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Characterization of two proteinase inhibitor (ATI) cDNAs from alfalfa leaves (*Medicago sativa* var. Vernema): the expression of ATI genes in response to wounding and soil microorganisms

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Abstract

cDNAs encoding two Bowman-Birk proteinase inhibitors were isolated from the leaves of alfalfa (*Medicago sativa*). The cDNAs are derived from a small gene family (3 to 10 genes) encoding alfalfa trypsin inhibitors (ATIs). Each cDNA clone encoded a mature ATI that was part of a larger, putative preprotein. ATI mRNAs are continuously expressed in flower parts, but are mechanically wound-inducible in the stems and leaves. ATI mRNA is shown to be continuously present in roots of soil-grown plants, but its presence is primarily in response to microorganisms present in the soil. Additionally, while mechanical wounding of the alfalfa roots induced ATI mRNA synthesis both in the roots and in the leaves, microbial infection of the roots triggered ATI mRNA synthesis in the roots but not in the leaves. These results suggest that both local and systemic signalling pathways for proteinase inhibitor synthesis are present in alfalfa plants.

Introduction

Proteinase inhibitors are found throughout the plant kingdom, where they serve a protective function against herbivorous insects [1]. The most intensively studied proteinase inhibitors are the Inhibitor I and Inhibitor II families found in solanaceous plants [2]. These inhibitors are expressed developmentally in the flowers [3, 4], and in response to mechanical wounding or microbial infection in the leaves [5, 6]. Microbe-induced proteinase inhibitor synthesis is restricted to the site of infection while the wound-induced synthesis is a systemic response. The signal transduction pathways regulating these responses have been the subject of intensive research [7].

A wound-inducible trypsin inhibitor had previously been isolated from alfalfa leaves and was

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X79879 (ATI 18) and X79880 (ATI 21).

shown to be a member of the Bowman-Birk family [8, 9]. Bowman-Birk inhibitors are small (M_r ca. 8000), cysteine-rich proteins typically found in seeds, where their expression is developmentally regulated [10]. The alfalfa trypsin inhibitor was the first published example of a wound-inducible Bowman-Birk inhibitor. More recently, another wound-inducible Bowman-Birk inhibitor has been isolated from maize coleoptiles [11].

In this report, we present the sequences of two cDNAs encoding alfalfa trypsin inhibitors (ATIs) that are homologous to a mature ATI protein previously isolated from alfalfa leaves [8, 9]. We show that ATI mRNA is expressed developmentally, and in response to wounding and to soil microorganisms. The induction of ATI in roots by soil microorganisms is not accompanied by a systemic response in leaves, indicating that both local and systemic signaling pathway are present that regulate ATI synthesis.

Materials and methods

Construction of cDNA library and isolation of ATI clones

Poly(A)$^+$ RNA was extracted from alfalfa leaves ca. 9 h after wounding and a cDNA library was prepared using the method of Gubler and Hoffman [12]. About 3000 clones were screened with a degenerate oligonucleotide probe corresponding to amino acids 4–9, as numbered from the amino terminus of the previously isolated, mature ATI [8, 9].

An ATI cDNA (ATI 7) was isolated, but sequencing revealed that it contained a cloning artifact, a long series of adenine residues preceded by a long series of thymidine residues, at the 5′ end. Consequently, a representative cDNA library was constructed in λ Zap (Stratagene, La Jolla, CA) using poly(A)$^+$ RNA extracted from alfalfa leaves ca. 7 h after wounding. Eighty thousand primary library clones were screened with the ATI 7 insert and 36 hybridizing clones were identified. The twelve longest clones were purified, of which eight clones hybridized to the ATI 7 insert at high stringency (0.1 × SSC, 65 °C), while the remaining four clones hybridized to the ATI 7 insert at moderate stringency (1 × SSC, 65 °C). The longest clone from each of the two groups was sequenced by the dideoxy method of Sanger *et al.* [13].

Isolation and analysis of RNA and genomic DNA

Genomic DNA was isolated using the CTAB method [14]. Electrophoresis was carried out on a 0.8% agarose gel and the separated DNA molecules were blotted onto nitrocellulose membrane (Schleicher and Schuell, Keene, NH). DNA fragments to be used as probes were labelled with ^{32}P by nick translation (NEN DuPont, Wilmington, DE). Hybridization was at 42 °C in 50% formamide.

Total RNA was isolated by freezing the tissue in liquid N_2 and grinding to a fine powder. The powdered tissue was extracted in the presence of Tris-buffered phenol pH 8.0 (Baker, Philipsburg, NJ) and 100 mM Tris-HCl pH 8.0 (1 g tissue = 0.5 ml phenol + 0.5 ml Tris-HCl).

