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Loss of Specific Sequences in a Natural Variant of Potato Proteinase Inhibitor II Gene Results in a Loss of Wound-Inducible Gene Expression

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Abstract : We have isolated several proteinase inhibitor II genes *pin2* from a Russet Burbank potato DNA library. One of these, *pin2T* was subcloned and a 1.8 kb XbaI/NsiI insert was sequenced. This fragment contained the complete Inhibitor II gene including 965 bp of flanking DNA upstream from the gene and 200 bp of flanking DNA downstream from the gene. The open reading frame encodes a protein that is similar to other reported proteinase Inhibitor II proteins. The DNA sequence of the 5' flanking region of pin2T from -714 to +1 is highly homologous (91% identity) with that of the previously isolated wound-inducible *pin2K*. There are, however, four small deletions in the *pin2T* promoter which are located at -221 to -200, -263 to -254, -523 to -426 and -759 to -708 relative to the transcription start site of the wound-inducible *pin2K*. Three of these deletions map to a portion of the promoter that controls the wound-inducibility of the proteinase inhibitor genes. Chimeric genes containing the promoter of the *pin2T* gene linked with the both CAT and GUS were constructed and transfered into tobacco plants. Analysis of these plants indicated that *pin2T* is not a wound-inducible gene but is expressed at low levels. Thus, wound-inducibility is lost with the concomitant natural deletion of three small regions of the promoter. Comparision of the sequences deleted in *pin2T* relative to the *pin2K* with Genebank sequences indicates that the deleted sequences contain a motif (consensus 5'-AGTAAA-3') that is found in many other wound-inducible genes but not easily found in the published promoter sequences of other plant genes. Nuclear proteins from unwounded and wounded potato leaves were bound to the proximal promoter region, downstream of the 5'-AGTAAA-3', of *pin2T*. The comparison of the *pin2T* gene with the *pin2K* gene indicates that the natural internal promoter deletions are likely responsible for loss of the wound-inducible phenotype in the *pin2T* gene(Received January 24, 1996; accepted March 4, 1996).

Introduction

In nature, plants are subjected to the frequent environmental harassment by herbivorous insects, fungi and other pathogens which attack plants both above and below the ground. To survive, they have developed intricate defense mechanisms to respond to these environmental pests.

One of the best studied of these defense systems is the proteinase inhibitor system of solanaceous plants. Proteinase inhibitors are found in the seeds of most plants; however, in some solanaceous plants these defense factors are expressed in the foliage. While the level of these proteinase inhibitors is normally low in unwounded foliage, the foliar proteinase inhibitors can be induced by mechanical injury such as the attack of chewing insects.[1,2] In potato and tomato, there are two major families of non-homologous proteinase inhibtors, called proteinase inhibitor I (PI-I) and proteinase inhibitor II (PI-II). They are active against trypsin-like and chymotrypsin-like proteases from a variety of sources.[3]

The cDNAs[4-6] and the genes[7-11] for both PI-I and PI-II have been isolated and characterized. These probes have permitted a greater understanding of the mechanisms of gene activation. For example, Graham *et al.*[12] determined that the induction following wounding is regulated at the transcriptional level and that the induction process included a lag phase of two to four hours after wounding before mRNA is synthesized.

Other studies have shown that the promoters can be used to express marker genes[10,13] or the native proteinase inhibitor[14] in transgenic plants. Theses studies have indicated that *pin2* regulartory sequences are capable of driving expression in two different plant tissues, wounded leaves and unwounded potato tubers.[13] Thus, the proteinase inhibitor genes are capable of responding to

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a developmental program as well as to environmental stimuli. Other studies have determined that the *pin2* promoter increases the level of expression of mRNA, probably by functioning as an very strong terminator.[15,16] In addition, several promoter deletion studies have identified a region between -500 and -130 of the *pin2* promoter which appears to be responsible for wound-inducible regulation of these proteinase inhibitor genes.[17,18] This region also binds nuclear proteins from both unwounded and wounded tomato leaves. It is also clear from these studies that the far upstream region of the promoter between -1340 and -800 contain an enhancer which is responsible for driving high levels of expression of the *pin2* gene in transgenic plants.[18]

In this report we describe the isolation and analysis of a natural proteinase inhibitor II gene varient (*pin2T*) that is not wound-inducible but is expressed constitutively at low levels in transgenic plants. Our molecular and functional characterization of this *pin2T* gene leads us to identify a repeated sequence that has been lost from the promoter of this gene, but is also repeated in the promoters of many other wound inducible genes. Our analysis indicates that loss of this sequence is responsible for loss of the wound-inducible phenotype.

