Abscisic acid and jasmonic acid affect proteinase inhibitor activities in barley leaves

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Summary

Proteinase inhibitor (PI) accumulation has been described as a plant defense response against insects and pathogens. The induction of PIs is known to be regulated by endogenous chemical factors including phytohormones. We studied the induction of barley chymotrypsin and trypsin inhibitory activities by aphid infestation, mechanical wounding, abscisic acid (ABA) and jasmonic acid (JA). Wounding experiments led to a minimal accumulation of PI activity (16 % over controls) compared to that found in barley seedlings infested by aphids, where chymotrypsin inhibitor activity showed a two-fold increment. No systemic induction could be detected in healthy leaves of an infested or mechanically injured plant. Exogenous ABA applied on barley leaves increased the chymotrypsin inhibitory activity, while JA only increased trypsin inhibitory activity locally and systemically when applied exogenously. Our data suggest that two different mechanisms may be regulating the induction of these two types of inhibitors.

Key words: Abscisic acid – aphids – barley – greenbug – jasmonic acid – protease inhibitors – *Schizaphis graminum* – wounding

Abbreviations: ABA = abscisic acid. – BAEE = N-benzoyl-L-arginine ethyl ester. – JA = jasmonic acid. – MEJA = JA methyl ester. – PI = proteinase inhibitor. – SA = salicylic acid. – TEE = tyrosine ethyl ester

Introduction

Proteinase inhibitors (PIs) have been recognized as an important group of proteins involved in plant defense (Koiwa et al. 1997). The accumulation of PIs has been described not only

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upon attack by herbivorous insects (Green and Ryan 1972) or by fungal infection (Rickauer et al. 1992), but also by wounding (Peña-Cortés et al. 1995). The induced expression of PIs is also systemic, which has led to the proposal of chemical elicitors and some plant hormones to participate in this process (Koiwa et al. 1997, and references therein). Abscisic acid (ABA) has been proposed to upregulate the expression

of the proteinase inhibitor II in potato and in ABA-deficient mutant tomato, where the mRNA levels are similar in ABA treated or wounded plants (Peña-Cortés et al. 1995). Jasmonic acid (JA) is also known to mediate the induction of PIs (Turner et al. 2002). JA and its volatile ester methyl jasmonate (MEJA) strongly induce the accumulation of inhibitors I and II when applied to potato and tomato leaves (Farmer et al. 1992).

Inhibitors of trypsin, chymotrypsin and microbial proteases are the most common PIs in barley and are present mostly in seeds, although they are also found in vegetative tissues (Boisen 1983). Nevertheless, there is little information on the regulation of PI induction in cereals compared to what is known for dicotyledonous plants. A Bowman-Birk trypsin inhibitor-related protein was found to accumulate by wounding in maize and translocation of the transcript has been demonstrated between organs of the maize seedling (Eckelkamp et al. 1993, Rohrmeier and Lehle 1993). Another proteinase inhibitor from maize (MPI) was shown to be induced both locally and systemically by fungal infection, mechanical wounding, ABA and MEJA (Cordero et al. 1994) and by insect feeding (Tamayo et al. 2000). Amino acid sequence of MPI reveals homology to the potato inhibitor I family, but the highest homology (60 %) is found with the barley chymotrypsin inhibitors CI-1 and CI-2. In barley, JA is known to play a role in defense responses by promoting the synthesis thionins, small polypeptides with antifungal activity involved in the defense against pathogens (Andresen et al. 1992).

We have previously described that aphid infestation promotes an increase of trypsin and chymotrypsin inhibitory activities in barley seedlings (Casaretto and Corcuera 1998). In this work, we study the effect of the phytohormones ABA and JA on the PI activity in barley leaves and compare the responses to those observed with aphid infestation or mechanical wounding. We report that chymotrypsin and trypsin inhibitory activities are increased by ABA and JA, respectively. The possibility of systemic induction of PIs is also analyzed.

Materials and Methods

Plant material

Barley (*Hordeum vulgare* L.) seedlings cv. Frontera were grown in pots with vermiculite and irrigated daily with a nutritive solution (Phostrogen®, Bayer). Plants were cultivated in a growth chamber at 22 °C (± 2 °C) and a photoperiod of 14 h of light.

Aphid infestation and mechanical wounding

Barley seedlings were infested with 40 nymphs of the aphid *Schizaphis graminum* Rondani biotype C per plant. Infestations were done in the whole primary leaf of seven-day-old plants, the lower half of the primary leaf leaving the upper half free of aphids, or in the first leaf of eleven-day-old plants leaving the second one (approximately 4 cm long) free of aphids. Mechanical wounding was performed by punch-

ing the surface of the leaves with the tip of a 100 µm glass needle (40 wounds per plant).

ABA and JA treatments

Detached leaves of seven-day-old plants were floated on water or on aqueous solution of different concentrations of (±)-abscisic acid (Sigma) or (±)-jasmonic acid (Sigma). Incubations were performed at 22 °C under constant light. For systemic induction assays, eleven-day-old seedlings were cut at the base and the first leaf was incubated with the hormones inside culture tubes placed horizontally and sealed with parafilm, leaving the second leaf outside the tube. Whole detached leaves were used including the tip, since no significant difference in PI activities were observed between the lower and upper halves.

