Stimulation of Barley Plasmalemma H\textsuperscript{+}-ATPase by Phytotoxic Peptides from the Fungal Pathogen \textit{Rhynchosporium secalis}\textsuperscript{1}

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Abbreviations: NIP, necrosis-inducing peptide; PM, plasma membrane.

A small family of necrosis-inducing peptides has been identified as virulence factors of \textit{Rhynchosporium secalis}, a fungal pathogen of barley (\textit{Hordeum vulgare} L.). Two members of this family, NIP1 and NIP3, were found to stimulate the phosphohydrolyzing activity of the Mg\textsuperscript{2+}-dependent, K\textsuperscript{+}-stimulated H\textsuperscript{+}-ATPase of plasma membrane vesicles isolated from barley leaves by partitioning in an aqueous two-phase system. Stimulation of enzyme activity was saturated by 10 to 15 μM fungal protein. Another member of the peptide family, NIP2, did not affect the enzyme, indicating that it has a different mode of action.

Leaf scald of barley (\textit{Hordeum vulgare} L.) is caused by the imperfect fungus \textit{Rhynchosporium secalis} (Oudem.) J.J. Davis. The pathogen grows subcuticularly, primarily above anticlinal epidermal walls during early stages of pathogenesis. Only at late stages of development could fungal hyphae be found in the leaf mesophyll (Ayres-Offei and Clare, 1970; Lehnackers and Knogge, 1990). Initial symptoms in both susceptible and resistant barley cultivars comprise the swelling of epidermal cells and a subsequent loss of rigidity of anticlinal epidermal walls. This is often accompanied by a separation of the plasmalemma from the cell wall (Ayres-Offei and Clare, 1970; Jones and Ayres, 1974; Hosemans and Branchard, 1985; Lehnackers and Knogge, 1990). Within the first few days postinoculation with fungal spores, an increased opening of stomata is observed (Ayres, 1972; Ayres and Jones, 1975; Branchard and Laffray, 1987; H. Lehnackers and W. Knogge, unpublished data). In addition, increases in transpiration, root-adsorbed solute accumulation, and the permeability of epidermal cells of infected susceptible barley leaves have been described (Jones and Ayres, 1972; Ayres and Jones, 1975).

During the subcuticular stage of development, the fungus does little damage to the plant cell walls. Therefore, fungal compounds affecting host cells are likely to diffuse across the plant cell walls. Recently, a small family of NIPS was identified in culture filtrates of \textit{R. secalis} as well as in infected leaves of a susceptible barley cultivar (Wevelsiep et al., 1991). Given the small sizes of these molecules (<10 kD), their movement across the cell wall should be unobstructed. The question was how do they affect the plant cells.

Increased opening of stomata, as seen in barley leaves infected with \textit{R. secalis}, is also a characteristic pathological symptom caused by the fungus \textit{Fusicoccum amygdali} Del., the causal agent of peach and almond canker (Marré, 1979). This effect has been attributed to a host-nonspecific fungal toxin, fusicoccin. Stomatal opening and impairment of water balance are the consequences of the stimulatory effect of fusicoccin on the activity of the plasmalemma-localized H\textsuperscript{+}-ATPase, not only of the host species but of a wide variety of nonhost plants as well. The present work addresses the possibility that the NIPS from \textit{R. secalis} influence the plasmalemma H\textsuperscript{+}-ATPase of barley leaves in a similar manner.

**MATERIALS AND METHODS**

**Plant Growth and Fungal Culture**

Origin and culture conditions of barley (\textit{Hordeum vulgare} L.) cv Atlas and cv Atlas 46 and of \textit{Rhynchosporium secalis}, race US238.1, were previously described (Lehnackers and Knogge, 1990). For the isolation of PM vesicles, primary leaves of 10-d-old seedlings were harvested.

**Isolation of NIPS**

The purification of NIPS from culture filtrates of \textit{R. secalis}, as well as the deglycosylation of NIP3 using N-glycosidase F, has been described elsewhere (Wevelsiep et al., 1991).

**Isolation and Characterization of Microsomes and PM Vesicles**

Isolation of microsomes by differential centrifugation and purification of PM vesicles by partitioning in an aqueous dextran-PEG two-phase system was performed as described by Kjellbohm and Larsson (1984). The composition of the phase system was 6.2% (w/w) dextran T500, 6.2% PEG 3350, 0.33 M sucrose, 3 mM KCl, 5 mM potassium phosphate buffer, pH 7.8. Contamination by other cellular membranes was quantified by marker measurements using freshly isolated membrane preparations.