Materials and Methods

Subcloning of Inhibitor IIT clone and sequence analysis

Our methods for screening the potato genomic library for *pin2* clones were previously described.[10] The recombinant lambda phage *Φ2T* was mapped with restriction enzymes and a 6.0 kb HindIII/PstI region was identified as containing the *pin2* gene and was selected for subcloning. This region was subcloned in various sites within pUC118 and pUC119 vectors as two fragments, a 2.6 kb HindIII/HindIII fragment and a 3.4 kb HindIII/PstI fragment. Single stranded DNA templates were prepared using M13KO7 phage[19] and sequencing was conducted on single stranded DNAs using the dideoxy chain termination method.[20] Sequence analysis was conducted with the GCG analysis package on a VAX computer.[21]

Construction of chimeric Inhibitor IIT-GUS and Inhibitor IIT-CAT genes

A pair of promoterless plant transformation vectors were constructed that containied that β-glucuronidase (GUS) and the chloramphenicol acetyl transferase (CAT) marker genes.[22] Both vectors are reconstructions of pBI 101.1 which have been modified to contain the strong *pin2K* terminator.[15] These vectors, pRT189(CAT) and pRT190 (GUS), have poly-linkers upstream of the marker genes for promoter fusions. The promoter of the *pin2T* gene was isolated as BamHI/BglII fragment and moved into the BamHI site of the promoterless transformation vectors. All constructions were sequenced through the junction between the pin2 promoter and reporter genes to confirm the structure.

Transformation of tobacco plants

The chimeric *pin2T*-GUS and *pin2T*-CAT genes were introduced into *Agrobacterium tumefacience* strain LBA 4404 by direct transformation method.[15] *Agrobacterium* cells containing the chimeric gene were co-cultivated for 2 days with leaf pieces from sterile *Nicotiana tabacum* cv. Xanthi plants as previously described.[10] The bacterial cells were washed away and transformed tobacco calli were screened on Murashige-Skoog[23] agar medium containing 3% sucrose, kanamycin (200 mg/*l*), cefotaxime (250 mg/*l*) and an appropriate amount of phytohormones, naphthaleneacetic acid and benzyladenine. The co-cultivated plant calli were incubated at 28℃ under light (3000 lux) for 12 hr/day. The tissues were transfered to another fresh medium. Plants were regenerated on Murashige-Skoog agar medium containing the same concentration of sucrose and antibiotics but lacking the phytohormones. Regenerated plants were transfered to pots and grown in a greenhouse.

Plant DNA isolation and polymerase chain reaction

To confirm that the transformed plants were indeed transgenic, the presence of the transgene was verified by polymerase chain reaction, PCR. Three different oligonucleotides were synthesized, which corresponded to a conserved region of the *pin2K* and *pin2T* promoter at -174 to -155 (5'-GAAGAAGCAAGCGTAAGTAC-3'), and reverse primers corresponded to the GUS coding region from +74 to +90 (5'-GAATTCCACAGTTTTCG-CGA-3') and to the CAT coding region from +15 to +34 (5'-CAACGGTGGTATATCCAGTG-3'). Plant DNA was isolated according to the published method2.[24] The oligonucleotides were combined with the plant DNA, and subjected to the PCR. Products were separated on a 1% agarose gel and hybridized with a 225 bp Sau3AI /BglII fragment (-198 to +27) of the *pin2T* promoter as a probe.

Enzyme assays

GUS activity was determined using spectrofluorometric assay as described by Jefferson.[25] Extracted leaf protein was determined according to Bradford.[26] In each case, 100 μg proteins were asssayed for enzyme activity. The product was quantified with Shimadzu fluorometer 2000 using 4-methyl-umbelliferone as a standard. CAT activity was determined by a published method.[27] Leaf proteins (100 μg) were assayed in the presence of 1 μl [^{14}C]-

chloramphenicol (3.7 Mbq/ml) as a substrate.