Analysis of ABA

ABA determination was performed according to a method described previously (Kettner and Dorffling 1987). Leaves cut in 1 cm segments (5 g) were extracted overnight in 25 mL of 85 % (v/v) ethanol. The extracts were passed through a Millipore filter (0.2 µm). ABA was separated using a Lichrosphere 100 RP 18 HPLC column (Lipp 1991). The mobile phase was an isocratic mixture of acetonitrile:water (45 : 55 v/v) with a flow of 1.5 mL min^{-1}. Quantification was done at 236 nm using (±) cis, trans-ABA (Sigma) as standard. The identity of ABA isolated by HPLC, was confirmed by direct injection into a Hewlett Packard HP 5989A (Palo Alto, CA, USA) mass spectrometer. Sequential extraction of the leaves showed 90 % recovery of endogenous ABA in the first extraction, 7 % in the second and about 3 % in the third. In addition, when different amounts of standard hormone (ABA) were added to the samples, ABA was recovered quantitatively with similar 90 % efficiency, also indicating that no interfering substances were present.

Proteinase inhibitor activity

The determination of chymotrypsin and trypsin inhibitory activity was performed as described before (Casaretto and Corcuera 1998). Briefly, leaves were ground in a cold mortar and pestle with 50 mmol/L Tris-HCl buffer, pH 8.0 (3 mL buffer g^{-1} tissue) containing 10 mmol/L 2-mercaptoethanol and 5 % (w/v) PVP-40, then filtered with a cheesecloth and centrifuged at 20,000 g for 30 min. The supernatant was used as a crude extract to measure inhibitory activity. PI activity was measured spectrophotometrically by means of inhibition of sterase activity of trypsin and chymotrypsin using the method described elsewhere (Schwert and Takenaka 1955). Both enzymatic activities were measured at 25 °C. The assays were initiated with the incubation of 0.1 mL of the extract with 5 µg of trypsin (Sigma) or 20 µg of chymotrypsin (Sigma) in 1 mmol/L HCl and 50 mmol/L Tris-HCl buffer (pH 8.0 for trypsin or pH 7.0 for chymotrypsin) for 10 min at room temperature, in a final volume of 0.2 mL. For trypsin assay, this incubation mixture was added to 3 mL of 1 mmol/L N-benzoyl-L-arginine ethyl ester (BAEE, Sigma) containing 20 mmol/L CaCl$_2$ in 50 mmol/L Tris-HCl buffer, pH 8.0. Absorbance was measured at 256 nm. For chymotrypsin assay, the incubation mixture was added to 3 mL of 1 mmol/L L-tyrosine ethyl ester (TEE, Sigma) containing 20 mmol/L CaCl$_2$ in 50 mmol/L Tris-HCl buffer, pH 7.0. Absorbance was measured at 235 nm. Inhibitor activ-

ities were expressed as inhibitor units per µg of protein. An inhibitor unit (UI) was considered as the amount of inhibitor that reduces the hydrolysis of 1 µmol of substrate per minute at standard conditions. Proteases incubated with the extraction buffer were used as control reactions. Protein content of extracts was determined using the method of Bradford (1976) using bovine serum albumin as standard. Student t-test was performed with all sets of data.

Results

PI activities induced by aphids and mechanical wounding

Aphid infestation of barley plants leads to the accumulation of PIs, especially inhibitors of chymotrypsin (Casaretto and Corcuera 1998). Thus, we studied the effect of mechanical wounding on PI activities in the leaves of young barley plants.
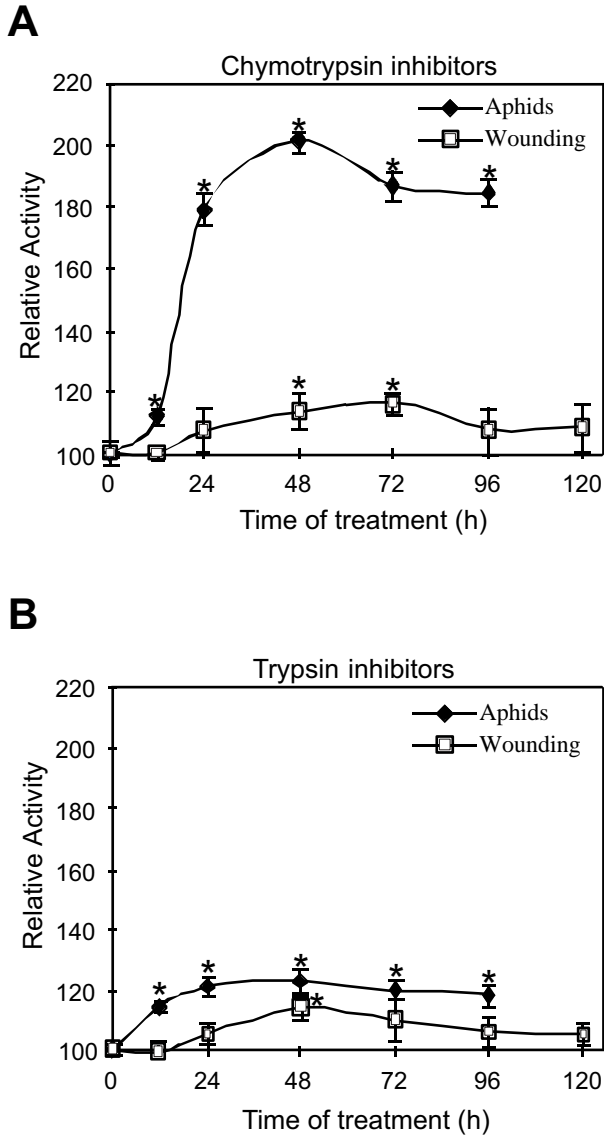

