Induction of Wound Response Genes in Tomato Leaves by Bestatin, an Inhibitor of Aminopeptidases

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Bestatin, an inhibitor of some aminopeptidases in plants and animals, is a powerful inducer of defense genes in tomato leaves; these genes are also induced by herbivore attacks, mechanical wounding, systemin, and methyl jasmonate. Unlike wounding and systemin, bestatin does not cause an increase in intracellular jasmonic acid concentrations, and inhibitors of the octadecanoid pathway do not inhibit induction by bestatin. Furthermore, defense genes were induced by bestatin in a mutant tomato line (JL-5) with a defect in the octadecanoid pathway. Bestatin therefore appears to be exerting its effects close to the level of transcriptional control of these genes, where it may be inhibiting a regulatory protease.

INTRODUCTION

Defense genes are induced in numerous plant species both locally and distally by signals generated in response to herbivore and pathogen attacks (Bowles, 1990; Ryan, 1992; Schaller and Ryan, 1995). Among these genes are those encoding serine proteinase inhibitors (inhibitors I and II; Graham et al., 1986), prosystemin (proSYS; McGurl et al., 1992), leucine aminopeptidase (LAP; Hildmann et al., 1992; Pautot et al., 1993), polyphenol oxidase (PPO; Constabel et al., 1995), an aspartic proteinase inhibitor (CDI; Hildmann et al., 1992), a cysteine proteinase inhibitor (CYS; Hildmann et al., 1992), and threonine deaminase (TD; Hildmann et al., 1992). Localized signals include oligosaccharides released from the cell walls of both plants and pathogens (Bishop et al., 1984; Darvill and Albersheim, 1984; Walker-Simmons and Ryan, 1984), whereas a systemically mobile signal has been identified as the 18-amino acid peptide systemin, which is released upon wounding by herbivore attacks (Pearce et al., 1991; Narváez-Vásquez et al., 1995). Both classes of signaling molecules have been proposed (Farmer and Ryan, 1992; Doares et al., 1995) to mediate gene induction via a lipid-based signaling pathway called the octadecanoid pathway (Vick and Zimmermann, 1984). In this pathway, linolenic acid, released from membranes in response to the wound signals, is converted to jasmonic acid (JA); this leads to the transcriptional activation of defensive genes.

We report here that bestatin, when supplied to tomato plants through their cut stems, is a powerful inducer of the same group of defense genes that are activated in response to wounding and systemin, and may be exerting its effects at or near the level of transcription of these genes.

RESULTS

The aminopeptidase inhibitor bestatin ([2S, 3R]-3-amino-2-hydroxy-4-phenylbutanoyl-L-leu), when supplied to excised, young tomato plants through their stems, induced the accumulation of proteinase inhibitors I and II in leaves to levels equal to those induced by systemin (Pearce et al., 1991). As shown in Figure 1, bestatin levels as low as 1.5 nmol per plant were sufficient to cause half-maximal induction of both inhibitors.

ProSYS, unlike other defense genes shown, is constitutively expressed to measurable levels but is induced to higher levels by wounding, systemin, and methyl jasmonate. Whereas bestatin specifically induced the same mRNAs as those transcriptionally activated by systemin, mRNAs of pathogenesis-related (PR) proteins, which do not respond to either wounding or methyl jasmonate, were not induced (Figure 2). The induction kinetics of proteinase inhibitor I mRNA, as a representative of the group of wound-inducible RNAs, in response to bestatin and systemin
Figure 1. Induction of Proteinase Inhibitor Proteins in Leaves of Young Tomato Plants by Bestatin.

Bestatin in a phosphate-buffered solution or in buffer alone was supplied to excised tomato plants through the cut stem. PI indicates the levels of proteinase inhibitor I (squares) and inhibitor II (circles) assessed 24 hr later. The levels of PI in buffer-treated control plants were 22 ± 2 μg per mL and 18 ± 4 μg per mL for proteinase inhibitor I and inhibitor II, respectively. Each point represents the average of at least 36 plants in six independent experiments. The standard error is indicated.

are compared in Figure 3 and were found to be similar. In both cases, an initial increase in inhibitor I mRNA was observed 2 hr after supplying the plants with the inducer, and mRNA levels continued to rise thereafter, up to 24 hr (Figure 3).