Activity of glucan synthase II was determined according to the method of Fink et al. (1987) in a total volume of 110 μL containing 40 μM Mops adjusted to pH 7.0 with Tris, 100 mM sucrose, 150 mM cellobiose, 0.04% (w/w) digitonin, 20 μM of membrane protein, and 0.2 mM UDP-[\textsuperscript{14}C]glucose (85.8 MBq/mmol). The assay for glucan synthase I was as above.
Table 1. Characterization of PM vesicles (P) isolated from two near-isogenic barley cultivars in comparison to microsomes (M) Values are the means from triplicate measurements of three or more independent membrane preparations. Specific enzyme activities are given in nmol min\(^{-1}\) mg\(^{-1}\) protein, Chl amounts in mg mg\(^{-1}\) protein, and NO\(_3\)-sensitivity in percentage of total ATPase activity.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Marker</th>
<th>Atlas</th>
<th>Atlas 46</th>
<th>-fold</th>
<th>-fold</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>P Enrichment</td>
<td>M</td>
<td>P Enrichment</td>
<td></td>
</tr>
<tr>
<td>Plasmalemma</td>
<td>H(^+)-ATPase (K(^+) increment)</td>
<td>32</td>
<td>184</td>
<td>5.8</td>
<td>27</td>
</tr>
<tr>
<td>Golgi apparatus</td>
<td>Glucan synthase II</td>
<td>2.5</td>
<td>13</td>
<td>5.2</td>
<td>2.8</td>
</tr>
<tr>
<td>IDPase</td>
<td>Glucan synthase I</td>
<td>5.1</td>
<td>2.7</td>
<td>0.5</td>
<td>4.9</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Cyt c oxidase</td>
<td>167</td>
<td>21</td>
<td>0.1</td>
<td>182</td>
</tr>
<tr>
<td>ER</td>
<td>Cyt c reductase</td>
<td>9</td>
<td>15</td>
<td>1.6</td>
<td>12</td>
</tr>
<tr>
<td>Tonoplast</td>
<td>NO(_3) sensitivity of ATPase</td>
<td>11</td>
<td>4.6</td>
<td>0.4</td>
<td>7</td>
</tr>
<tr>
<td>Chloroplasts</td>
<td>Chl</td>
<td>98.3</td>
<td>2.3</td>
<td>0.02</td>
<td>91.8</td>
</tr>
</tbody>
</table>

except that it contained only 4 \(\mu\)M UDP-[\(^{14}\)C]glucose (8.4 GBq/mmol) and also 50 mm MgCl\(_2\). Reaction products were quantified according to the method of Kaus et al. (1983). IDPase was measured essentially according to the procedure of Nagahashi and Kane (1982) in a total volume of 100 \(\mu\)L containing 50 mm Tris/HCl (pH 7.2), 5 mm MgCl\(_2\), 100 mm KCl, 1% (w/w) Triton X-100, 1 mm inosine diphosphate, and 10 \(\mu\)g of membrane protein. Released phosphate was quantified according to the method of Fiske and Subbarow (1925). Assays for Cyt c oxidase and NADPH-Cyt c reductase (antimycin A-insensitive) were as described by Hodges and Leonard (1974) using 10 \(\mu\)g of membrane protein. Chl was quantified according to the method of Bruinsma (1961).

**ATPase Assay**

For membrane characterization a photometric assay was used. The enzyme reaction was performed according to the method of Hodges and Leonard (1974) using 1 \(\mu\)g of membrane protein in a total volume of 50 \(\mu\)L containing 40 mm Mes adjusted to pH 6.5 with Tris, 0.25 mm sucrose, 50 mm KCl, and 3 mm MgSO\(_4\) unless otherwise stated, and 1 mm ATP. To monitor ATPase activity regardless of vesicle orientation, membranes were routinely permeabilized with 0.01% (w/w) Triton X-100. The proportion of right-side-out vesicles was determined by subtracting K\(^+\)-stimulated ATPase activity found in the absence of detergent (inside-out vesicles) from total K\(^+\)-stimulated ATPase activity (Larsson et al., 1984). In routine assays, the following inhibitors were included: 1 mm NH\(_4\) molybdate, 1 mm NaNO\(_3\) (Gallagher and Leonard, 1982), 100 mm NH\(_4\)NO\(_3\) (Mandala and Taiz, 1985). After 20 min, 10% (v/v) TCA was added, the protein was precipitated by centrifugation, and the supernatant was transferred to a microtiter plate. After the addition of 20 \(\mu\)L of 1-amino-2- naphthol-4-sulfonic acid (Fiske and Subbarow, 1925) and 100 \(\mu\)L of 4 n HClO\(_4\), released phosphate was quantified photometrically using an ELISA reader. In this manner, a large number of assays could be easily performed. The influence of fungal proteins on ATPase activity was analyzed using a radiometric assay. Membranes (2 \(\mu\)g of protein) were preincubated with effector peptides (5 \(\mu\)g unless otherwise specified) for 15 min at room temperature. The reaction was started by the addition of 37 kBq of \([\gamma-32P]\)ATP (61 TBq/ mmol). Released phosphate was quantified by the method of Shacter (1983).