A

B

Figure 1. Accumulation of chymotrypsin **(A)** and trypsin **(B)** inhibitory activity by infestation or mechanical wounding. Seven-day-old barley plants cv. Frontera were infested for 48 h with *S. graminum* (40 aphids per plant; solid symbols), or damaged with 40 punctures per plant per day (open symbols). Results are expressed as percentage of activity compared to control plants (3 measurements ± s.e.). The activities of non-infested controls at time 0 were as follows: chymotrypsin inhibitors, 85 ± 3 UI µg^{-1} protein; trypsin, 83 ± 2 UI µg^{-1} protein. PI activity in seven to twelve-day-old control plants did not varied more than 4 % for chymotrypsin and 3 % for trypsin inhibitors (Casaretto and Corcuera 1998). Asterisks denote significant difference (p < 0.01) from control plants (time 0).
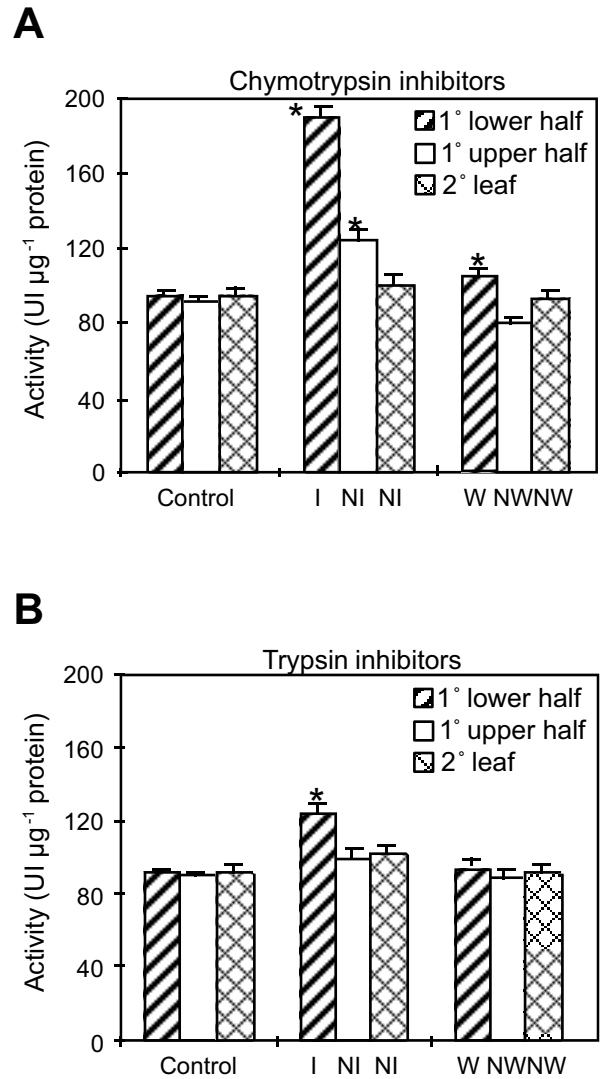