Preparation of nuclear proteins and DNA gel retardation assay

Nuclei were isolated from unwounded and wounded potato leaves as well as potato tubers as described in the published paper.[28] Nuclear protein was quantified according to Bradford[26] using BSA as a standard. Labelling of DNA fragments and extraction of labeled DNA fragments were followed the published method.[29] DNA gel retardation assay was performed according to the published method.[30] DNA binding proteins were identified by electrophoresis on 4% acrylamide gel. After electrophoresis, the gel was dried at 50℃ for 30 min and exposed to the Kodak X-Omat film for various time.

Results and Disscusion

It has been previously demonstrated that the *pin2* cDNAs hybridize to many sequences (12 to 20) within the *S. tuberosum* genome indicating that this is a medium sized gene family.[24] To increase our understanding the regulation of the inhibitor II genes within this species, we choose to identify other genes from the *pin2* family which was altered in their mode of expression. The potato genomic library was initially screened with the tomato proteinase inhibitor II cDNA[5] by Dr. T. E. Cleveland as a postdoctoral fellow in Dr. C. A. Ryan's laboratory of the Washington State University. To confirm the restriction enzyme map of the recombinant lambda phage termed *Φ2T*, the Southern hybridization of phage 2T DNA with the tomato proteinase inhibitor II cDNA was carried out using standard methods.[29] The recombinant lambda phage *Φ2T* was mapped with restriction enzymes and a 6.0 kb HindIII/PstI region was identified as containing the *pin2* gene and was selected for subcloning. The restriction maps of *Φ2T* and *Φ2K* are toatally different from each other (data not shown), it was reasoned that the *Φ2T* might contain a potato proteinase inhibitor II gene (*pin2T*) that was regulated differently from *pin2K*.

The primary structures of the *pin2T* gene and of its flanking regions were determined by sequencing the entire 1777 bp XbaI/NsiI fragment of the Inhibitor IIT gene. This XbaI/NsiI fragment was subcloned into 20 different subclones to sequence completely in both directions and to read through all of the restriction sites. This fragment contained the complete Inhibitor II gene including 965 bp of flanking DNA upstream from the gene and 200 bp of flanking DNA downstream from the gene. The nucleotide sequence of the *pin2T* gene and flanking sequences are shown in the Fig. 1. The protein encoded by *pin2T* is composed of two exons separated

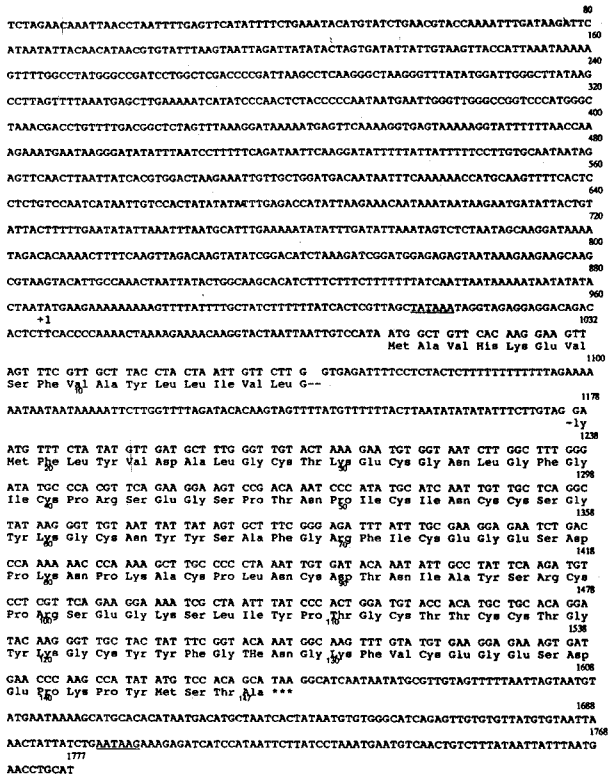

Fig. 1. A nucleotide sequence of a 1.8 kb fragment of the Inhibitor IIT gene and flanking sequences. The TATA box and polyadenylation signal AATAAG are underlined. +1 indicates the site of transcription initiation in the homologous tomato inhibitor II cDNA sequence. The numbering of the amino acids begins at the amino acid terminus of the transit sequence.

by a single 113 bp intron and is a typical of other proteinase inhibitors that has been previously published.[5,6,9-11] The deduced amino acid sequence of the PI-IIT protein exhibits approximately 80% amino acid identity with previously published sequences of PI-II.

