyr Tyr Phe Asp
 115          120          125
Gln Asp Gly Asp Phe Val Cys Glu Gly Glu Ser Pro Glu Pro Lys Thr
 130          135          140
Thr Ala Tyr Phe
145

```

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY:

(ii) MOLECULE TYPE: DNA

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Cauliflower mosaic virus

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

caatcccact atccttcgca agacc

25



**WHAT IS CLAIMED IS:**

1. An isolated nucleic acid molecule having a nucleotide sequence of SEQ ID NO:1 or a nucleotide sequence that encodes SaPIN2a having an amino acid sequence of  
5 SEQ ID NO:2.
2. An isolated nucleic acid molecule having a nucleotide sequence that hybridizes under stringent conditions to the complement of a nucleotide sequence of SEQ ID NO:1, wherein the stringent conditions comprise hybridization in 50% deionized  
10 formamide, 6xSSC, 5x Denhardt's, 1% SDS, 100 µg/ml denatured salmon sperm DNA at 42°C overnight (about 4-16 hours), and washing in 0.1xSSC, 0.1% SDS at 65°C.
3. An isolated SaPIN2a polypeptide having the amino acid sequence of SEQ ID NO:2 or a fragment thereof.  
15
4. An isolated polypeptide encoded by the nucleic acid molecule of claim 2 or a fragment thereof.
5. A recombinant vector comprising a nucleotide sequence of SEQ ID NO:1  
20 or a nucleotide sequence that encodes SaPIN2a having an amino acid sequence of SEQ ID NO:2.
6. A recombinant vector comprising a nucleotide sequence that hybridizes under stringent conditions to the complement of a nucleotide sequence of SEQ ID NO:1,  
25 wherein the stringent conditions comprise hybridization in 50% deionized formamide, 6xSSC, 5x Denhardt's, 1% SDS, 100 µg/ml denatured salmon sperm DNA at 42°C overnight (about 4-16 hours), and washing in 0.1xSSC, 0.1% SDS at 65°C.
7. The recombinant vector of claim 5 or 6 further comprising a regulatory  
30 nucleic acid operably linked to said nucleotide sequence.
8. The recombinant vector of claim 7 wherein the regulatory nucleic acid is 35S promoter of cauliflower mosaic virus (CaMV 35S).
- 35 9. A recombinant cell comprising the vector of claim 5 or 6.



10. The recombinant cell of claim 9 wherein the cell is a plant cell.
11. The recombinant cell of claim 10 wherein the plant is a leafy vegetable  
5 crop.
12. The recombinant cell of claim 11 wherein the crop is lettuce.
13. A recombinant cell comprising the vector of claim 7.
- 10
14. The recombinant cell of claim 13 wherein the cell is a plant cell.
15. The recombinant cell of claim 14 wherein the plant is a leafy vegetable  
crop.
- 15
16. The recombinant cell of claim 15 wherein the crop is lettuce.
17. A method for producing a transformed plant comprising: (i)  
transforming a plant with a recombinant vector comprising a nucleotide sequence of SEQ  
20 ID NO:1 or a nucleotide sequence that encodes SaPIN2a having an amino acid sequence of  
SEQ ID NO:2; and (ii) selecting a transformed plant in which said nucleotide sequence is  
expressed.
18. A method for producing a transformed plant comprising: (i)  
25 transforming a plant with a recombinant vector comprising a nucleotide sequence that  
hybridizes under stringent conditions to the complement of the nucleotide sequence of SEQ  
ID NO:1, wherein the stringent conditions comprise hybridization in 50% deionized  
formamide, 6xSSC, 5x Denhardt's, 1% SDS, 100 µg/ml denatured salmon sperm DNA at  
42°C overnight (about 4-16 hours), and washing in 0.1xSSC, 0.1% SDS at 65°C; and (ii)  
30 selecting a transformed plant in which said nucleotide sequence is expressed.
19. The method of claim 17 or 18 wherein an endogenous protease activity  
of the transformed plant is inhibited.

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20. The method of claim 19 wherein the endogenous protease activity is a trypsin-like activity and/or chymotrypsin-like activity.

21. A transformed plant produced by the method of claim 17 or 18.

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22. The transformed plant of claim 21 wherein the plant is a leafy vegetable crop.

23. The transformed plant of claim 22 wherein the crop is lettuce.

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24. The transformed plant of claim 23 wherein an endogenous protease activity of the transformed lettuce is inhibited.

25. The transformed plant of claim 24 wherein the endogenous protease activity is a trypsin-like activity and/or chymotrypsin-like activity.

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26. The transformed plant of claim 23 wherein the transformed lettuce is resistant to an insect.

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27. The transformed plant of claim 26 wherein the insect is *Trichoplusia ni*.

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Figures: 1a, b, 2, 3a, b, 4, 5, 6, 7, 8, 9

Pages: \_\_\_\_\_

Unscannable items  
received with this application  
(Request original documents in File Prep. Section on the 10<sup>th</sup> floor)

Documents reçu avec cette demande ne pouvant être balayés  
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10<sup>ème</sup> étage)