A

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Figure 2. Local and systemic induction of chymotrypsin **(A)** and trypsin **(B)** inhibitory activity in barley leaves. Barley seedlings cv. Frontera were infested for 48 h with *S. graminum* (40 aphids per plant), or damaged with 40 punctures per plant per day. PI activity was measured in lower (hatched bars) and upper (open bars) halves of the primary leaf and in the second leaf (crossed bars). I, infested lower half; NI, non-infested upper half or second leaf; W, wounded lower half; NW, non-wounded upper half or second leaf. Each bar represents the mean of 3 measurements ± s.e. Asterisks denote significant difference (p < 0.005) from control leaves.
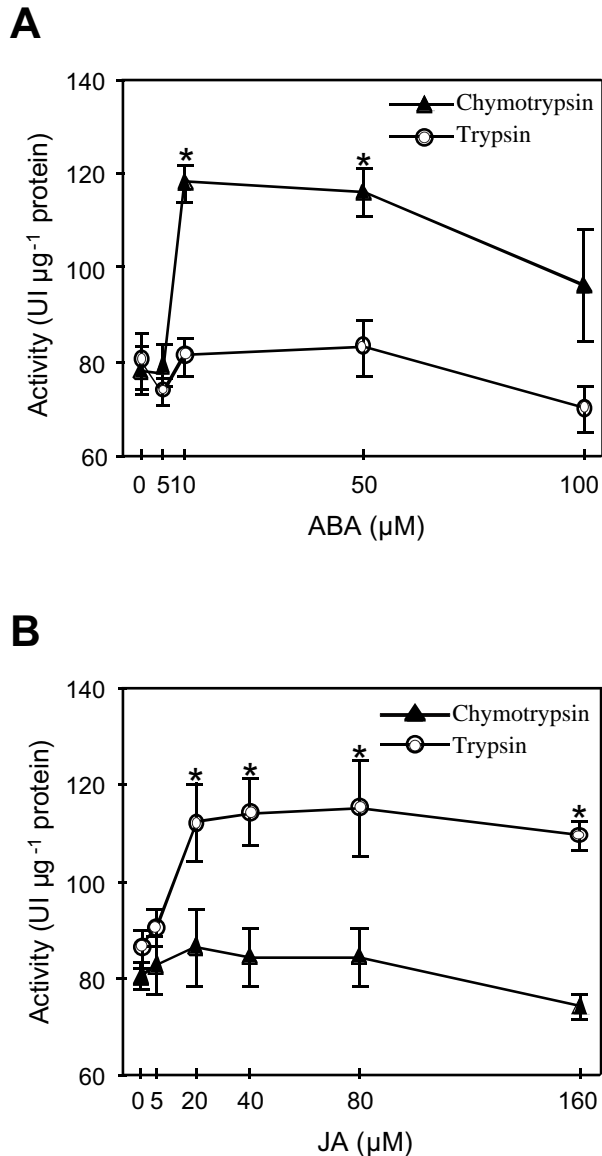
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A

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Figure 3. Effect of ABA **(A)** and JA **(B)** on proteinase inhibitor activities in barley leaves. Chymotrypsin (triangles) and trypsin (circles) inhibitor activities were measured in detached leaves after been incubated for 24 h with different concentration of the hormones or with water (0 μmol/L). Each point represents the mean of 3 measurements ± s.e. Asterisks denote significant difference (p < 0.01) from untreated leaves.

In all treatments, increments of PI activity never exceeded 16 % over the controls for chymotrypsin inhibitors or 14 % for anti-trypsin activity (Figs. 1 A and 1 B, respectively). During the time course of the experiment, PI activity due to mechanical wounding was always lower than the activity observed in infestation experiments. In addition to the puncture assays in the whole leaves, other means of mechanical wounding using clamps were tried, with no different outcome. As previously observed (Casaretto and Corcuera 1998), the peak activity (a

100 % increase) of chymotrypsin inhibitor activity occurred between 48 and 72 h of infestation with aphids (Fig. 1 A).

Increased PI activities were not detected systemically, but only in infested leaves. Chymotrypsin inhibitor activity increased not only in the infested zones, but also in upper half of leaves infested in the lower half (Fig. 2 A). Accumulation of trypsin inhibitor activity was detected only in infested zones (Fig. 2 B). Artificial wounds led to a small response of chymotrypsin inhibitors (24 % over controls) only in the wounded part of the leaf (Fig. 2 A). No systemic induction of PI activity was observed in the second untreated leaf, when the first leaf from the same plant was infested or wounded (Fig. 2).

ABA and JA enhance different PI activities in barley

The signal transduction pathway that regulates the production of PIs includes the participation of phytohormones such as ABA and JA (Koiwa et al. 1997). To test whether these hormones could play similar roles in barley, chymotrypsin and trypsin inhibitory activities were monitored in barley leaves treated with ABA or JA.

Following ABA treatment, chymotrypsin inhibitory activity increased up to 51 % over the untreated plants, while trypsin inhibitor activity remained virtually unchanged (Fig. 3 A). JA treatment, however, resulted in an increased trypsin inhibitory activity (ca. 40 % over the control plants), with no changes of anti-chymotrypsin activity (Fig. 3 B). In both cases, the maximum activity was reached after 24 h of treatment with the hormones (Fig. 4). Similar PI activity levels were also detected in non-exposed leaves of plants with only the first leaf treated with JA (Fig. 5). Chymotrypsin inhibitory activity was not induced systemically by ABA (data not shown). Comparable results were obtained either by incubating detached barley leaves or spraying whole plants with both hormones. For instance, 114 ± 10 UI μg^{-1} protein (44 % over control plants) of chymotrypsin inhibitory activity and 109 ± 8 UI μg^{-1} protein (31 % over control plants) of trypsin inhibitory activity were observed 24 h after been sprayed with 50 μmol/L ABA and 40 μmol/L JA, respectively (data not shown). These results suggest that these two classes of PIs may be differentially regulated by ABA and JA.