The comparison of nucleotide sequence of the *pin2T* promoter with that of the wound-inducible *pin2K*[10] is shown in the Fig. 2. The 5' flanking region of the *pin2T* is highly homologous (91% identity) with that of *pin2K* from -780 to +1 relative to the transcription start site of *pin2K* with the exception of four small deletions. These deletions are shown relative to +1 of *pin2K* as △1 (△22 bp, -221 to -200), △2 (△20 bp, -273 to -254), △3 (△65 bp, -526 to -462), and △4 (△52 bp, -759 to -708) in the Fig. 2.

In order to map the controlling regions of the *pin2T* promoter, full length promoter was linked to promoterless plant transformation vector containing either CAT or GUS (pRT190 or pRT189) coding sequences and the *pin2K* terminator. The junctions between the reporter genes and the *pin2T* promoter fragment were sequenced to confirm the orientation of the promoter. Following transfer of the *pin2T*-CAT and *pin2T*-GUS vectors into

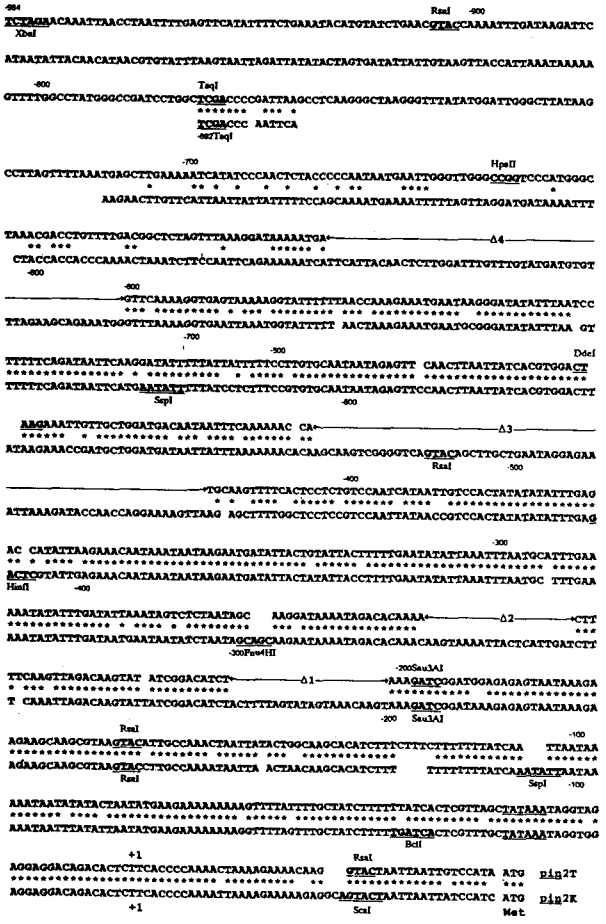

Fig. 2. Comparison of the nucleotide sequence of the 5' flanking region of *pin2T* with that of *pin2K*. The TATA boxes are underlined and +1 indicates the site of transcription initiation site. The translation start codon (ATG) is also shown. The deleted regions of pin2T are shown as △1, △2, △3, and △4. Asterisks between the sequences indicates identity. Gaps were introduced for best fit of alignment. The restriction enzyme sites are underlined and labeled.

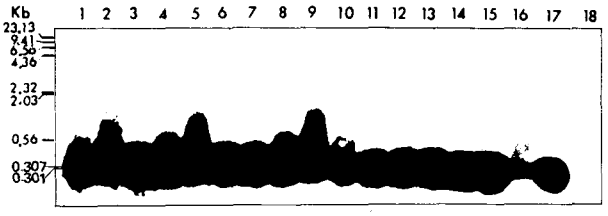

Fig. 3. Autoradiogram of Southern analysis of PCR products from transformed and untransformed plants. Lanes #1-17 are from transgnic tobacco leaf DNAs and lane #18 is from untransformed plants leaf DNA as a negative control. PCR products were separated on a 1% agarose gel and hybridized with 225 bp Sau3AI/BglII fragment (-198 to +27) of the *pin2T* promoter as a probe. PCR products of *pin2T*-GUS and *pin2K*-GUS constructions should be 301 bp and 307 bp, respectively.

