Plant Cell Growth and Ion Flux Responses to the Streptomyctete Phytotoxin Thaxtomin A: Calcium and Hydrogen Flux Patterns Revealed by the Non-invasive MIFE Technique

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Thaxtomin A, a key phytotoxin produced by plant pathogenic Streptomyces sp., is implicit in common scab disease expression in potato. Primary targets and modes of action of thaxtomin A toxicity in plant cells are not well understood. In this work, early signalling events associated with thaxtomin A toxicity were studied using the ion-selective microelectrode ion flux estimation (MIFE) technique. Thaxtomin A-induced changes in net ion fluxes were measured across the plasma membrane (PM) of root and pollen tube tissue in Arabidopsis thaliana and tomato. Within a minute after toxin application, a rapid and short-lived Ca²⁺ influx was observed. Well ahead of the marked inhibition of root growth, a significant shift towards net H⁺ eflux across the PM occurred in all tissues. Similar to root tissues, thaxtomin A significantly modified ion flux profiles from growing pollen tubes. Thaxtomin A was more effective in young, physiologically active tissues (root elongation zone or pollen tube apex), suggesting a higher density of thaxtomin A-binding sites in these regions. Overall, our data provide the first evidence that thaxtomin A triggers an early signalling cascade, which may be crucial in plant–pathogen interactions. It also suggests a possible interaction between thaxtomin A and PM auxin receptors, as revealed from experiments on the auxin-sensitive ucu2-2/gi2 A. thaliana mutant.

Keywords: Calcium — Common scab disease of potato — Hydrogen — Ion flux — Pollen tube growth — Thaxtomin A.

Abbreviations: [Ca²⁺]c, cytosolic free calcium; CCCP, carbonylcyanide m-chlorophenylhydrazone; CNG, cyclic nucleotide-gated non-selective ion channel; MIFE, microelectrode ion flux estimation; NSCC, non-selective cation channel; PM, plasma membrane.

Introduction

Thaxtomin A is a dipeptide phytotoxin produced by all plant-pathogenic Streptomyces sp. responsible for common scab disease (King et al. 1989, Loria et al. 1997). Based on economic losses, common scab is ranked the fourth most important disease affecting potatoes in North America (Loria et al. 1997) and one of the three most significant in Australia (Wilson et al. 1999). Thaxtomin A has been shown to induce common scab-like disease symptoms, in the absence of the pathogen, when applied to developing tubers (Lawrence et al. 1990). Furthermore, mutation within thaxtomin A biosynthesis genes eliminating thaxtomin A production by the pathogen Streptomyces acidiscabies rendered the strain non-pathogenic (Healy et al. 2000). These data clearly indicate a central role for thaxtomin A in common scab pathogenesis.

The major cellular targets and mode of action of thaxtomin A toxicity in plant cells are not fully understood. Initial studies have suggested it ultimately targets the plant cell wall, causing inhibition of cellulose biosynthesis or deposition (Fry and Loria 2002), similar to a group of common herbicides (King et al. 2001). The ability of Arabidopsis thaliana thaxtomin A-resistant mutants (txr1) to lay down a cell wall in the presence of thaxtomin A with altered cell wall components (Scheible et al. 2003) also implicates a cell wall-mediated response.

Whilst these and others studies have contributed to a greater understanding of how thaxtomin A may operate, they all have focused on the later stages of plant responses to toxin (e.g. specific gene activation, synthesis of specific metabolites and enzymes and, ultimately, a morphological response). The events preceding these responses have not been studied, yet they are an essential component of the disease induction response. The mode of action of thaxtomin A will be more clearly elucidated with the identification of ion channels or candidate receptor sites mediating the interaction between the toxin and a host plant plasma membrane (PM).

Calcium signalling in response to plant-pathogenic extracts (including phytotoxins and elicitors) has been reported across a range of species including tobacco (Lecourieux et al. 2002, Kadota et al. 2004), soybean (Ebel et al. 1995), carrot (Bach et al. 1993) and parsley (Nürnberg et al. 1994, Jabs et al. 1997, Blume et al. 2000). With plant defence elicitors, a transient increase in the cytosolic Ca²⁺ level is crucial for the induction of the oxidative burst and thus defence responses (Clough et al. 2000). In the case of thaxtomin A, it is unknown as to whether the interaction between toxin and plant is characterized by a rapid Ca²⁺ flux change, and whether this recognition is responsible for subsequent downstream toxic effects.

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A range of techniques has been used to study Ca\(^{2+}\) signaling events in response to plant-pathogenic extracts. Whilst providing valuable knowledge, there are some deficiencies associated with each of these techniques. For example, \(^{45}\text{Ca}^{2+}\) uptake experiments (Bach et al. 1993, Nürnberger et al. 1994) are only capable of measuring cell-associated (not localized) Ca\(^{2+}\) uptake (Grant et al. 2000) with relatively low time resolution. This allows neither accurate quantification of Ca\(^{2+}\) influx into the cell nor adequate resolution of the fast kinetics associated with early signalling events. Pharmacological evidence obtained by using lanthanides (La\(^{3+}\) and Gd\(^{3+}\)) to block Ca\(^{2+}\) influx (Atkinson et al. 1990, He et al. 1993, Nürnberger et al. 1997) also cannot be interpreted unambiguously as, being used at millimolar external concentrations (Shimizu et al. 1997), these lanthanides may enter the cell thus affecting the PM Ca\(^{2+}\)-ATPase (Quiquampoix et al. 1990).