The observation that the same array of defense genes that are activated by wounding and systemin are also induced by bestatin suggested that bestatin probably activates these genes by interacting with the same signal transduction pathway. Inhibitors of the octadecanoid pathway were therefore employed to identify possible sites of bestatin action. A systemin analog (Ala-17-systemin), which is totally inactive as an inducer but is a potent competitor of systemin (Pearce et al., 1993), inhibited systemin activity but had no effect on bestatin-induced proteinase inhibitor I protein accumulation, which is shown in Figure 4. Bestatin, therefore, appears to activate the signaling pathway downstream of systemin perception.

Several elicitors of the wound response in tomato leaves, including oligouronides, chitosan, and systemin, have been shown to cause transient increases in leaf JA levels that precede defense gene activation (Doares et al., 1995a). Table 1 shows that no increase in JA levels was observed in leaves of bestatin-treated tomato plants. This suggests a site of bestatin action downstream of the octadecanoid pathway. This view was supported by the use of the tomato mutant JL-5 (Lightner et al., 1993), which is blocked in the octadecanoid pathway (G. Howe and C.A. Ryan, manuscript in preparation). JL-5 does not respond to systemin, but proteinase inhibitor accumulation was induced by bestatin in this mutant line, as shown in Figure 5.

DISCUSSION

Bestatin was first isolated from culture filtrates of Streptomyces olivoreticuli and has been shown to be a competitive inhibitor of aminopeptidase B, LAP, alanine aminopeptidase, and dipeptidyl, tripeptidyl, and tetrapeptidyl aminopeptidases (Umezawa et al., 1976). This inhibitor has been shown to inhibit flower induction in Lemna paucicostata (Tanaka et al., 1993). When supplied to young tomato plants through their cut stems, bestatin specifically induced the accumulation of several mRNAs of wound response genes in leaves. These mRNAs include proteinase inhibitor I, proSYS, PPO, CDI, CYS, LAP, and TD.
Figure 3. Time Course of Proteinase Inhibitor I mRNA Induction.
RNA was isolated from tomato leaves at the times shown after treatment of the excised plants, as given in Figure 1, with either systemin (2.5 pmol per plant) or bestatin (45 nmol per plant), and then subjected to gel blot analysis. Blots were probed with a proteinase inhibitor I cDNA. Duplicate, ethidium bromide-stained gels are shown at bottom.

CDI, CYS, and TD mRNAs are known to be wound inducible in potato; whether they are wound inducible in tomato has not yet been shown. Also, whereas all of these mRNAs are wound inducible (Graham et al., 1986; Hansen and Hannapel, 1992; Hildmann et al., 1992; McGurl et al., 1992; Pautot et al., 1993; Constabel et al., 1995), only proteinase inhibitor I and PPO had been shown to be induced by systemin (Pearce et al., 1991; Constabel et al., 1995). Clearly, systemin has a broad role in wound signaling and activates a spectrum of inducible defense-related genes.

Although a role for systemin in defense signaling in response to wounding is well established, it is not clear how bestatin, an inhibitor of aminopeptidases, leads to the activation of the same set of wound-inducible genes. One possibility is that LAP, which itself is an inducible component of the wound response, is the target of bestatin inhibition. The modulation of systemin activity has in fact been suggested as a possible function for LAP (Pautot et al., 1993) as well as for a systemin-cleaving protease in plasma membranes of tomato (Schaller and Ryan, 1994). It seems conceivable, therefore, that the half-life of systemin may be increased by the inhibition of the degradative activity of LAP by bestatin. This hypothesis could not be tested directly because a sensitive quantitative assay for endogenous systemin is not available. However, if bestatin led to an increase in endogenous systemin concentrations, two scenarios would be probable: (1) competitive inhibitors of systemin and inhibitors of the octadecanoid pathway would inhibit bestatin action; and (2) bestatin would raise intracellular levels of JA. Bestatin, unlike systemin, did not cause an elevation of intracellular JA levels, and the competitive inhibitor of systemin, Ala-17-systemin, had no effect on bestatin-induced proteinase inhibitor I accumulation. Addition, the JL-5 mutant (Lightner et al., 1993) that is blocked in the octadecanoid pathway (G. Howe and C.A. Ryan, manuscript in preparation) was induced to accumulate proteinase inhibitors by bestatin but was unresponsive to systemin. Maximum levels of proteinase inhibitors induced by bestatin were slightly lower in JL-5 than in the tomato cultivar Castlemart (Figure 5), but reduced accumulation of inhibitor I compared with that of the wild type was also observed in response to methyl jasmonate and had been ascribed to a general reduction in fitness due to the genetic background of this genotype (Lightner et al., 1993).