Table 1. CAT activity[a] driven by *pin2K* and *pin2T* promoters

Transgenic plant	Promoter	Unwounded plant	Wounded plant
Tr25 (n=6)[b]	*pin2K*	2.25± 1.05	32.5± 10.3
Tr198 (n=14)	*pin2T*	0.76± 0.06	0.65± 0.05
Untransformed (n=4)		0.40± 0.11	0.50± 0.08

[a]CAT activity: % conversion of chloramphenicol to chloramphenicol acetates, [b]number of plants assayed

Table 2. GUS activitya driven by *pin2K* and *pin2T* promoters

Transgenic plant	Promoter	Unwounded plant	Wounded plant
Tr210 (n=2)[b]	*pin2K*	61± 52	1406± 97
Tr193 (n=15)	*pin2T*	20± 10	26± 13
Untransformed (n=4)		12± 3	14± 4

[a]GUS activity: pmol 4-MU/min·mg protein, [b]number of plants assayed

Agrobacterium, tobacco plants were transformed to kanamycin resistance and regenerated. The presence of the transgene in the transformed tobacco plants was determined by performing PCR on isolated DNA from kanamycin-resistant plants. Fig. 3 shows one of the Southern blot hybridizations of PCR products from transgenic plants probed with a small fragment (-198 to +27) of the *pin2T* promoter. PCR products of *pin2T*-CAT, *pin2T*-GUS, *pin2K*-GUS constructions should be 265 bp, 301 bp, and 307 bp, respectively.

Table 1 and 2 show the comparison of CAT and GUS activities driven by *pin2* promoters. CAT activity driven by the *pin2K* promoter was well induced by wounding, while CAT activity driven by the *pin2T* promoter was not induced by wounding. However the *pin2T*-CAT construct expressed little CAT activity in both wounded and unwounded leaves. Similar results were obtained in GUS assay of Tr210 (*pin2K*) and Tr193 (*pin2T*) plants. GUS activites driven by the wound-inducible *pin2K* promoter showed that wounding causes 23 times higher expression of the GUS gene than that of corresponding unwounded plants. On the other hand, the expression of the *pin2T*-GUS construct showed low but above background levels. Therefore, we concluded that the *pin2T* promoter is functional but has lost the wound-inducible phenotype.

It has been previously demonstrated that nuclear proteins specifically interact with sequences in the *pin2* promoter.[17,18] To determine whether the *pin2T* promoter was deficient in its interactions with nuclear proteins, DNA gel retardation assays were carried out on several small fragments from the *pin2T* promoter. The radiolabeled DNA fragments were incubated with the nuclear proteins from 3 different tissues; unwounded (U) and wounded (W) potato leaves as well as tubers (T), and the incubation mixture was run on a polyacrylamide gel. An autoradiogram of the gel retardation assay is shown in the Fig. 4. While the nuclear proteins from both un-