Total RNA to be used for isolation of poly(A)$^+$ RNA was precipitated by the addition of 1/4 volume of 8 M lithium chloride, incubation at 4 °C overnight then centrifugation at 12000 × *g* for 30 min. Poly(A)$^+$ RNA was purified using oligo-dT columns (Pharmacia, Piscataway, NJ). RNA was quantified by measuring absorbance at 260 nm using a Beckman Model 2400 spectrophotometer.

For northern blot analysis 5 μg total RNA was fractionated by electrophoresis on a 1.4% agarose/formaldehyde gel prior to blotting [15]. Blotting, nick-translation of probes and hybridization were as described above. All northern blots were probed with a 370 bp *Pst* I-*Hinc* II coding region fragment of ATI 18. This fragment did not distinguish between individual ATI gene family members.

Plant treatments

Alfalfa plants were about eight weeks old and were flowering. In experiments involving leaf

wounding, most of the leaves in the lower third of the plant were wounded once with a hemostat. In experiments involving root wounding, the plant was removed from the pot and the root mass (including soil) was randomly crushed with pliers.

A magenta tissue culture box (Sigma Chemical Company, St. Louis, MO), one third filled with soil (Soils Incorporated, Puyallup, WA), was attached to a second tissue culture box with an adaptor. The boxes and soil were autoclaved for 30 min then left at room temperature for 3 days then reautoclaved. Three surface-sterilized alfalfa seeds were planted in each box in a tissue culture hood. The soil was inoculated with microorganisms at the time of planting. Plants were allowed to grow for three weeks before RNA extraction.

Identification of soil microorganisms

Greenhouse soil in which alfalfa plants were growing was streaked onto YMB agar plates [16] and incubated at room temperature for 2 to 3 days. Bacterial colonies with different morphologies were streaked to purity and typed by analysis of their lipid profiles (Five Star Laboratories, Branford, CT).

Results and discussion

cDNAs encoding two members of an alfalfa trypsin inhibitor (ATI) gene family were isolated from a wounded alfalfa leaf cDNA library. The sequence of the longer clone, ATI 18, is shown in Fig. 1. ATI 18 contains an open reading frame which encodes a 113 amino acid protein, composed of a 58 amino acid ATI preceded by a relatively long putative leader sequence (Fig. 2). Since there are five methionine residues clustered at the 5′ end of the open reading frame, the putative leader sequence could be from 44 amino acids to 55 amino acids, depending on which methionine is used as the translation initiation site.

The leader sequence may target the ATI preprotein to the endoplasmic reticulum, a first step

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ATI 18   1 cataagtacatagtactaagagagagtaAGtgAGAaAGaGattatATGGAGTTGATGATG 60
ATI 21     .........................gAGaaAGAgAGgGtgataATGGAGTTGATGATG 33
                                                     MET

ATI 18  61 AACAAGAAGGCAATGATGATGAAGTTAGCTTTGTTGGTTTTCCTTCTGGGCTTCACTTCA 120
ATI 21  34 AACAAGAAGGCAATGATGATGAAGTTAGCTTTGTTGGTTTTCCTTCTGGGCTTCACTTCA 93

ATI 18 121 ACAGtGGTTGATGCTCGTTTCGATCaAGCATCCTTCATCAgTCAACTGCTCTTCAAtGGT 180
ATI 21  94 ACAGgGGTTGATGCTCGTTTCGATCgAGCATCCTTCATCAcTCAACTGCTCTTCAAcGGT 153

ATI 18 181 GAAGCTGCTAATTACGATGTCAAATCCACAACAACAGCATGCTGTAATTTCTGCCCTTGC 240
ATI 21 154 GAAGCTGCTAATTACGATGTCAAATCCACAACAACAGCATGCTGTAATTTCTGCCCTTGC 213

ATI 18 241 ACAAgATCAATCCCTCCTCAGTGTCGTTGTaCtGATATTGGAGAgACATGTCACTCtGCT 300
ATI 21 214 ACAAaATCAATCCCTCCTCAGTGTCGTTGTtCcGATATTGGAGAaACATGTCACTCaGCT 273

ATI 18 301 TGCAAAAGTTGCcTTTGCACAAGaTCTatTCCTCCACAGTGCCGTTGTACTGATATCACA 360
ATI 21 274 TGCAAAAGTTGCaTTTGCACAAGgTCTtaTCCTCCACAGTGCCGTTGTACTGATATCACA 333