Aphid infestation promotes dehydration of barley seedlings (Cabrera et al. 1995). To test whether ABA accumulation due to infestation or any dehydration condition can trigger increase of PI activity, ABA levels and chymotrypsin inhibitory activity in barley seedlings under different conditions were measured. Infested plants with high ABA levels (567 ± 32 pmole g^{-1} f.wt., a five-fold increment over controls) presented over two-fold increase of chymotrypsin inhibitory activity (Table 1). ABA also increased in the non-infested part (upper half) of an infested leaf (52 % over controls) that presented a moderate increment (50 %) of chymotrypsin inhibitory activity. However, water stressed plants, also with high ABA concentration (668 pmole g^{-1} f.wt.), showed no induced
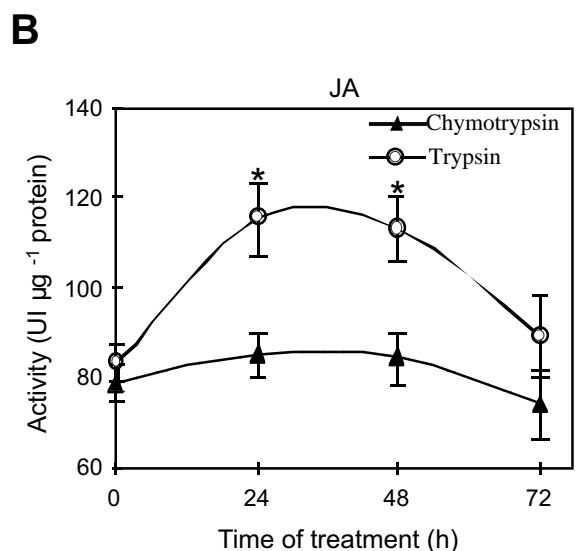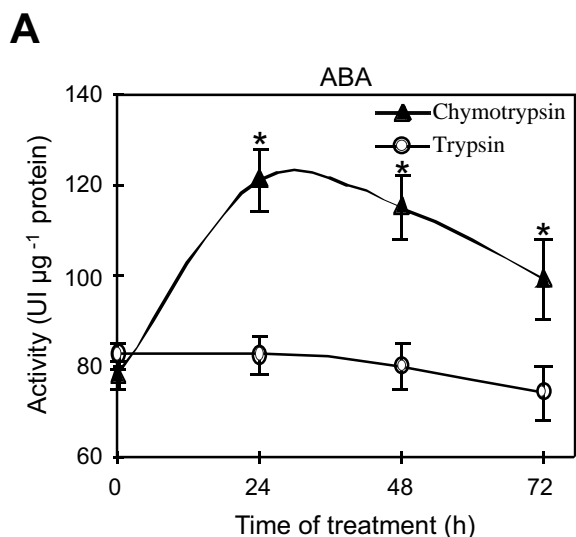
A

Figure 4. Time course of ABA- **(A)** and JA- **(B)** induced chymotrypsin (triangles) and trypsin (circles) inhibitory activities. Seven-day-old barley leaves were incubated with 20 µmol/L ABA or 40 µmol/L JA for 24, 48 or 72 h. Both inhibitor activities in control plants were never more than 83 UI µg^{-1} protein and did not varied more than 8 % of the activities observed at time 0. Each point represents the mean of 3 measurements ± s.e. Asterisks denote significant difference (p<0.01) from untreated leaves.

PI activity. In addition, treatment of barley leaves with exogenous ABA led to similar endogenous ABA concentrations to that found in infested plants, but although anti-chymotrypsin activities increased significantly, they were always lower than that detected in infested leaves. These data demonstrate that the aphid-induced PI activity is independent of dehydration and suggest that, in addition to ABA, aphid-induced damage may promote a signal event that is required for a higher activity of chymotrypsin inhibitors.
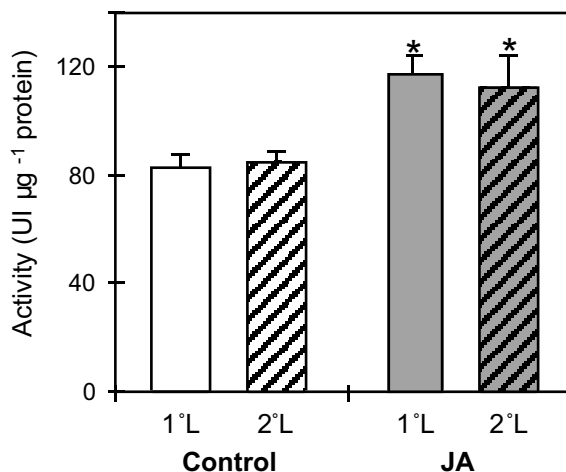
Figure 5. Systemic JA-induced trypsin inhibitory activity in barley seedlings. Primary leaves of eleven-day-old barley plants were incubated with (JA) or without (control) 40 µmol/L JA and trypsin inhibitory activity was measured in the first (1L) and second (2L) leaves. Each point represents the mean of 3 measurements ± s.e. Asterisks denote significant difference (p<0.01) from untreated leaves.