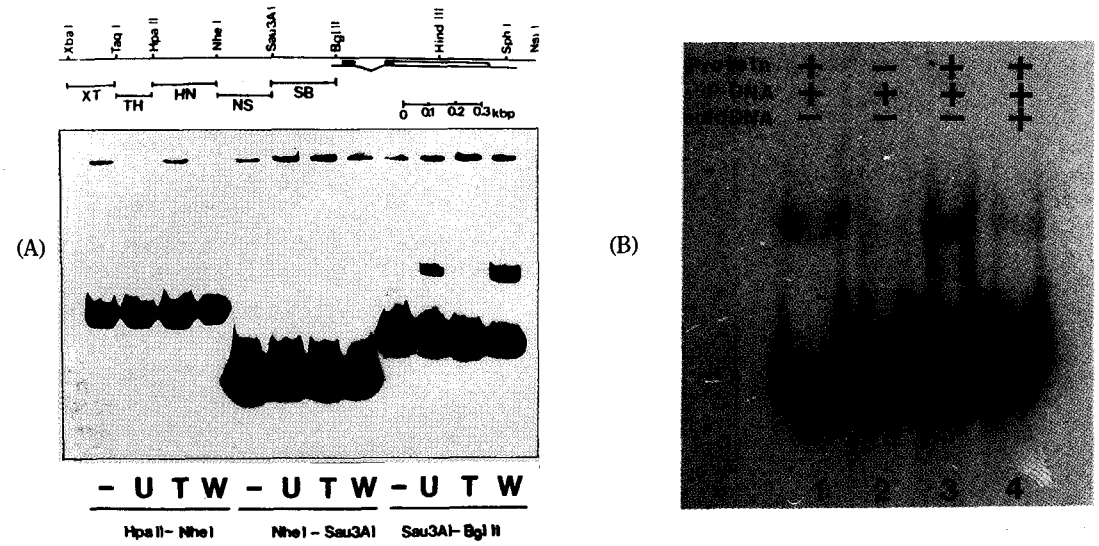

Fig. 4. Autoradiogram of DNA gel retardation assay. (Panel A) The symbols at the bottom of each lane represent additions of nuclear proteins to the labeled DNA fragments. (−) indicates no additions while U, T, and W indicate nuclear proteins isolated from unwounded leaf, tuber, and wounded leaf, respectively. Three different *pin2T* promoter fragments were incubated with the isolated nuclear proteins and subjected to 4% polyacrylamide gel. After drying, the gel was exposed to X-Omat film. (Panel B) Competetition of DNA binding proteins with unlabeled DNA fragment. Lane 1 contains a reduced amount (0.5X) of labeled Sau3AI/BglII fragment; Lane 2 contains labeled DNA fragment only; Lane 3 contains labeled DNA fragment and nuclear proteins from unwounded leaf; Lane 4 contains labeled and unlabeled (10X) DNA fragment plus nuclear proteins.

wounded and wounded foliage showed strong interactions with the proximal Sau3AI-BglII fragment (-198 to +27), the proteins from tuber showed little or no interaction with any of promoter fragments. The binding of the nuclear proteins to the proximal promoter fragment was specific because the signal from the DNA-protein complex was reduced when a 10 fold excess of unlabeled Sau3AI-BglII fragment was added to the binding reaction (Panel B, lane 4). These results are identical with those previousely reported for the *pin2K* promoter[17] and they indicate the deletions in the *pin2T* promoter did not cause the loss of protein binding ability.

Meanwhile, Dr. Paul Staswick, University of Nebraska, indicated to us that the AGTAAA was frequently located in the promoters of jasmonic acid inducible genes (personal communication to Dr. R. W. Thornburg). Because methyl jasmonate is one of the inducers of *pin2* genes,[31] we examined the promoters of *pin2K* and *pin2T* genes for this sequence. We noticed that this same squence was located three times in *pin2K* but all three sequences were deleted in *pin2T* (Table 3). The wound-inducible *pin2K* promoter contains this sequence 5'-AGTAAA-3' once in the △2 region and twice in the △1 region of *pin2T* promoter. We therefore undertook to search through the GeneBank to determine if this sequence was found in other wound-induced genes.

Table 3 shows the comparison of the promoter sequence of *pin2T* with that of the wound-inducible *pin2K* in the two deleted regions as well as those of several other wound-induculble genes. The sequence 5'-AGTAAA-3' could be found easily in other wound-inducible genes. The hexanucleotide sequence, however, could not be easily found in the promoter region of other plant genes. Only one of twenty different promoters of the published ribulose-1,5-bisphosphate carboxylase small subunit genes[32] contains this sequence. Only one of eleven different promoters of auxin-inducible genes[33] contains this sequence. However, chlorophyll a/b binding protein genes from pea, wheat, and Arabidopsis[34-36] and 7 different ribulose-1,5-bisphophate carboxylase large subunit genes[37] do not have this hexanucleotide in their sequenced promoter regions.

The comparison of the promoter sequences of the *pin2T* with that of the wound-inducible *pin2K* leads to conclusion that deletion of several small sequences in the *pin2T* promoter region relative to the *pin2K* promoter is responsible for the loss of wound-inducibility in the *pin2T* gene. The true test of this hypothsis would be to add the deleted regions to the *pin2T* gene or delete them from the *pin2K* gene and observe the effect on the expression. Therefore the deletion test on the *pin2K* gene using oligonucleotide-directed mutagenesis will be carried out.