A second possible mode of action for bestatin is that of blocking a branch pathway and thus redirecting an intermediate into the octadecanoid pathway. The plant octadecanoid pathway is generally analogous with the eicosanoid pathway in animals, which leads to the production of leukotrienes and prostaglandins from arachidonic acid. Bestatin has been shown to be an inhibitor of leukotriene A4 (LTA4) hydrolase, which in animals catalyzes the terminal, rate-limiting step in LTB4 biosynthesis (Orning et al., 1991). This inhibition has been explained by structural similarities between LTA4 hydrolase and aminopeptidases (Malfroy et al., 1989; Orning et al., 1991). The substrate of this enzyme, LTA4, is an unstable 5,6-epoxide.
which is structurally related to allene oxide, the substrate of allene oxide cyclase in the octadecanoid pathway. If a similar enzyme—an allene oxide hydrolase—exists in plants, it could compete with allene oxide cyclase for their substrate, inhibition of this enzyme by bestatin would increase the flow through the octadecanoid pathway, providing that a source of allene oxide can be found. This would then lead to an increase in intracellular levels of JA and its downstream transcriptional activators. Regulated degradation has been observed for numerous nuclear proteins (Ciechanover and Schwartz, 1994). A good example is the NF-KB family of transcription factors, which is important for stress- and pathogen-induced transcriptional activation of defense-related genes in animals (Grilli et al., 1993). Proteolysis is a necessary step in the activation of these transcription factors (Lin et al., 1995) and is also involved in their inactivation (Cressmann and Taub, 1994). Bestatin was shown to partially inhibit NF-KB degradation in liver nuclei (Cressmann and Taub, 1994). The activation of NF-κB has been shown to be inhibited by salicylic acid (SA; Kopp and Ghosh, 1994). Doares et al. (1995b) recently showed that one point of SA inhibition of defense gene induction in tomato is after JA synthesis. It is possible that SA acts at the transcriptional level to inhibit systemin-mediated induction of defensive genes. The opposite effects of bestatin and SA on defense gene expression in tomato possibly may be explained by their differential effects on the activity of a NF-κB-like transcriptional activator.

A further possibility is that bestatin N interacts with a protein of unknown function or directly with a transactivating factor. However, until such interactions are identified, the logical possible target is an aminopeptidase. The nature of the target of bestatin and its function in the signaling cascade remain to be elucidated.

### Table 1. Accumulation of JA in Response to Bestatin and Systemin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>JA (pmol/g)</th>
<th>Inhibitor I (μg/mL)</th>
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<tbody>
<tr>
<td>Buffer</td>
<td>105 ± 25</td>
<td>40</td>
</tr>
<tr>
<td>Bestatin</td>
<td>89 ± 7</td>
<td>157</td>
</tr>
<tr>
<td>Systemin</td>
<td>2066 ± 98</td>
<td>184</td>
</tr>
</tbody>
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* The levels of endogenous JA in leaves of young tomato plants supplied with systemin (2.5 pmol per plant) and bestatin (45 nmol per plant) were compared with control plants supplied with buffer alone. Three grams of leaf tissue was harvested 135 min after the beginning of the treatment and analyzed for JA content. Values given are the average of four samples from two independent experiments. The standard error is indicated. The levels of proteinase inhibitor I were analyzed in six plants from the same experiments as the control to determine the effectiveness of the inducers.

