Do strigolactones contribute to plant defence?

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SUMMARY

Strigolactones are multifunctional molecules involved in several processes outside and within the plant. As signalling molecules in the rhizosphere, they facilitate the establishment of arbuscular mycorrhizal symbiosis, but they also act as host detection cues for root parasitic plants. As phytohormones, they are involved in the regulation of plant architecture, adventitious rooting, secondary growth and reproductive development, and novel roles are emerging continuously. In the present study, the possible involvement of strigolactones in defence responses was investigated. For this purpose, the resistance/susceptibility of the strigolactone-deficient tomato mutant Slccd8 against the foliar fungal pathogens Botrytis cinerea and Alternaria alternata was assessed. Slccd8 was more susceptible to both pathogens, pointing to a new role for strigolactones in plant defence. A reduction in the content of the defence-related hormones jasmonic acid, salicylic acid and abscisic acid was detected by high-performance liquid chromatography coupled to tandem mass spectrometry in the Slccd8 mutant, suggesting that a reduction in homeostasis is altered in the mutant. Moreover, the expression level of the jasmonate-dependent gene PinII, involved in the resistance of tomato to B. cinerea, was lower than in the corresponding wild-type. We propose here that strigolactones play a role in the regulation of plant defences through their interaction with other defence-related hormones; especially with the jasmonic acid signalling pathway.

Strigolactones (SLs) are plant hormones that were initially identified as signalling molecules in the rhizosphere. They are mainly produced in the roots and have been detected in the root exudates of a wide range of monocot and dicot plant species, which suggests their importance in nature (Xie et al., 2010). In the rhizosphere, SLs act as host detection cues for symbiotic arbuscular mycorrhizal fungi and root parasitic plants of the Orobanchaceae (reviewed in López-Ráez et al., 2011). They are derived from the carotenoids through sequential oxidative cleavage by carotenoid cleavage dioxygenases (CCD7 and CCD8) (Gomez-Roldan et al., 2008; López-Ráez et al., 2008; Matusova et al., 2005; Umehara et al., 2008), thus belonging to the apocarotenoid class, which includes the plant hormone abscisic acid (ABA).

As phytohormones, it has been suggested that SLs play a pivotal role in plants as modulators of the coordinated development of roots and shoots in response to nutrient-deficient conditions (Koltai and Kapulnik, 2011). Accordingly, their biosynthesis and exudation into the rhizosphere is induced under such adverse nutritional conditions (López-Ráez et al., 2008; Yoneyama et al., 2007). They have been shown to regulate above-ground architecture by the inhibition of lateral shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008). Since then, extensive efforts have been devoted to examine the novel roles and functions of SLs in plant physiology. Thus, it has been shown that they stimulate secondary stem growth, acting as positive regulators of cambial activity (Agusti et al., 2011). They also regulate below-ground plant architecture by affecting primary root length, lateral root initiation and root hair development (Kapulnik et al., 2011a; Ruyter-Spira et al., 2011). More recently, it has been shown that they repress adventitious rooting and positively affect reproductive development (Kohlen et al., 2012; Rasmussen et al., 2012). In all of these functions, a cross-talk between SLs and other signalling pathways regulated by phytohormones, such as auxin, ethylene (ET) and ABA, seems to play a prominent role (reviewed in Kohlen et al., 2011). Novel functions for SLs are emerging at a fast pace, broadening our understanding of their relevance in plant physiology. However, no relationship with defence responses has been addressed so far.