Unless otherwise stated, the results are the means obtained from five or more independent membrane preparations and NIPs from different purifications. All measurements were performed with three replicates.

**Protein Determination**

Protein concentrations were determined according to the procedure of Bradford (1976) using BSA as a standard.

**RESULTS**

**Membrane Purification**

PM vesicles were purified by aqueous two-phase partitioning (Kjellbom and Larsson, 1984) from primary leaves of near-isogenic barley cv Atlas and cv Atlas 46. Based on the activities of two marker enzymes, Mg\(^{2+}\)-dependent, K\(^+\)-stimulated H\(^+\)-ATPase and glucan synthase II, an approximately 6-fold enrichment was achieved in the PM fraction (U, in Kjellbom and Larsson, 1984) relative to the microsomal fraction (Table I). As shown by the activity of antimycin A-insensitive NADPH-Cyt c reductase, the PM vesicles were contaminated by ER. However, the amounts of all other
cellular membranes were significantly reduced relative to microsomal membranes.

**Characterization of ATP-Hydrolyzing Activity**

ATP hydrolysis in plant membrane preparations can be due to several different enzymes. The stimulation of ATPase activity by K⁺ is characteristic of the plasmalemma-localized H⁺-ATPase. The K⁺ increment of ATPase activity made up 41.9% of total ATP hydrolysis in PM vesicle preparations and was completely inhibited by 100 μM vanadate (Fig. 1). Further studies with different inhibitors of ATP-hydrolyzing activities revealed that about 4.8% of total phosphohydrolysis was caused by the tonoplast ATPase (nitrate inhibited). Molybdate inhibited 16.2% of the activity, indicating the presence of acid phosphatases. No activity of mitochondrial ATPase (azide inhibited) was detectable. The remaining 37.1% may have been due to membrane-associated kinases or other phosphohydrolyses acting on ATP. Yields and specific activities of PM ATPase were very similar in PM vesicles isolated from the near-isogenic barley cv Atlas and cv Atlas 46 (Table I). K⁺-stimulated ATPase activities measured in the absence and presence of 0.01% (w/w) Triton X-100 were utilized to determine vesicle orientation (Larsson et al., 1984). In all experiments, 80 to 90% of the vesicles were found to be in the right-side-out orientation (Fig. 2).

**Influence of NIPs**

When PM vesicle preparations were preincubated with NIP1 or NIP3, ATPase activity was stimulated by 35 to 75% (Fig. 3). The relatively high variation was due to different PM vesicle preparations and not to a high se of the enzyme assay. When preparations showed a high stimulation of ATPase activity by NIP1, a high stimulation by NIP3 was always found as well. Vice versa, preparations with low stimulation by NIP1 also exhibited low stimulation by NIP3. Pretreatment for 15 min was necessary to obtain maximum stimulation. No significant differences were found between vesicles from the two barley cultivars. NIP3 is a glycoprotein the carbohydrate moiety of which could be enzymically removed without altering its toxicity (Wevelsiep et al., 1991). This was also found to be the case for its stimulatory effect on the ATPase (Fig. 3). Stimulation of enzyme activity was dependent on the concentrations of the NIPs and reached saturation at approximately 60% in the presence of 10 to 15 μM fungal protein (Fig. 4). No significant stimulation by NIP1 (Fig. 3) or by NIP3 was found in the absence of K⁺ or in the presence of 100 μM vanadate.

The stimulatory effect was specific, because neither BSA nor most fractions from fungal culture filtrates affected enzyme activity (data not shown). However, among the proteins eluting from a cation exchange column during purification of the NIPs, another ATPase-stimulating protein, ASP, was identified but not characterized further. This protein, which also had a relative molecular mass of <10 kD, stimulated the ATPase activity to an extent similar to that of NIP1 and NIP3 (Fig. 3). NIP2 was not found to influence ATPase activity, although it exhibited the same toxicity in bioassays as NIP3 (Wevelsiep et al., 1991).