Aequorin technology (Knight et al. 1991) recently has documented rapid transient elevation and/or sustained [Ca\(^{2+}\)]\(_{cyt}\) levels from many plant species in response to a range of specific and non-specific elicitors and phytotoxins (Felix et al. 1999, Blume et al. 2000, Grant et al. 2000, Lecourieux et al. 2002). However, the fact that it cannot distinguish between transient [Ca\(^{2+}\)]\(_{cyt}\) changes occurring as a result of increased Ca\(^{2+}\) uptake from the external medium (e.g. PM mediated) and changes resulting from Ca\(^{2+}\) release from internal stores (such as the vacuole or endoplasmic reticulum) does not allow localization of the specific elicitor/phytotoxin target(s) in plant cells. As a result, some of the reported data are inconsistent. For example, a rapid increase in [Ca\(^{2+}\)]\(_{cyt}\) was monitored in aequorin-transformed tobacco cells treated with elicitors derived from yeast or Gliocladium deliquescence (Knight et al. 1991). In contrast, harpin, a protein from the pathogenic bacterium Erwina amylovora, which induces an oxidative burst and hypersensitive cell death in tobacco, did not affect [Ca\(^{2+}\)]\(_{cyt}\) in this plant (Chandra et al. 1997).

Direct electrophysiological evidence reporting elicitor-induced activation of PM Ca\(^{2+}\)-permeable channels comes from patch-clamp experiments (Gelli et al. 1997, Zimmermann et al. 1997). However, this technique cannot be used if the toxin end target is located in the plant cell wall (absent in protoplasts), such as in the case of thaxtomin A (Fry and Loria 2002). Therefore, in vivo studies of Ca\(^{2+}\) fluxes, mediating early interaction between the host and pathogen, are crucial.

Fluxes of other ions such as H\(^{+}\), K\(^{+}\) and Cl\(^{-}\) are also implicated in early pathogen recognition (Zimmermann et al. 1999, Clough et al. 2000, Lecourieux et al. 2002). For H\(^{+}\) literature reports are rather controversial, suggesting that effects may be plant or tissue specific. In most cases, elicitor-induced H\(^{+}\) influx or extracellular alkalization is reported (Atkinson et al. 1990, Nürnberger et al. 1994, Kuchitsu et al. 1997, Scheel 1998). However, Vera-Estrella et al. (1994) found a 4-fold increase in PM H\(^{+}\)-ATPase activity in elicitor-treated tomato cells. This is consistent with the acidification of the extracellular medium (increased H\(^{+}\) efflux) observed elsewhere (Blumwald et al. 1998), and suggests that the mode of action of this specific elicitor may differ from that observed with non-specific elicitors or pathogenic toxins (Vera-Estrella et al. 1994). It remains to be determined if the above specificity is also the case for thaxtomin A.

A convenient way of measuring early electrophysiological responses at the PM to pathogens or toxin action is the use of non-invasive microelectrode ion flux estimation (MIFE). This technique previously has been applied successfully to study plant adaptive responses to various abiotic stresses such as salinity (Shabala 2000, Shabala et al. 2003), cold (Shabala and Shabala 2002), osmotic (Lew 1998, Shabala et al. 2000, Shabala and Lew 2002) and acid stress (Shabala et al. 1997, Babourina et al. 2001). In this work, the MIFE technique has been applied to study early events associated with thaxtomin A perception and signalling in A. thaliana roots and tomato roots and pollen tubes. We provide the first evidence that thaxtomin A triggers an early signalling cascade, causing rapid and tissue-specific changes in net Ca\(^{2+}\) and H\(^{+}\) ion flux profiles, which are crucial in plant-pathogen interactions. We also provide the evidence for a possible interaction between thaxtomin A and PM auxin receptors, as revealed from experiments on the auxin-sensitive aux2-2/rid2 A. thaliana mutant. These data will be critical for the elucidation of possible cellular targets and toxicity mechanisms for thaxtomin A.

**Results**

**Thaxtomin A-induced effects on root growth**

Thaxtomin A reduced the total root length of A. thaliana, tomato and potato (Fig. 1) in a dose-dependent manner. Tomato and potato were less sensitive than A. thaliana, with higher doses required to inhibit root growth by 50%. At higher thaxtomin A concentrations, root growth inhibition was more pronounced in A. thaliana than in tomato and potato. These results suggest that thaxtomin A induces a dose-dependent inhibition of root growth, with A. thaliana being more sensitive than tomato and potato.
thaxtomin A concentrations, all species showed necrosis and eventual death (data not shown). In addition to reducing total root length, thaxtomin A treatment had a significant effect on other morphological root parameters (Table 1). A progressive increase in root diameter in both the elongation (data not shown) and morphological root parameters (Table 1). A progressive increase in root diameter in both the elongation (data not shown) and morphological root parameters (Table 1). A progressive increase in root diameter in both the elongation (data not shown) and morphological root parameters (Table 1).

Fig. 2: Effect of 24 h exposure to thaxtomin A on net H⁺ efflux (data not shown). In addition to reducing total root length, thaxtomin A treatment had a significant effect on other morphological root parameters (Table 1). A progressive increase in root diameter in both the elongation (data not shown) and mature zone (Table 1) was observed. Thaxtomin A treatment also increased (up to 10-fold) the root hair density in A. thaliana roots, reduced the length of the root hair-free apical zone and significantly (>50%) shortened the length of the elongation zone (Table 1). The average length of the root hair was not significantly (P > 0.05) affected. Similar effects were also observed in other plant species studied (data not shown).