Table 1. Effect of ABA on chymotrypsin inhibitor activity in barley leaves. Barley plants were infested as indicated in Figure 2. Water stress assays consisted in suppressing the daily watering of seven day-old barley seedlings for the next 4 days. Determination of ABA and PI activity was done from cut primary leaves (whole leaf, upper or lower halves). Exogenous ABA was applied by incubating leaves in ABA solutions for 24 h. Chymotrypsin inhibitory activity of whole control leaf was 80 ± 2 UI µg^{-1} protein. Values represent the mean of 3 measurements ± s. e.

Treatment	ABA in leaf[1] (pmol g^{-1} fr. wt)	Chymotrypsin inhibitory activity[2] (relative activity)
Whole control leaf	90±3[a]	100±2[a]
Control lower half	68±1[b]	100±2[a]
Control upper half	93±3[a]	99±2[a]
Lower half infested	567±32[c]	220±6[b]
Upper half non-infested	141±4[d]	150±5[c]
Exogenous ABA 1 µmol/L	140±4[d]	97±3[a]
Exogenous ABA 10 µmol/L	252±5[f]	151±4[c]
Exogenous ABA 50 µmol/L	376±10[g]	149±3[c]
Exogenous ABA 100 µmol/L	592±4[c]	125±2[d]
Water stress	668±17[e]	99±2[a]

[1] Different letter represents significant different values (p<0.03).
[2] Different letter represents significant different values (p<0.005).

Discussion

Proteinase inhibitor activities increase in response to aphid feeding on barley leaves (Casaretto and Corcuera 1998). In

this work we describe a differential regulation of chymotrypsin and trypsin inhibitors in barley. Chymotrypsin inhibitory activity increased upon ABA treatment, while trypsin inhibitory activity was induced by exogenous JA. Furthermore, only a small increment of PI activities was found in response to mechanical stress.

Even though most hormone-induced genes in barley are not induced by wounding as it occurs in many dicot species (Lee et al. 1996), few cases of wound response in barley have been reported. Expression of the *MLO* defense regulator barley gene increases in response to wounding and fungal infection (Piffanelli et al. 2002). In addition, transcription of the lipoxygenase1 (*LoxA*) gene has been shown to be MEJA- and wound-inducible in barley leaves (Rouster et al. 1997). In the same way as many JA-induced barley genes do not respond to wounding, PIs are probably barely affected by such stress. This response may also be less evident throughout different developmental stages of the plants. Whether part of the response to aphid infestation is due to insect regurgitants rather than physical damage is unknown and much difficult to determine. Besides disrupting leaf tissues with their stylets, aphids release several potential elicitors in their saliva (Miles 1999). Damage from phloem-feeding aphids is perceived by activating not only the JA-dependent, wounding related signaling pathway, but also the SA-dependent pathway (Walling 2000, Moran and Thompson 2001, Kessler and Baldwin 2002). Although SA accumulation has been reported in aphid-infested plants, including wheat (Mohase and van der Westhuizen 2002) and barley (Chaman et al. 2003), the possibility that SA-dependent responses are correlated with resistance to aphids is still debatable (Moran and Thompson 2001, Mohase and van der Westhuizen 2002, Chaman et al. 2003).

ABA and JA elevated PI activities in barley (Fig. 3). ABA treatment increased only anti-chymotrypsin activity, the same activity that augmented the most during infestation, though ABA-treated leaves with high endogenous ABA did not presented high PI activity as infested plants did. It is likely that ABA accumulation in infested barley plants may be partly responsible for the increment of chymotrypsin inhibitory activity. A simulation of the high levels of endogenous ABA by means of water stress did not result in increased PI activities (Table 1), which indicates that chymotrypsin inhibitory activity induced by aphid infestation is independent of dehydration. It has been shown that the level of endogenous ABA increases upon dehydration and with increasing aphid density (Cabrera et al. 1995). With 20 aphids per plant, for example, endogenous ABA starts to elevate 24 h after infestation and peaks at 72 h, and the water potential is comparable to that of water stressed plants (Cabrera et al. 1995). With 40 aphids per plant, ABA levels could peak sooner, which would correlate with the peak of chymotrypsin inhibitory activity (Fig. 1 A). It has been suggested that ABA may act directly over osmotic stress responses, and simultaneously, it may be involved in defense genes transcription along with other factors such as JA (Hildmann et al. 1992). Evidence on the participation of

ABA in the induction of PIs derives from molecular and genetic analyses (Xu et al. 1993, Peña-Cortés et al. 1995, De Leo et al. 2001). Birkenmeier and Ryan (1998) have shown, however, that ABA does not induce systemic wound-response genes in tomato. These authors suggested that ABA is involved in maintaining the physiological conditions of plants so that they can respond to wounding.

On the other hand, JA treatment could only affect the anti-trypsin activity even at higher levels than those observed in infestation experiments (Figs. 1 and 3). Moreover, exogenous JA also increased trypsin inhibitory activity in untreated leaves (Fig. 5), what was not detected in infestation assays. This may be explained by a rise in SA levels in infested plants that counteracts a JA induction of PIs, since pathogen-induced SA is known to inhibit wound-induced JA production and JA-elicited gene expression (Kessler and Baldwin 2002, and references therein).