**DISCUSSION**

During the early stages of the interaction of *R. secalis* and its host plant, barley, fungal mycelia are confined beneath
the cuticle. Neither haustoria formation, substantial degradation of plant cell walls, nor intracellular fungal growth occur during this period (Ayesu-Offei and Clare, 1970; Lehncackers and Knogge, 1990). Therefore, to release nutrients from host cells, the fungus is thought to produce and secrete toxic compounds capable of moving across cell walls. Fungal race US238.1 is avirulent on barley cv Atlas 46 carrying resistance gene Rs1 but virulent on the near-isogenic cv Atlas lacking this gene (Lehnackers and Knogge, 1990). In culture filtrates of this race, proteins of low relative molecular mass (<10 kD) were detected that caused necrosis upon injection into leaves of both barley cultivars. During pathogenesis on the susceptible cultivar, the appearance in significant concentrations of two of these, NIP1 and NIP3, correlated with the development of necrotic lesions, indicating a function in the killing of host cells (Wevelsiep et al., 1991).

Both peptides, NIP1 and NIP3, were found to stimulate the activity of the plasmalemma-localized Mg\textsuperscript{2+}-dependent, K\textsuperscript{+}-stimulated H\textsuperscript{+}-ATPase from leaves of both near-isogenic barley cultivars, indicating a mode of action similar to that of the host-nonselective toxin of F. amygdali, fusicoccin. ATPase activity was stimulated by about 60\%, which is similar in extent to the stimulation by fusicoccin of phosphohydrolytic activity and H\textsuperscript{+} pumping of the ATPase isolated from radish seedlings (Rasi-Caldogno and Pugliarello, 1985). Cell collapse is presumed to be a consequence of the impairment of a number of metabolic processes that are controlled by enzymes sensitive to changes in intracellular ion concentrations. These are controlled by the ATPase-generated electrochemical proton gradient, probably making the H\textsuperscript{+}-pumping ATPase a major consumer of ATP in plant cells.

Another similarity was observed between the NIPs from R. secalis and fusicoccin. In membrane preparations from corn roots, ATPase activity and a fusicoccin-binding protein could be separated (Stout and Cleland, 1980). Furthermore, the stimulation by fusicoccin of the ATPase from Vicia faba was lost after solubilization of the enzyme (Blum et al., 1988), indicating the presence of a fusicoccin-binding protein, which is not identical with the ATPase. This has been confirmed by photoaffinity labeling (Feyerabend and Weiler, 1989; Meyer et al., 1989). When the ATPase from barley PM vesicles was partially purified by centrifugation in a glycerol gradient (cf. Serrano, 1984), no stimulation of enzyme activity by the NIPs was detectable (data not shown). Therefore, in a manner similar to fusicoccin, the NIPs do not seem to interact directly with the ATPase. Preliminary data from photoaffinity labeling experiments and affinity chromatography using NIP3 indicate the presence of an NIP3-binding protein of about 65 kD. Functions of the fusicoccin- and the NIP3-binding proteins are unknown. However, the modulation of ATPase activity by protein kinases is currently being discussed as a regulatory mechanism (Serrano, 1989).

The induction of necrosis by the NIPs from R. secalis was not restricted to barley but also occurred in other cereal...
species. Analysis of a dicotyledonous species, bean (Phaseolus vulgaris), revealed that NIP3 was not only toxic to bean leaf tissue but also stimulated ATPase activity in PM vesicles isolated from this plant (W. Knogge, unpublished data). It is typical for host-selective toxins to affect fundamental processes that are widespread or even common to all plants. The plasmalemma H+-ATPase is such a target that is stimulated by the NIPs from R. secalis, by fuscoxacin, or by syringomycin, the toxin from a bacterial plant pathogen, Pseudomonas syringar (Bidwai et al., 1987). The opposite effect on this enzyme is exerted by a toxin from another fungal pathogen, Ceratospora beticola, which inhibits H+ pumping and hydrolytic activity in membranes isolated from several plants (Ballio, 1991).

R. secalis seems to utilize various tools to affect host cells by different means. NIP2, the third member of the small family of NIPs detected in fungal culture filtrates, was as toxic to barley leaf tissue as was NIP3 (Wevelsiep et al., 1991). Nevertheless, it had no influence on ATPase activity, suggesting a different mode of action.

The observed ATPase-stimulating activities of NIP1 and NIP3 suffice to explain the toxicity of these molecules in both near-isogenic barley cultivars. It is not yet proven, however, that these compounds are indeed involved in symptom expression during pathogenesis. In particular, during the early phase, before their appearance in infected susceptible leaves, epidermal cell collapse remains to be explained. Toxicity is only one characteristic of NIP1. In addition, it was shown to be a race-specific elicitor with activity only in barley cultivars carrying resistance gene Rps1 (M. Hahn, S. Jingling, and W. Knogge, unpublished data). The data concerning ATPase stimulation, therefore, clearly indicate that the eliciting activity of NIP1 is not due to the effect on the ATPase, because NIP3 had no elicitor activity. Future studies must ascertain the mechanism through which NIP1 activates plant defense responses.

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LITERATURE CITED