Effects of long-term (24 h) thaxtomin A incubation on root H⁺ fluxes

A 24 h incubation with 0.2 µM thaxtomin A enhanced the active H⁺ export in all functional root zones (meristematic, elongation and mature) of A. thaliana [significant (P = 0.05) reduction in net H⁺ influx; Fig. 2A]. Functionally more active zones, such as elongation and meristem regions, were more sensitive to thaxtomin A (Fig. 2A). Treatment of tomato with 0.5 µM thaxtomin A also resulted in enhanced H⁺ extrusion within the elongation zone, but not in the meristem or mature zones (Fig. 2B).

Short-term transient ion flux responses to thaxtomin A

Transient H⁺ flux responses to thaxtomin A application were variable (Fig. 3A). For ~50% of plants tested, net H⁺ influx was unchanged or slightly increased (two upper traces in Fig. 3A), while other plants showed a significant (P < 0.05) shift towards net efflux after addition of thaxtomin A (final concentration = 3 µM) to the bath (two lower traces in Fig. 3A). In the latter cases, root pre-treatment with PM H⁺-ATPase inhibitors orthovanadate or carbonylcyanide m-chlorophenyl-hydrazone (CCCP) reduced (P < 0.05) the magnitude of thaxtomin A-induced shift towards net H⁺ efflux (data not shown). These results implicate H⁺ pump involvement in observed root responses to thaxtomin A. Overall, the average net H⁺ flux was barely changed after thaxtomin A application (closed circles in Fig. 3B). At the same time, there was a pronounced (P < 0.01) net Ca²⁺ efflux (Fig. 3B, open circles), most likely as a result of Ca²⁺ displacement from the cell wall (see below), the magnitude of which progressively decreased 8–10 min after the treatment.

Table 1: Effect of thaxtomin A treatment on growth and development of A. thaliana (wild-type Columbia) root hairs

<table>
<thead>
<tr>
<th>Thaxtomin A concentration (µM)</th>
<th>Root length, mm</th>
<th>Root diameter, µm</th>
<th>Length of elongation zone, µm</th>
<th>Root hair density (hairs mm⁻¹)</th>
<th>Tip zone length free of root hairs (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22.60 ± 0.44 **</td>
<td>135 ± 4</td>
<td>251 ± 8</td>
<td>16.7 ± 1.2</td>
<td>1178 ± 43</td>
</tr>
<tr>
<td>0.025</td>
<td>15.60 ± 0.28 h</td>
<td>139 ± 5</td>
<td>250 ± 10</td>
<td>19.6 ± 1.1 h</td>
<td>912 ± 28 b</td>
</tr>
<tr>
<td>0.05</td>
<td>13.50 ± 0.21 c</td>
<td>141 ± 5</td>
<td>221 ± 7</td>
<td>27.4 ± 2.0 c</td>
<td>804 ± 25 c</td>
</tr>
<tr>
<td>0.1</td>
<td>7.80 ± 0.19 d</td>
<td>150 ± 4</td>
<td>196 ± 6</td>
<td>37.7 ± 1.9 de</td>
<td>580 ± 30 d</td>
</tr>
<tr>
<td>0.15</td>
<td>4.80 ± 0.11 e</td>
<td>159 ± 5</td>
<td>177 ± 6</td>
<td>41.8 ± 2.2 d</td>
<td>542 ± 29 d</td>
</tr>
<tr>
<td>0.2</td>
<td>2.90 ± 0.11 f</td>
<td>161 ± 5</td>
<td>125 ± 8</td>
<td>67.6 ± 2.3 c</td>
<td>220 ± 12 e</td>
</tr>
<tr>
<td>0.3</td>
<td>1.80 ± 0.07 g</td>
<td>203 ± 7</td>
<td>108 ± 7</td>
<td>72.7 ± 3.4 c</td>
<td>199 ± 11 e</td>
</tr>
<tr>
<td>0.5</td>
<td>1.10 ± 0.08 h</td>
<td>203 ± 10</td>
<td>n/m</td>
<td>174.0 ± 7.1 a</td>
<td>n/m</td>
</tr>
<tr>
<td>1.0</td>
<td>0.93 ± 0.08 h</td>
<td>n/m</td>
<td>n/m</td>
<td>159.0 ± 6.3 b</td>
<td>n/m</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td>0.55</td>
<td>14.7</td>
<td>20.4</td>
<td>10.3</td>
<td>72.2</td>
</tr>
</tbody>
</table>

Data are the average ± SEM (n = 10).

*Values followed by the same letter within each column do not differ significantly (P = 0.05)
LSO, least significant difference; n/m, not measured.

Fig. 2: Effect of 24 h exposure to thaxtomin A on net H⁺ flux (inward positive) measured near the surface of Arabidopsis thaliana and tomato roots (both for meristematic, elongation and mature zones). (A) A. thaliana (0.2 µM thaxtomin A); (B) tomato (0.5 µM thaxtomin A). Control (clear), treated with thaxtomin A (shaded). Data are the average ± SEM (n = 5).
Rapid Ca\textsuperscript{2+} signalling in response to thaxtomin A

The unpredictable H\textsuperscript{+} flux transition and the contrary to expected (from the literature) Ca\textsuperscript{2+} flux kinetics necessitated the examination of Ca\textsuperscript{2+} flux transients in a pH-buffered solution. This was to prevent H\textsuperscript{+}/Ca\textsuperscript{2+} exchange in the Donnan system and separate any thaxtomin A-induced changes in Ca\textsuperscript{2+} transport across the PM from the Ca\textsuperscript{2+} fluxes originating from the cell wall (Shabala and Newman 2000). Accordingly, net Ca\textsuperscript{2+} flux responses were measured in a pH-buffered (2 mM MES + 4 mM Tris, pH 5.7) solution. Under these conditions, application of thaxtomin A (final concentration in bath equal to 3 µM) caused an immediate and rapidly diminishing spike of Ca\textsuperscript{2+} influx (Fig. 4A). This thaxtomin A-induced Ca\textsuperscript{2+} uptake was fully suppressed by known blockers of Ca\textsuperscript{2+}-permeable channels at the PM, such as La\textsuperscript{3+} (Fig. 4B) and Gd\textsuperscript{3+} (Fig. 4C), with efficient concentrations of 100 and 30 µM, respectively. No inhibitory effect of La\textsuperscript{3+} or Gd\textsuperscript{3+} in non-buffered solution was observed (data not shown).