Our data suggest that different regulatory mechanisms may participate in the induction of these two classes of inhibitors in barley. Different signaling pathways mediated by ABA and JA has been described for barley (Wasternack et al. 1995, Lee et al. 1996, Ortel et al. 1999). The present work proposes PI regulation as another example of ABA- or JA-dependent signaling. The existence of similar independent pathways regulating PIs has also been suggested for other species. In tomato the negative regulator abi1 can block the ABA induction, but not the JA induction of the *pin2* gene (Carrera and Prat 1998). A soybean Cys PI (Botella et al. 1996) and a rice Bowman-Birk PI (Rakwal et al. 2001) have been shown to be induced by wounding and JA, but not by ABA.

The lack of systemic induction of PI activity upon infestation or mechanical wounding is not surprising. Although acquired resistance induced by insects or pathogens has been demonstrated in most cereals (Mohase and van der Westhuizen 2002), in barley and wheat only local acquired resistance has been reported (Kogel et al. 1994). Similarly, induced PI activity was only detected in infested leaves (Fig. 2). This observation however, does not rule out the possibility that a systemic signal may exist in barley since JA was able to induce PI activity in untreated leaves (Fig. 5). Systemic induction of PIs has also been demonstrated in rice (Xu et al. 1993) and maize (Eckelkamp et al. 1993). In addition, because very low levels of endogenous JA have been found in barley leaves (Andresen et al. 1992), airborne communication via JA is unlikely. PI activities in barley leaves were not affected by exogenous ethylene (data not shown). Unexpectedly, MEJA did not affect PI activities as JA did when sprayed over barley leaves, perhaps because MEJA was not absorbed efficiently. It would be interesting to study the endogenous levels of jasmonates when cereal plants undergo physical damage such as an insect infestation.

Different PIs have shown some toxicity towards sap-sucking pests when reared on artificial diets (Tran et al. 1997, Casaretto and Corcuera 1998, Foissac et al. 2002), and recently,

protease digestion has been detected in rice sap-sucking insects (Foissac et al. 2002), suggesting that the accumulation of PIs may play a role in protecting cereals from insects such as aphids. Further studies will help to elucidate the regulatory mechanisms of PI accumulation in several crop species including cereals.

References

Andresen I, Becker W, Schluter K, Burges J, Parthier B, Apel K (1992) The identification of leaf thionin as one of the main jasmonate-induced proteins of barley (*Hordeum vulgare*). Plant Mol Biol 19: 193–204

Birkenmeier GF, Ryan CA (1998) Wound signaling in tomato plants. Evidence that ABA is not a primary signal for defense gene activation. Plant Physiol 117: 687–693

Boisen S (1983) Protease inhibitors in cereals. Occurrence, properties, and physiological role, and nutritional influence. Acta Agric Scand 33: 369–381

Botella MA, Xu Y, Prabha TN, Zhao Y, Narasimhan ML, Wilson KA, Nielsen SS, Bressan RA, Hasegawa PM (1996) Differential expression of soybean cysteine proteinase inhibitor genes during development and in response to wounding and methyl jasmonate. Plant Physiol 112: 1201–1210

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of dye binding. Anal Biochem 72: 248–254

Cabrera HM, Argandoña VH, Zúñiga GE, Corcuera LJ (1995) Effect of infestation by aphids on the water status of barley and insect development. Phytochemistry 40: 1083–1088

Carrera E, Prat S (1998) Expression of the *Arabidopsis abi1-1* mutant allele inhibits proteinase inhibitor wound-induction in tomato. Plant J 15: 765–771

Casaretto JA, Corcuera LJ (1998) Proteinase inhibitor accumulation in aphid-infested barley leaves. Phytochemistry 49: 2279–2286

Chaman ME, Copaja SV, Argandoña VH (2003) Relationships between salicylic acid content, phenylalanine ammonia lyase (PAL) activity, and resistance of barley to aphid infestation. J Agric Food Chem 51: 2227–2231

Cordero MJ, Raventos D, San Segundo B (1994) Expression of a maize proteinase inhibitor gene is induced in response to wounding and fungal infection: systemic wound-response of a monocot gene. Plant J 6: 141–150

De Leo F, Ceci LR, Jouanin L, Gallerani R (2001) Analysis of mustard trypsin inhibitor-2 gene expression in response to developmental or environmental induction. Planta 212: 710–717

Eckelkamp C, Ehmann B, Schopfer P (1993) Wound-induced systemic accumulation of a transcript coding for a Bowman-Birk trypsin inhibitor-related protein in maize (*Zea mays* L.) seedlings. FEBS Lett 323: 73–76

Farmer EE, Johnson RR, Ryan CA (1992) Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. Plant Physiol 98: 995–1002