ATI 18 361 AACTTCTGTTATCCAAAATGTAATTAACTATGTTAGTATGTACCATATGCATGTGATGAA 420
ATI 21 334 AACTTCTGTTATCCAAAATGTAATTAACTATGTTAGTATGTACCATATGCATGTGATGAA 393
                                  STOP

ATI 18 421 ACAAAGTCCCTTAGGCTTTGTTTTTTGTGTATGGAAATGAATAAATAATCGATGGAATAA 480
ATI 21 394 ACAAAGTCCCTTAGGCTTTGTTTTTTGTGTATGGAAATGAATAAATAATCGATGGAATAA 453

ATI 18 481 AAATTACTGCTTGGCCATGGAGTTTGTTTTTCACAAACTATATGGTCAAAGTGTTTGTTT 540
ATI 21 454 AAATTACTGCTTGGCCATGGAGTTTGTTTTTCACAAACTATATGGTCAAAGTGTTTGTTT 513

ATI 18 541 T
ATI 21 514 T
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Fig. 1. The nucleotide sequences of two alfalfa trypsin inhibitor cDNAs, ATI 18 and ATI 21. Nucleotide residues which differ between the two clones are printed in small type. The putative initiation and termination codons are labelled as MET and STOP, respectively.

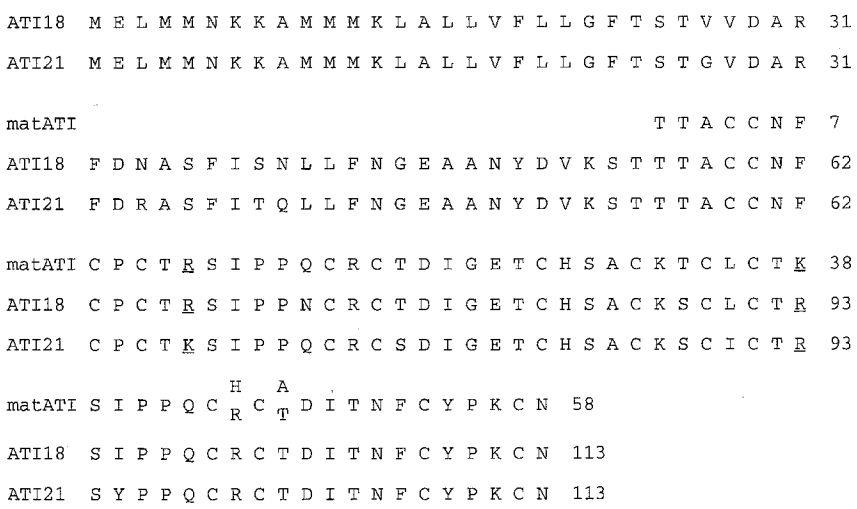

Fig. 2. Deduced amino acid sequences of the proteins encoded by the alfalfa trypsin inhibitor cDNAs, ATI 18 and ATI 21. The initiating methionine is assumed to be the first methionine in the open reading frame encoding the ATI. The sequences are aligned with that of the published, mature ATI protein (matATI) [2]. The active site residues are underlined.

in the pathway leading to the eventual deposition of the mature protein in the central vacuole of the cell, which is known to be the storage site for proteinase Inhibitors I and II in tomato [17]. An 80 amino acid Bowman-Birk inhibitor isolated from cowpea (*Vigna unguiculata*) is also processed from a preprotein with a relatively long leader sequence of 66 amino acids [18].

The ATI protein encoded by the ATI 18 cDNA differs by three amino acid residues from the published, mature ATI protein sequence (matATI, Fig. 2), including a lysine to arginine substitution at one of the two putative active sites (ATI 18 amino acid 93) [10]. This substitution would not affect the trypsin specificity of the protein product of the ATI 18 mRNA.

The other alfalfa trypsin inhibitor cDNA, designated ATI 21, is composed of 514 bp compared to the 541 bp of ATI 18, the difference being an additional 27 bp at the 5′ end of ATI 18 (Fig. 1). There are 23 bp differences between the overlapping regions of ATI 18 and ATI 21 (95.6% homology), and 9 amino acid differences between the preproteins encoded by the two cDNAs (Fig. 2). There are also six amino acid differences between the published, mature ATI protein sequence and that of the mature ATI moiety of the preprotein encoded by ATI 21 (Fig. 2), including substitutions at each of the two, putative active sites (ATI 21 amino acids 67 and 93). As in the case of ATI 18, the substitutions would not affect the trypsin specificity of the active sites of the ATI 21 mRNA protein product.