Effect of thaxtomin A on pollen tube growth and ion fluxes

Tomato pollen tubes are a convenient model system for studying the effects of various factors on the tip-based mechanisms of growth of plant axial organs. In this study, it was used (for the first time) to look at electrophysiological and growth effects of thaxtomin A. Pollen tube growth was reduced with the application of increasing levels of thaxtomin A (Fig 5A), with 50% reduction observed in the presence of 0.5 µM thaxtomin A. When net hydrogen fluxes were measured near the tip of the growing pollen tube, a large oscillating H\textsuperscript{+} influx was observed (Fig. 5B, from 3 to 6 min). Such ion flux oscillations were present in every growing tube and had a period within the 1–2 min range (Fig. 5B). Application of thaxtomin A caused a significant shift towards net H\textsuperscript{+} efflux (Fig. 5B) consistent with our previous data on roots (Fig. 2). Remarkably, fast oscillations in H\textsuperscript{+} transport activity were still present in growing pollen cells treated with thaxtomin A (Fig. 5C).

As the pollen tube growth is limited to the tip only (Hepler et al. 2001), studies of thaxtomin A effects on ion flux kinetics, measured from various parts along the growing pollen tube, was an efficient way of locating potential thaxtomin A

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**Fig. 3** Short-term transient ion flux responses to thaxtomin A (3 µM final concentration in the chamber). (A) Typical examples of net H\textsuperscript{+} flux transition in response to thaxtomin A application, measured from the elongation zone of *Arabidopsis thaliana* (wild-type Columbia) roots. (B) Average transient H\textsuperscript{+} and Ca\textsuperscript{2+} flux responses to thaxtomin A in non-buffered solution from the elongation zone. Data are the average ± SEM (n = 6).

**Fig. 4** Evidence for rapid Ca\textsuperscript{2+} signalling in response to thaxtomin A. (A) Average net Ca\textsuperscript{2+} flux transients in an H\textsuperscript{+}-buffered medium (MES + Tris). (B) Effect of the La\textsuperscript{3+} block (100 µM). (C) Effect of the Gd\textsuperscript{3+} block (30 µM). All measurements were taken from the surface of *Arabidopsis thaliana* (wild-type Columbia) root in the middle of the elongation zone. Data are the average ± SEM (n = 5).
targets in plant cells. Accordingly, net H\(^+\) fluxes were measured from the tip and base regions of growing and non-growing pollen tubes (Fig. 6). In controls, growing pollen tubes were characterized by polarized ion flux patterns (large net H\(^+\) influx at the tip, and large net H\(^+\) efflux at the base; both significantly different from zero at \(P < 0.01\)) (Fig. 6A). In contrast, only a small net H\(^+\) efflux was measured from non-growing pollen grains (Fig. 6A). Application of thaxtomin A had a significant, but inverse effect in both apical and basal part of the pollen cell (Fig. 6A), but only in growing cells. No effect of thaxtomin A on non-growing pollen grains was found, although non-zero fluxes were still measured from the imbibed but non-growing pollen grains (Fig. 6A). It is important to note that fast oscillations of 2–5 min were still measured from non-growing pollen grains, regardless of thaxtomin A treatment (Fig. 6B).

**Increased sensitivity of an A. thaliana auxin-sensitive mutant to thaxtomin A incubation**

The *A. thaliana* double mutant (*ucu2-2* and *gi2*) (CS3397–ABRC) is regarded as hypersensitive to auxin based on research from our laboratory (data not shown) and others (Pérez-Pérez et al. 2004). It was compared with the wild-type Columbia genotype by measuring net H\(^+\) flux responses after 24 h exposure to 0.2 \(\mu\)M thaxtomin A (Fig. 7). While both wild type and *ucu2-2/gi2* mutant species showed a significant shift towards net H\(^+\) efflux in both meristematic and elongation zones (the most sensitive zones to thaxtomin A application; Fig. 2), the magnitude of reduction was 4- to 5-fold greater (significant at \(P < 0.05\)) for *ucu2-2/gi2* plants (Fig. 7).
Discussion

This work provides the first evidence that thaxtomin A triggers an early signalling cascade, causing rapid and tissue-specific changes in net Ca\(^{2+}\) and H\(^+\) ion flux profiles, which may be crucial in plant–pathogen interactions. The use of the MIFE technique revealed the importance of the Donnan exchange and cell wall acidification as key factors determining the mode of action of thaxtomin A in plant tissues. The pollen tube and H\(^+\) ion flux patterns measured from it were an ideal model system for studying thaxtomin A action. The possible interaction between thaxtomin A and PM auxin receptors was also identified.

Thaxtomin A effects on root morphology

Growth and development of roots in *A. thaliana*, tomato and potato plants were all significantly affected by thaxtomin A treatment (Fig. 1; Table 1), as has been described previously (Leiner et al. 1996, Loria et al. 1997, Fry and Loria 2002, Scheible et al. 2003). This information was used to derive effective working concentrations of thaxtomin A (0.2 and 0.5 \(\mu\)M, respectively, for *A. thaliana* and tomato) for subsequent experiments.