### METHODS

#### Growth of Plants and Bioassay

Tomato plants (Lycopersicon esculentum cv Castlemart) were grown under light for 17 hr at 28°C at >300 μE m⁻² sec⁻¹ and in darkness for 7 hr at 18°C. Twelve to 14 days after planting, the plants were excised and supplied with systemin or bestatin (Sigma) through their cut stems and assayed for proteinase inhibitor induction as described previously (Pearce et al., 1993). Briefly, plants were excised at the base of the stem and transferred into 10 mM potassium phosphate buffer, pH 6.5, containing the inducing compound. After 2 hr, during which 90 μL of the solution had been imbibed, plants were transferred to water. After 24 hr under continuous light, proteinase inhibitor concentrations were determined in expressed leaf juice by radial immunodiffusion assay (Ryan, 1967).

#### RNA Gel Blot Analyses

RNA was isolated from tomato tissue by a procedure based on phenol extraction of frozen tissue ground in liquid N₂. Five micrograms of total RNA was fractionated by electrophoresis in formaldehyde agarose gels and transferred to nitrocellulose membranes using standard laboratory procedures (Sambrook et al., 1989). Prehybridization was in 50% formamide, 5 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, 2 x Denhardt's solution (1 x Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), and 200 μg/mL of denatured salmon sperm DNA at 42°C for 4 hr. Hybridization was performed in the same buffer with the addition of 2 ng/mL of radiolabeled DNA probe. Blots were washed twice for 30 min in 1 x SSC, 0.5% SDS at 60°C. Membranes were then exposed to Kodak XAR films at -80°C with intensifying screen for the time periods indicated.

#### Figure 5. Induction of Proteinase Inhibitors in the Mutant JL-5

The levels of proteinase inhibitors I (PI-I) and II (PI-II) were analyzed in control plants (cultivar Castlemart) supplied with systemin (2.5 pmol per plant), bestatin (45 nmol per plant), or buffer alone (control). These inhibitor levels are compared with levels obtained by the same inducers in JL-5, a tomato mutant blocked in the octadecanoid signaling pathway. Twenty-four plants were analyzed in four independent experiments for each data point. Error bars indicate the standard error.
Blots were probed with the following cDNAs: proteinase inhibitor I (Graham et al., 1985), prosystemin (proSYS; McGurl et al., 1992); leucine aminopeptidase (LAP; a gift from J. Boll, University of California, Riverside, CA; Pautot et al., 1993), polyphenol oxidase (Constabel et al., 1995), tobacco pathogenesis-related proteins (PR-3 and PR-3-a; a gift from J. BOI, University of Leiden, The Netherlands), and ubiquitin (a gift from A. Conconi, Washington State University, Pullman, WA). A cathespind D inhibitor (CDI) probe was obtained from a wound-induced tomato leaf cDNA library in λZAP (Stratagen). The sequences of oligonucleotide primers 5'-CGGAATTCGAYACNAAYG-GNAANGA-3' and 5'-CGGAATTCACNGTNGGDATRTTRAA-3' were derived from the published potato CDI sequence (Hildmann et al., 1992) and included EcoRI restriction sites to facilitate subsequent cloning of the amplified product into pBluescript SK- (Stratagen).

The threonine deaminase (TD) probe was amplified by polymerase chain reaction (PCR) as given above with primers 5'-CGGAATTCATCATCGAATGG-TGG-3' derived from the published potato sequence (Samach et al., 1991). A cysteine proteinase inhibitor (CYS) probe was amplified by PCR as given above using oligo(dT) as the 3' primer. The sequence of the 5' primer, 5'-CGGAATTCATCATCGAATGG-TGG-3' derived from the published potato sequence (Samach et al., 1991; Werner et al., 1993). The CYS cDNA was homologous with the potato CYS cDNA (Hildmann et al., 1992; Waldron et al., 1993). The CYS cDNA had not been cloned from tomato previously. The sequence of this cDNA is reported elsewhere.

Quantification of Jasmonic Acid in Tomato Leaves

A competitive enzyme-linked immunoassay based on a monoclonal antibody directed against methyl jasmonate (Albrecht et al., 1993) was employed to determine the levels of jasmonic acid (JA) (3R,7R- and 3R,7S-isomers) in extracts of tomato leaves.

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