The original ATI protein isolated from alfalfa leaves [8, 9] and the ATIs encoded by cDNAs 18 and 21 appear, therefore, to be highly homologous members of an ATI family. During the original purification of the published ATI, several protein fractions were recognized by anti-ATI antibodies and were assumed to be ATI isoforms [8]. Southern blot analysis of alfalfa genomic DNA suggested that the ATI gene family contains between three and ten members (data not shown).

ATI mRNA expression

Northern blot analysis of the expression pattern of ATI mRNA in both unwounded and wounded alfalfa plants revealed that ATI mRNA is expressed throughout the flowers and in the roots of unwounded plants, but not in the stems or leaves (Fig. 3). ATI mRNA is systemically induced in the stems and leaves in response to wounding of the leaves. The level of ATI mRNA expression is

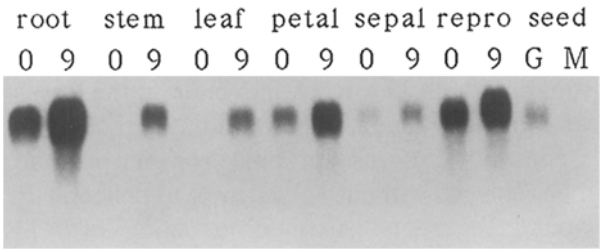

Fig. 3. Spatial distribution of ATI mRNA before and after wounding. About 30% of the leaves on an 8-week old flowering alfalfa plant were wounded (Materials and methods). Total RNA was isolated before wounding ($t = 0$) and 9 h after wounding ($t = 9$) from the following tissues: root; stem; leaf; sepal; petal; stamen + pistil (repro.). Total RNA was also extracted from green, developing seed (G) and mature seed (M). The RNA extracted from leaves was taken from the unwounded, systemically induced leaves. RNA extraction and blotting was as described in Materials and methods. The blots were probed with an ATI coding-region fragment as described in Materials and methods.

also increased in the flowers and roots in response to leaf wounding (Fig. 3). In addition, ATI mRNA is detected in immature seeds but not in the mature seeds (Fig. 3). It is important to note that the cDNA fragment used to probe the northern blot hybridized to both ATI 18 and ATI 21, and that no attempt was made to determine which members of the ATI gene family are expressed developmentally, and which are expressed in response to wounding.

The observed expression of ATI mRNA in immature seeds is consistent with the fact that Bowman-Birk inhibitors were originally identified as seed storage proteins which are synthesized during seed development [10]; their role as inducible proteins has been characterized relatively recently [9–11]. The constitutive expression of ATI mRNA throughout the flowers is consistent with the previously reported observations that proteinase inhibitor II mRNA is expressed in the flowers of potato, tomato and tobacco plants [3, 4].

An unexpected feature of ATI mRNA expression was the high level found in the roots (Fig. 3), since the well-studied proteinase inhibitors from tomato and potato are not expressed in unwounded roots [3]. To determine if microorganisms in the soil were stimulating ATI mRNA expression in the roots, we germinated and grew alfalfa plants within sterilized tissue culture boxes containing each of the following: sterile soil that was infected with a small amount of unsterilized greenhouse soil in which alfalfa plants had been growing; sterilized soil that had been infected with bacterial monocultures isolated from greenhouse soil in which alfalfa plants had been growing; sterilized soil alone. Total RNA was extracted from the roots and leaves of three week old plants and northern blots of this RNA were probed with an ATI cDNA-coding region fragment which hybridized to both ATI 18 and ATI 21.

Inoculation with unsterilized greenhouse soil clearly induced ATI mRNA expression above the basal level of expression found in the roots of alfalfa plants grown in sterile soil (Fig. 4). Seven of the fifteen monocultures also induced ATI mRNA expression in the roots. The induction of ATI mRNA by one of the most potent microbial inducers is shown in Fig. 4. This microorganism was identified as *Pseudomonas putida*. These results suggest that microorganisms in the soil are responsible for most of the 'constitutive' expression of ATI mRNA in alfalfa roots. This response is not found in tomato or potato plants, since their roots do not accumulate proteinase inhibitors, even when roots or leaves are wounded. No attempt was made to determine which members of the ATI gene family are expressed in response to microbial infection. It is important to note that

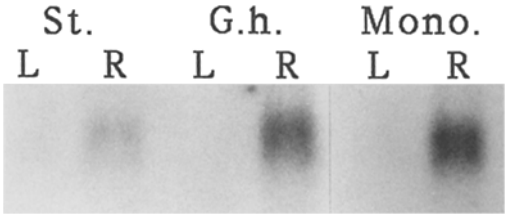

Fig. 4. Induction of ATI mRNA expression in the roots in response to microorganisms. Alfalfa plants were grown in sterilized soil within tissue culture boxes which had been inoculated with: greenhouse soil (G.h.); a monoculture of *Pseudomonas putida* (Mono.), or which had been left uninoculated (St.). At three weeks after germination the roots (R) and leaves (L) were harvested separately and total RNA was extracted, blotted and probed as described in Materials and methods.

the plants appeared to be entirely healthy, suggesting that none of the microorganisms living in the soil were pathogenic to alfalfa.