Apart from its effect on general root growth, thaxtomin caused a significant and proportional increase in the root hair density (Table 1), suggesting a pivotal role for this toxin in root development. Root hair growth is tip based and is achieved by deposition of new PM and cell wall material on the expanding tip (Peterson and Farquhar 1996). Regulation of the direction in which the secretory apparatus operates appears to be linked intimately to the \([\text{Ca}^{2+}]_{\text{cyt}}\) concentrations in the apex (Gilroy and Jones 2000). This fact provided good justification for further electrophysiological experiments using the MIFE technique, in which Ca\(^{2+}\) fluxes were measured in response to thaxtomin A.

Physiologically active plant tissue has increased sensitivity to thaxtomin A

The observed enhancement of H\(^+\) extrusion after 24 h incubation in thaxtomin A (Fig. 2) suggests that root growth inhibition may be partially mediated by H\(^+\) flux patterns. The greater effect of thaxtomin A on physiologically active plant tissues, such as the elongation zone of both *A. thaliana* and tomato roots (Fig. 2) or tips of the growing pollen tubes (Fig. 6), is consistent with this idea. Our electrophysiological findings support the morphological studies of Fry and Loria (2002) who found that tobacco cell cultures going through the active expansion phase of cell cycle growth were much more susceptible to thaxtomin A-induced hypertrophy than other phases of the cell cycle. The reported findings are also supported by other electrophysiological data, suggesting that elongation and mature root zones respond differently (both qualitatively and quantitatively) to other (abiotic) stresses such as salinity (Shabala et al. 2004), reactive oxygen species (Demidchik et al. 2004) and signalling molecules (Ludidi et al. 2004), with a higher sensitivity attributed to the elongation zone. Thus, it is logical to suggest that the primary perception site for thaxtomin A action in plants may be within these physiologically active zones.

Electrophysiological aspects of thaxtomin A signalling

As mentioned in the Introduction, despite a vast bulk of literature, no direct measurements of Ca\(^{2+}\) flux through the PM in response to phytotoxins or elicitors has been reported in vivo at the single cell level. Our work was tailored to these intact plant systems as thaxtomin A is proposed to have an end target intimately to the \([\text{Ca}^{2+}]_{\text{cyt}}\) concentrations in the apex (Gilroy and Jones 2000). This fact provided good justification for further electrophysiological experiments using the MIFE technique, in which Ca\(^{2+}\) fluxes were measured in response to thaxtomin A.

In non-buffered solution, H\(^+\) flux responses to thaxtomin A were variable (Fig. 3A) while Ca\(^{2+}\) flux was always pushed to net eflux (Fig. 3B). This reflects an interaction between H\(^+\) and Ca\(^{2+}\) (Donnan effect; Richter and Dainty 1989, Shabala and Newman 2000) whereby thaxtomin A induction of the PM H\(^+\)-ATPase causes acidification of the extracellular space and a release of Ca\(^{2+}\) from the cell wall as a result of the shift in chemical equilibrium (Fig. 8). This also explains the variability of measured thaxtomin A-induced transient H\(^+\) fluxes in non-buffered solution: depending on the ‘buffering patterns’ of the cell wall, some H\(^+\) ions were able to pass through (two lower traces in Fig. 3A), or the extruded H\(^+\) was exchanged for Ca\(^{2+}\) (top two traces in Fig. 3A). This may also explain the failure to detect an acidification response in previous studies (Fry and Loria 2002). When experiments were carried out in a pH-
buffered solution, we were able to resolve the rapid thaxtomin A-induced transient Ca\textsuperscript{2+} spike (Fig. 4A) which rapidly decayed (within 1.5–2 min). Notably the thaxtomin A-induced Ca\textsuperscript{2+} influx peak occurs 1 or 2 min earlier than other reported [Ca\textsuperscript{2+}]	extsubscript{cyt} peaks for a range of plant species in response to various elicitors (Blume et al. 2000, Grant et al. 2000, Lecourieux et al. 2002, Kadota et al. 2004). That may indicate that initial and rapid Ca\textsuperscript{2+} influx across the PM may be required to trigger further Ca\textsuperscript{2+} release from some internal stores (such as the endoplasmic reticulum or vacuole; Sanders et al. 1999) via a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release mechanism (Fig. 8).

The specific nature of the Ca\textsuperscript{2+}-permeable channel mediating thaxtomin A-induced Ca\textsuperscript{2+} influx measured in our experiments (Fig. 4A) remains to be determined. A series of recent publications suggested that several Arabidopsis mutants, such as dmd (Clough et al. 2000, Jurkowski et al. 2004) or hlm (Balague et al. 2003), that fail to produce the hypersensitive response, are defective in proteins encoding cyclic nucleotide-gated non-selective ion channels (CNGs). Such CNGs are known to be permeable to all physiologically relevant cations, including Ca\textsuperscript{2+} (Verry and Sentenac 2002), and sensitive to Gd\textsuperscript{3+} and La\textsuperscript{3+}. The fact that in our experiments thaxtomin A-induced Ca\textsuperscript{2+} influx was completely inhibited by La\textsuperscript{3+} and Gd\textsuperscript{3+} (Fig 4B, C) suggests that CNGs may be likely candidates mediating thaxtomin A interaction with the cellular membrane (Fig. 8).