Foissac X, Edwards MG, Du JP, Gatehouse AM, Gatehouse JA (2002) Putative protein digestion in a sap-sucking homopteran plant pest (rice brown plant hopper; *Nilaparvata lugens*: Delphacidae)-identification of trypsin-like and cathepsin B-like proteases. Insect Biochem Mol Biol 32: 967–978

Green TR, Ryan CA (1972) Wound-induced proteinase inhibitors in plant leaves. Science 175: 776–777

Hildmann T, Ebneth M, Peña-Cortés H, Sanchez-Serrano JJ, Willmitzer L, Prat S (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell 4: 1157–1170

Kessler A, Baldwin IT (2002) Plant responses to insect herbivory: the emerging molecular analysis. Annu Rev Plant Biol 53: 299–328

Kettner J, Dorffling K (1987) Abscisic acid in *Ceratocystis coerulescens*. Physiol Plant 69: 278–282

Kogel K-H, Beckhove U, Dreschers J, Münch S, Rommè Y (1994) Acquired resistance in barley. Plant Physiol 106: 1269–1277

Koiwa H, Bressan RA, Hasegawa PM (1997) Regulation of protease inhibitors and plants defense. Trends Plant Sci 2: 379–384

Lee J, Parthier B, Löbler M (1996) Jasmonate signalling can be uncoupled from abscisic acid signalling in barley: identification of jasmonate-regulated transcripts which are not induced by abscisic acid. Planta 199: 625–632

Lipp J (1991) Detection of ABA and proline in pollen. Biochem Physiol Pflanzen 187: 211–216

Miles PW (1999) The aphid saliva. Biol Rev 74: 41–85

Mohase L, van der Westhuizen A (2002) Salicylic acid is involved in resistance responses in the Russian wheat aphid-wheat interaction. J Plant Physiol 159: 585–590

Moran PJ, Thompson GA (2001) Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. Plant Physiol 125: 1074–1085

Ortel B, Atzorn R, Hause B, Feussner I, Miersch O, Wasternack C (1999) Jasmonate-induced gene expression of barley (*Hordeum vulgare*) leaves – the link between jasmonate and abscisic acid. Plant Growth Regul 29: 113–122

Peña-Cortés H, Fisahn J, Willmitzer L (1995) Signals involved in wound-induced proteinase inhibitor II gene expression in tomato and potato plants. Proc Natl Acad Sci USA 92: 4106–4113

Piffanelli P, Zhou F, Casais C, Orme J, Jarosch B, Schaffrath U, Collins NC, Panstruga R, Schulze-Lefert P (2002) The barley MLO modulator of defense and cell death is responsive to biotic and abiotic stress stimuli. Plant Physiol 129: 1076–1086

Rakwal R, Kumar Agrawal G, Jwa NS (2001) Characterization of a rice (*Oryza sativa* L.) Bowman-Birk proteinase inhibitor: tightly light regulated induction in response to cut, jasmonic acid, ethylene and protein phosphatase 2A inhibitors. Gene 263: 189–198

Rickauer M, Bottin A, Esquerre-Tugaye M-T (1992) Regulation of proteinase inhibitor induction in tobacco cells by fungal elicitors, hormonal factors, and methyl jasmonate. Plant Physiol Biochem 30: 570–584

Rohrmeier T, Lehle L (1993) WIP1, a wound-inducible gene from maize with homology to Bowman-Birk proteinase inhibitors. Plant Mol Biol 22: 783–792

Rouster J, Leah R, Mundy J, Cameron-Mills V (1997) Identification of a methyl jasmonate-responsive region in the promoter of lipoxygenase 1 gene expressed in barley grain. Plant J 11: 513–523

Schwert GW, Takenaka Y (1955) A spectrophotometric determination of trypsin and chymotrypsin. Biochim Biophys Acta 16: 570–575

José A. Casaretto, Gustavo E. Zúñiga, Luis J. Corcuera

Tamayo MC, Rufat M, Bravo JM, San Segundo B (2000) Accumulation of a maize proteinase inhibitor in response to wounding and insect feeding, and characterization of its activity toward digestive proteinases of *Spodoptera littoralis* larvae. Planta 211: 62–71

Tran P, Cheesbrough TM, Keickhefer RW (1997) Plant proteinase inhibitors are potential anticereal aphid compounds. J Econ Entomol 90: 1672–1677

Turner JG, Ellis C, Devoto A (2002) The jasmonate signal pathway. Plant Cell 14: S153–S164

Walling LL (2000) The myriad plant responses to herbivores. J Plant Growth Regul 19: 195–216

Wasternack C, Atzorn R, Leopold J, Feussner I, Rademacher W, Parthier B (1995) Synthesis of jasmonate-induced proteins in barley (*Hordeum vulgare*) is inhibited by the growth retardant tetcyclacis. Physiol Plant 94: 335–341

Xu D, McElroy D, Thornburg RW, Wu R (1993) Systemic induction of a potato pin2 promoter by wounding, methyl jasmonate, and abscisic acid in transgenic rice plants. Plant Mol Biol 22: 573–588