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Table 3. Comparison of nucleotide sequence of *pin2T* △1 and *pin2T* △2 with other wound-inducible genes

Plant	Gene	Sequence	Reference
Potato	*pin2K*	$^{-294}$AATAAAATAGACACAAACA**AGTAAA**ATTACTCATTGATCTTT^{-253}	10)
Potato	*pin2T*	$^{-254}$GATAAAATAGACACAAAA——————△2——————CTTT^{-233}	this work
Potato	*pin2K*	$^{-232}$GGACATCTACTTTTAGTAT**AGTAAA**CA**AGTAAA**GATCGGATA^{-191}	10)
Potato	*pin2T*	$^{-212}$GGACATCTA——————△1——————AAGATCGGATG^{-193}	this work
Potato	*win1*	$^{-804}$TGCAAAAATGAATGAAATT**AGTAAA**TTTTGGCCATAGATTTC^{-763}	38)
Potato	*win2*	$^{-1324}$TATAGTAGAAGTTAGGAAA**AGTAAA**GGTAGACCGAAATAGAC^{-1283}	38)
Potato	*win2*	$^{-900}$ATCAAACATACGCATATCA**AGTAAA**AAATGAAGGTTTGATGT^{-859}	38)
Potato	*wun1*	$^{-478}$GAAATATAATTTTTTATTT**AGTAAA**ATAATATGAGAATTAAT^{-427}	39)
Poplar	*win3*	$^{-153}$TTTGTCGGAAATCAGCACG**AGTAAA**ACACTCCAATTTGTATT^{-112a}	40)
parsley	*chs1*[b]	$^{-87}$CCTCCCTTGAATTTCTATC**AGTAAA**TTTTCAACCCTCTCTCT^{-46}	41)
Soybean	*chs2*	$^{-309}$ATGAGCGTTAACATAATCA**AGTAAA**AATGATATTAAAAAAAA^{-267}	43)
Parsley	*pall*[c]	$^{-303}$CAGGTCATTCTAATCTAGG**AGTAAA**AAACTCCTGTTCAAACC^{-261}	43)
Arabidopsis	*pall*	$^{-659}$AGTTATAACAAAATGGTAC**AGTAAA**TTAAACAGAACATCAAG^{-617}	44)
Bean	*ch5b*[d]	$^{-583}$GTAATAAAATTAATAAAAAT**AGTAAA**TAAAAAAAATTATACTT^{-531}	45)
Tobacco	*pr1*[e]	$^{-290}$AAAAGAGATATAATATGGA**AGTAAA**AATTAATCAGATCAAA^{-249}	46)
Carrot	extensin	$^{-352}$AGAAACACCGACTCTGAAA**AGTAAA**AATTATACAATGAAAAT^{-311}	47)

[a]from ATG, [b]chalcone synthase, [c]phenylalanine ammonia-lyase, [d]chitinase, [e]pathogenesis-related protein.

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감자의 단백질 분해효소 억제제 II 유전자의 특별한 염기서열의 자연적 제거로 인한 상처 유발성 발현의 소실

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초록 : 감자의 genomic DNA library로 부터 분리한 proteinase inhibitor II (*pin2*) 유전자들의 제한효소 지도를 작성하였던바 이미 분리된 상처 유발 (wound-inducible) *pin2K* 유전자의 것과 상이성이 있는 *pin2T*를 분리하여 염기서열을 결정하였다. 두 유전자의 염기서열은 전체적으로 약 86%의 동일성을 보였으며 특히 promoter 부위의 염기서열은 *pin2K* 유전자의 전사개시 부위의 상대적인 위치인 -714까지 네부분의 결손(20 내지 65 bp)을 제외하면 약 91% 수준의 동일성을 보였다. 분리한 유전자들의 promoter 부위를 표지 유전자인 CAT와 GUS 유전자에 연결 시킨후 담배에서의 발현을 추적하였던바, *pin2K* 유전자의 promoter에 의한 표지유전자의 발현은 상처에 의해 발현 되었으나 *pin2T* 유전자의 promoter에 의한 표지유전자의 발현은 상처 유무와 관계없이 낮은 수준으로 나타났다. 또한 *pin2T* 유전자의 promoter 내의 결손은 핵 단백질의 promoter에의 결합에 영향을 주지 않았으며 상처 유발 *pin2K* 유전자의 promoter 염기서열과 비교하였을때 *pin2T* 유전자의 promoter 부위내에 5'-AGTAAA-3'라는 특별한 염기부위가 자연적으로 제거된것을 알수 있었다. 또한 5'-AGTAAA-3'의 염기부위가 다른 상처 유발 유전자들에서는 흔히 발견되고, 다른 식물 유전자들의 promoter에서는 쉽게 발견이 되지 않았다. 따라서 상처 유발 *pin2K* 유전자의 promoter내에 상처 유발과 관련있는 특별한 염기부위가 자연적으로 결실되어 *pin2T* 유전자의 발현이 상처 유발성을 잃은것으로 짐작된다.

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