From our data, the observed Ca\textsuperscript{2+} influx signature to thaxtomin A application is likely to be the most rapid event, triggering the cascade of events, leading to the plant responses observed, including root growth inhibition and plant death. Specific details of this signalling remain obscure and require further experimentation. At the same time, the observed thaxtomin A-induced activation of the H\textsuperscript{+}-pump (see below) and resultant Donnan exchange between H\textsuperscript{+} and Ca\textsuperscript{2+} in the cell wall might be one of the reasons for increased plant susceptibility to the pathogen. Exchange of H\textsuperscript{+} for Ca\textsuperscript{2+} will significantly acidify the cell wall and activate a large number of wall enzymes (primarily expansins; Cosgrove 2000) involved in the cleavage of molecular links between cell wall components, weakening cell wall structure. This may provide a partial explanation for the observed cell hypertrophy phenotype (Fry and Loria 2002) and reported disruption of cellulose synthesis/ deposition (Scheible et al. 2003). It also may explain the ability of the pathogen to penetrate through plant tissue successfully, a rare attribute of bacterial pathogens (Loria et al. 2003).

Given the effect of thaxtomin A in mediating Donnan exchange in the cell wall, and the measurement of cell wall acidification, which would be critical for pathogen/toxin efficiency, it would be interesting to note whether known phyto-toxins or elicitors (whereby a Ca\textsuperscript{2+} signature has been noted) would promote similar phenomena. With little or no other work being reported under in vivo conditions, this represents another area of future work which would improve our understanding of the plant–toxin interface in physiologically intact systems.

**Thaxtomin A activates the plasma membrane proton pump in plant root epidermis**

The activation of the energy-requiring PM proton pump has been reported previously in a few cases when elicitors have been applied to plant tissues (Vera-Estrella et al. 1994). The long-term effects of root exposure to thaxtomin A, with the observed significant shift towards net H\textsuperscript{+} efflux in all the structures and plant types studied [A. thaliana roots (Fig. 2A), tomato roots (Fig. 2B) and pollen tubes (Fig. 6A)], suggest that this H\textsuperscript{+} efflux might be mediated by the H\textsuperscript{+}-ATP pump (Fig. 8). This was confirmed further in direct pharmacological experiments, when H\textsuperscript{+} pump activity was suppressed by orthovanadate and CCCP (data not shown). Thus, our study suggests that the PM H\textsuperscript{+}-ATP pump is either a primary target of thaxtomin A itself or, more probably, mediates rapid cell responses to thaxtomin A treatment via a Ca\textsuperscript{2+}-mediated pathway (Fig. 8).

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![Fig. 8](https://example.com/fig8.png)   
**Fig. 8** A possible model of thaxtomin A binding and signalling. Thaxtomin A triggers Ca\textsuperscript{2+} influx, leading to an increase in [Ca\textsuperscript{2+}]	extsubscript{cyt}. This process is then amplified by the positive feedback (Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from internal stores such as the endoplasmic reticulum or a vacuole). Elevated [Ca\textsuperscript{2+}]	extsubscript{cyt} then enhances the activity of the PM H\textsuperscript{+}-pump, causing cell wall acidification and displacement of wall-bound Ca\textsuperscript{2+} ions for H\textsuperscript{+}. The cell wall will be weakened, increasing the ability of the pathogen to penetrate through plant tissue successfully.
Tomato pollen tube: a convenient model system that improves understanding of thaxtomin A action

Because both a tip-based Ca\(^{2+}\) gradient (Pierson et al. 1994, Malhó et al. 1995) and the PM H\(^+\) pump (Briskin and Hanson 1992) are implicated in tube growth, the pollen tube represents an ideal model system for studying mechanisms of toxin action on cell growth. Surprisingly, our data appear to be the first electrophysiological report in the literature on the effects of bacterial or fungal toxins on ion fluxes from pollen tubes.

In the growing pollen tube, there is a pronounced pH gradient between apical (acidic domain) and basal (constitutive alkaline) parts of the cell (Feijó et al. 1999, Holdaway-Clarke and Hepler 2003), with extracellular H\(^+\) fluxes measured from growing pollen tubes almost perfectly matching the cytoplasmic H\(^+\) distribution (Feijó et al. 1999). Our results on tomato pollen tubes mirror these observations (Fig. 5, 6).

There appears to be a large difference in distribution of key H\(^+\) transporters between cell apex and base, with a higher density of H\(^-\)ATP pumps in the base region, and a larger number of H\(^-\)-permeable non-selective cation channels (NSCCs) in the cell apex (Holdaway-Clarke and Hepler 2003). Both inward and outward H\(^+\) fluxes were affected by thaxtomin A treatment in our experiments (Fig. 6A). However, the effect on H\(^+\) flux was much more pronounced in the pollen apex, consistent with the idea that PM H\(^-\)-ATPase is affected by thaxtomin A. This is supported further by the absence of any effect of toxin on H\(^+\) flux patterns from non-growing pollen cells (Fig. 6).

Another remarkable feature of pollen tube growth is its oscillatory pattern (Hepler et al. 2001, Holdaway-Clarke and Hepler 2003). Ultradian growth oscillations (in the range of minutes) are usually accompanied by oscillations in extracellular Ca\(^{2+}\), H\(^+\), K\(^+\) and Cl\(^-\) fluxes as well as cytosolic pH and Ca\(^{2+}\) oscillations (Holdaway-Clarke et al. 1997, Feijó et al. 1999, Hepler et al. 2001, Zonia et al. 2002, Holdaway-Clarke et al. 2003). So far, it remains to be proven which of these oscillations are primary and which are secondary (Holdaway-Clarke and Hepler 2003). The fact that H\(^+\) flux oscillations were observed from non-growing pollen tubes and were not affected by thaxtomin A (Fig. 6B) suggests that, at least in tomato, H\(^+\) flux oscillations are unlikely to have a leading role and act as a pacemaker.