The presence of microorganisms in the soil did not account for all of the ATI gene expression in the roots, however, since there was a low, basal level of expression when the plants were grown under sterile conditions (Fig. 4). It may be that mechanical damage suffered by the roots as they grow through the soil is sufficient to induce some ATI mRNA expression, or that the plants were stressed in the enclosed environment of the tissue culture boxes.

Neither unsterilized greenhouse soil nor any of the monocultures induced ATI mRNA expression in the leaves. Microbial infection of the roots apparently did not result in the release of a mobile signal capable of inducing ATI mRNA synthesis in the leaves. To determine if damage to the roots can systemically induce ATI mRNA synthesis in the leaves, an alfalfa plant was removed from its pot, the root mass was crushed and, eight hours later, total RNA was extracted from both roots and leaves for northern blot analysis (Fig. 5).

Wounding the roots significantly increased the level of ATI mRNA in the roots and induced ATI mRNA synthesis in the unwounded leaves. To be sure that the ATI mRNA induction in the leaves was not due to inadvertent wounding of the leaves, a control plant was uprooted from its pot and then replaced without any attempt to deliberately wound the roots. A slight induction of ATI mRNA was observed in the leaves of the control plant (Fig. 5), which may have been due to some tearing of the root system. The ATI mRNA induction in the leaves of the control plant was, however, much less than in the leaves of the plant which had part of its root mass deliberately crushed.

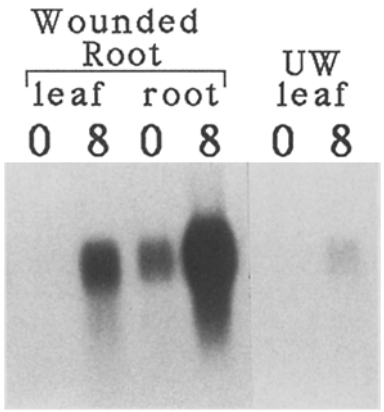

Fig. 5. Systemic induction of ATI mRNA expression in leaves in response to root wounding. An alfalfa plant was removed from its pot and the root mass was crushed (Materials and methods). RNA was extracted from roots and leaves before wounding ($t = 0$) and 8 h after wounding ($t = 8$). As a control, RNA was extracted from the leaves of a plant which had been removed from its pot then replaced, but not intentionally wounded (UW leaf). Total RNA was extracted, blotted and probed as described in Materials and methods.

This experiment demonstrated that alfalfa roots can release a mobile wound signal that induces ATI mRNA synthesis in the leaves. The results of the wounding experiment presented in Fig. 3 showed that a mobile wound signal can also travel from the leaves to the roots.

An explanation for the inability of soil-living microorganisms to systemically induce ATI mRNA synthesis in the leaves may be that the root damage caused by the microorganisms did not exceed a threshold level necessary to generate a systemic wound signal. It is also possible, however, that localized, microbe-induced, ATI mRNA synthesis in the roots proceeds by a mechanism distinct from that which regulates the systemic response to mechanical wounding.

Oligosaccharides derived from plant or microbial cell walls are known to induce a variety of localized plant defense responses [19, 20], including the local induction of proteinase inhibitor synthesis in leaves in response to wounding or pathogen infection [21]. Similarly, certain soil microorganisms may secrete cell wall-degrading enzymes which release pectic fragments that can act as local, but not systemic, elicitors of ATI synthesis in alfalfa roots. It has been hypothesized that local induction by pathogens results from release of oligogalacturonides by polygalacturonases and pectic lyases secreted by the microorganisms, and that systemic induction is the result of release of a mobile signal, systemin, from the wounded cells [22]. The results described here fit this model.

This paper reports the sequences of cDNAs encoding two members of the Bowman-Birk trypsin inhibitor family from alfalfa. In common with the well-characterized Inhibitors I and II from tomato and potato, mature ATIs are apparently derived from larger preproteins, are encoded by a small gene family and are systemically wound inducible in the leaves. Unlike Inhibitors I and II, ATIs are continuously expressed in the roots, mainly in response to soil-living microorganisms. It will be interesting to determine if the interaction between alfalfa roots and the nitrogen-fixing symbiont *Rhizobium meliloti* also results in the induction of defense-related proteins such as ATIs.

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