Overall, our experiments suggested that pollen tubes represent a very convenient system to study mechanisms of toxic pathogenic effects on cell growth and localized thaxtomin A perception to the tube tip. The usefulness of the pollen tube for studying thaxtomin A action is not surprising. In tomato, a pollen tube growth assay in the presence of the toxins from Stemphyllum solani and Alternaria solani was used in breeding programmes to detect resistance to these pathogens (Bell et al. 2001, Melian and Balashova 1994) and select superior toxin-tolerant tomatoes.

Increased sensitivity of A. thaliana auxin-sensitive mutant to thaxtomin A

Preliminary evidence has indicated a possible interaction between thaxtomin A and auxin. In vitro studies currently under investigation in our laboratory have indicated competition between exogenously applied auxin and thaxtomin A (data not shown). H\(^+\) flux patterns in the auxin-sensitive A. thaliana mutant (ucu2-2 and gi2) were affected to a greater extent than in Columbia wild-type roots (Fig. 7). Other morphological observations on this mutant (root growth inhibition, necrosis and root hair production) also showed its increased susceptibility to thaxtomin A compared with wild-type plants (data not shown). It may be suggested that thaxtomin A, which is structurally quite similar to indole acetic acid and also has a proposed similar biosynthetic pathway (King and Lawrence 1996), may interact with an auxin-type receptor.

The increase in root hair density with thaxtomin A treatments (Table 1) and root hair production patterns in the ucu2-2/gi2 mutant may also provide evidence of a thaxtomin A–auxin interaction. It is known that some root epidermal cells, called trichoblasts, are destined to develop root hairs (Peterson and Farquhar 1996, Gilroy and Jones 2000). Recent molecular studies have suggested that there are at least two factors that might control the cell decision to become a trichoblast or not. One is the degree of vacuolation (Galway et al. 1997), and the other is cell hormonal status (specifically, ethylene and auxin balance) (Masucci and Schiefelbein 1994). The fact that the auxin-sensitive mutant ucu2-2/gi2 was more affected by thaxtomin A (Fig. 7) is supportive of the ‘hormonal’ scenario in mediating thaxtomin A effects on root hair initiation.

Conclusions

The use of a non-invasive ion flux measuring technique allowed the characterization of specific ion responses to thaxtomin A in plant tissues. Our data provide the evidence that thaxtomin A triggers an early signalling cascade, causing rapid and tissue-specific changes in net Ca\(^{2+}\) and H\(^+\) ion flux profiles, which may be crucial in plant–pathogen interactions. In a non-buffered system, the production of the Ca\(^{2+}\) flux signature and proton pump activation was masked by a Donnan exchange in the cell wall. Thaxtomin A was more effective in young, physiologically active tissues, suggesting a higher density of thaxtomin A-binding sites in these regions. There is also some evidence that thaxtomin A interacts with auxin receptors at the PM, at least in Arabidopsis cells.

Materials and Methods

Plant material, media and root growth experiments

For all root experiments, plants were grown in the growth chamber at ambient temperature 22 ± 1\(^{\circ}\)C and 16 h day length (60 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)). Arabidopsis thaliana seeds were obtained from the Arabidopsis Biological Resource Center (ABRC), Ohio State University, Columbus, OH, U.S.A. and the Nottingham Arabidopsis Stock Centre (NASC), University of Nottingham, Loughborough, Leicestershire,
U.K. Tomato seeds (*Lycopersicon esculentum* var. *esculentum* cv. ‘Chandler’s English’) were kindly supplied by E. Chandler (Chandler’s Nursery, Hobart, Tasmania). Potato plants (*Solanum tuberosum* ‘Russet Burbank’) were obtained from the Department of Primary Industry, Devonport, Tasmania.

*Arabidopsis thaliana* seeds were surface-sterilized for 15 min in bleach solution (available Cl: 1.5% m/v). Twenty seeds were plated directly in two rows into Petri dishes containing Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 8 g l⁻¹ agar and 10 g l⁻¹ sucrose. After a vernalization period of 2 d at 4°C, plates were transferred into the growth chamber and oriented in an upright position of about 85°, enabling roots to grow along the agar surface essentially without penetrating it. After 5 d, root length was measured and plants were transferred to the above medium containing thaxtomin A. After 3 d of incubation, root length was re-measured, and the effect of thaxtomin A on root growth quantified. Each treatment had two replicates with 20 seeds per plate (n = 40 in total). The effect of thaxtomin A on root hair growth and development was assessed using *A. thaliana* seeds germinated directly onto treatment media, following a 2 d vernalization period (4°C). Root characteristics were measured after 5 d growth. Five representative plants per plate were assessed over two replicates (n = 10 in total).

Tomato seeds were germinated in Petri dishes on a sterile filter paper moistened with distilled water. After 5 d, evenly germinated seedlings were selected and exposed to various concentrations of thaxtomin A. Root lengths were measured twice: immediately prior to treatment, and 24 h after thaxtomin A application. Each treatment had two replicates with 10 seeds per plate (n = 20 in total).

Potato plantlets grown in tissue culture were used. Single-node sections, containing one leaf and a lateral bud, were excised from plantlets and placed in Petri dishes, with filter paper moistened with Potato Media Solution (PMS) containing MS, 3% sucrose, 0.05% casein hydrolysate and 0.004% ascorbic acid. After 5 d, cuttings with a single root were selected and transferred to Petri dishes with PMS ameliorated with thaxtomin A. Potato root lengths were measured twice: immediately prior to treatment, and 24 h after thaxtomin A application. Each treatment had two replicates with five plants per dish (n = 10 in total).

### Pollen growth experiments

Pollen was collected from greenhouse-grown tomato plants 40–60 d after planting and dried overnight. The dried pollen was transferred to 0.2 ml of growth solution containing 15% sucrose, 0.003% H₂BO₃, 1 mM CaCl₂, 0.2 mM KCl and thaxtomin A treatments, and placed in a 96-well microtitre plate (Selby Biolab, Australia). The homogenous distribution of pollen grains was achieved by shaking the wells for several seconds using a vortex. Then the plate was inverted and pollen grains germinated at 29°C for 3 h in the dark (the hanging drops method; Melian and Balashova 1994). At the end of that period, 50 μl of 10% formalin was added to each well to stop the growth process and fix germinated pollen tubes. Germinated pollen for each treatment was pooled (eight wells per treatment) and examined microscopically (Olympus CH biological microscope, Tokyo, Japan). The length of 50–100 randomly chosen pollen tubes for each treatment was measured and averaged.

### Non-invasive ion flux measurements

Net fluxes of H⁺ and Ca²⁺ were measured non-invasively using ion-selective vibrating microelectrodes (the MIFE technique; University of Tasmania, Hobart, Australia), generally as described in our early publications (Shabala et al. 1997, Shabala et al. 2000). Briefly, electrodes with a tip diameter of about 2 μm were pulled from borosilicate glass capillaries, dried in an oven, and silanized with tributylchloro-

rosiliane (Sigma-Aldrich, Milwaukee, WI, U.S.A.). Electrodes were first back-filled with an appropriate solution, and then the electrode tips were front-filled with commercially available ion-selective cocktail (H⁺, 95297; Ca²⁺, 21048; all from Sigma-Aldrich). After conditioning, the electrodes were calibrated in a known set of pH buffers (from 5.1 to 7.5) and standard Ca²⁺ solutions (0.1–1.0 mM range). Electrodes with a response of <50 mV per decade for H⁺ and 25 mV per decade for Ca²⁺, and correlation R < 0.999, were discarded. The reference electrode was a glass capillary filled with 500 mM KCl in 2% agar.

The microelectrodes were mounted on an electrode holder (MMTF-5; Narishige, Tokyo, Japan) that allowed precise three-dimensional positioning of electrode tips. The electrodes were initially positioned 20 μm from the root surface. During measurements, electrodes were moved in a slow square-wave manner between two positions—close (20 μm) and distant (50 μm)—radially outwards from the measured surface. The duration of each half-cycle of the electrode movement was 5 s; the actual electrode movement between two positions took about 0.4 s. The first 2 s after the movement began were ignored to allow both the movement and the electrochemical settling of the electrodes (Newman 2001). The recorded voltage gradients between positions were then converted into concentration differences using the calibrated Nernst slopes of the electrodes. Net flux values (influx positive) were calculated assuming cylindrical diffusion geometry as described in Shabala et al. (2000). No mixing of the bath medium occurred due to electrode movement as indicated by zero net fluxes measured in the absence of plant tissue in the chamber (data not shown).

### Experimental protocols for ion flux measurements

Both growth conditions and media used to grow plants for ion flux experiments were as described above. All experiments with *A. thaliana* were conducted on 5-day-old plants. Short-term transition experiments were conducted on excised root segments as described by Demidchik et al. (2003). Briefly, excised roots were immobilized on liquid agar in a holding chamber placed in a bathing medium (0.2 mM KCl, 0.1 mM CaCl₂). Experiments commenced 15–30 min after immobilization, after steady-state conditions were reached. Net ion fluxes were measured in control (prior to treatment) for 5–10 min, and then thaxtomin A was added to the chamber to give a final concentration of 3 μM thaxtomin A in the chamber. When channel blockers were used, these were added into the bathing medium at the time of immobilization. Long-term experiments involved the transfer of whole plants onto MS medium ameliorated with thaxtomin A (0.2 μM for *A. thaliana*; 0.5 μM for tomato), followed by incubation and subsequent growth for 24 h. Roots were then excised and fluxes in different root zones (meristem, elongation and mature) measured for several minutes.

For ion flux measurements on pollen, a small amount of freshly collected and dried pollen was immobilized on a surface of the thin (1 mm OD) glass capillary essentially as described by Shabala et al. (2001). The capillary was then mounted in a horizontal position in the Perspex holder within the measuring chamber filled by solution, used for pollen growth experiments. Depending on the aim of the experiment, the chamber was either left in the growth cabinet (at 29°C) for 1–2 h (when fluxes were measured from already germinated tubes), or placed in the Faraday cage for measurements immediately after pollen imbibition. In both cases, the room temperature during MIFE experiments was maintained at 29 ± 1°C to provide optimal conditions for pollen growth. Ion fluxes were measured from either the tip of the germinated pollen tube or the pollen grain base, depending on experimental purposes. Due to the small size of the pollen tube, only H⁺ flux was measured. All measurements were taken from the single pollen cells,
with no other tubes present in the field of view (∼400 magnification) to ensure that ion fluxes were measured from the single tube only.

Data analysis
Data were subjected to analysis of variance using Genstat 6 (Rothamsted Experimental Station, Harpenden, Hertfordshire, U.K.). For root growth data, significance was calculated at $P = 0.05$ and least significant difference (LSD) was used for comparison of mean values. For ion flux data, the standard t-test was used for comparison of mean values.

Acknowledgments
We would like to thank Dr. Alieta Eyles, Dr. Greg Luckman and Annabel Wilson for thaxtomin A production and tissue culture procedures. This work was partially supported by grants from Horticulture S.S.). R.T. was supported by a University of Tasmania Strategic Scholarship.

References


(Received October 3, 2004; Accepted February 4, 2005)