Plants are exposed to ever-changing and often unfavourable environmental conditions, which cause both abiotic and biotic stresses. Consequently, plants have evolved sophisticated mechanisms to flexibly adapt themselves to overcome such stress conditions. Most of these mechanisms—if not all—are regulated by plant hormones (Robert-Seilaniantz et al., 2011). In recent years, significant progress has been made in the identification of the key components in plant responses to biotic stress. The phytohormones salicylic acid (SA), jasmonic acid (JA) and derivatives—known as jasmonates (JAs)—and ABA are the major players in the signalling networks regulating plant defence responses against pathogens and pests, although others, such as ET, brassinosteroids, gibberellins and auxins, are now known to contribute to modulate the response (Robert-Seilaniantz et al., 2011; Ton et al., 2009). In the present work, the potential involvement of SLs in plant defence responses has been investigated.
For this purpose, leaves of the SL-deficient tomato mutant Slccd8 L09 and the corresponding wild-type plants (cv. Craigella) were infected with the pathogenic fungi Botrytis cinerea CECT2100 (Spanish Collection of Type Cultures, Universidad de Valencia, Valencia, Spain) and Alternaria alternata (isolated from an infected tomato field) in a detached leaf assay. Slccd8 L9 is an antisense CCD8 RNA interference (RNAi) line displaying a 95% reduction in SLs (Kohlen et al., 2012). Botrytis cinerea is a necrotrophic fungus causing grey mould in the leaves and fruits of many plants, including tomato, potato, grapes and strawberry (Dean et al., 2012). This pathogen is responsible for one of the most prominent, widespread and destructive diseases in agriculture at both pre- and post-harvest stages (Dean et al., 2012). Alternaria alternata is an opportunistic pathogen on a number of host plants, such as tomato, causing a range of diseases with a high economic impact in agriculture (Thomma, 2003). For the infection assay, plants were grown as described previously (López-Ráez et al., 2010a) and watered three times a week with Hewitt nutrient solution (Hewitt, 1966) containing 25% of the standard phosphorus concentration to promote SL production. Disease bioassays with B. cinerea and A. alternata were carried out using detached leaves from 10 plants per genotype. Conidia were collected from 10–15-day-old potato dextrose agar (PDA) plates supplemented with 20% of lyophilized tomato leaves. In the case of B. cinerea, suspensions of spores were incubated for 2 h in the dark in Gamborg's B5 medium (Gamborg et al., 1968) supplemented with 10 mM sucrose and 10 mM KH2PO4 (Vicedo et al., 2006). Detached leaves from 4-week-old tomato plants were challenged by the application of 5 μL of a suspension containing 106 spores/mL. Leaves were maintained at 20 °C and 100% relative humidity. Fungal hyphae grew concentrically, resulting in visible necrosis 48 h after inoculation. A disease index from '0' (healthy leaf) to '3' (heavily infected leaf) was established for infection quantification (Fig. 1). Data were subjected to one-way analysis of variance (ANOVA) using the software SPSS Statistics v. 20 for Windows (IBM, Armonk, NY, USA). Fisher’s least-significant difference (LSD) test was applied to determine statistical significance.

Slccd8 showed more severe symptoms of B. cinerea infection than the corresponding wild-type. An increase in disease development was observed in Slccd8 leaves relative to the wild-type 5 days after inoculation (Fig. 1A). Indeed, heavily infested leaves were only observed in Slccd8 at this stage. This increased susceptibility in Slccd8 was maintained over time. After 9 days, 41% of the leaves from Slccd8 were heavily infested (disease index 3), whereas only 15% of wild-type leaves reached this stage (Fig. 1A). The same trend was observed after inoculation with A. alternata. In this case, 5 days after inoculation, 40% of Slccd8 leaves were heavily infected, but only 12% of the wild-type (Fig. 1C). As SLs stimulate hyphal branching in germinating spores of arbuscular mycorrhizal fungi (Akiyama et al., 2005) and have been proposed to affect fungal pathogen development (Dor et al., 2011), we aimed to test whether the results observed were dependent on the inoculum source. Detached leaves from Slccd8 and wild-type plants were inoculated with 5-mm-diameter PDA plugs containing actively growing B. cinerea hyphae (1 week old). As for the previous experiment, leaves from 10 plants per genotype were used. Interestingly, the same disease pattern was observed (Fig. S1, see Supporting Information). The data confirm, therefore, that the

![Fig. 1](image)
SL-deficient tomato line Slccd8 is more susceptible to fungal pathogens, regardless of the inoculum source (germinating spores or actively growing hyphae), supporting an additional role of SLs in plant defence.

As mentioned, an effect of SLs on fungal pathogen development has been proposed previously (Dor et al., 2011). In order to evaluate a possible direct effect of SLs on fungal development, an in vitro assay with GR24 and B. cinerea was performed. Five-millimetre-diameter PDA plugs containing actively growing B. cinerea hyphae were applied to the middle of a Petri dish with PDA medium. Four different concentrations of GR24 (Chiralix) were tested, 0, 10−6, 10−7 and 10−8 M, all within the physiological concentration range (Xie et al., 2010). Four sterilized 5-mm-diameter fibreglass discs were applied to each plate, and 10 μL of the different GR24 solutions were added to each disc (Fig. 2B). Plates were incubated at 25 °C and high humidity for 5 days, and 10 individual plates were used. No effect on the B. cinerea growth pattern was observed by any of the GR24 concentrations tested (Fig. 2). This observation is in agreement with a previous study in which no alterations in the growth pattern of B. cinerea after 10−6 M GR24 application were detected (Steinkellner et al., 2007).

Table 1 Levels of defence-related hormones in the strigolactone (SL)-deficient tomato mutant Slccd8 and the wild-type (WT).

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<tr>
<th>Hormone (ng/g dry weight)</th>
<th>Genotype</th>
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<tr>
<td></td>
<td>WT</td>
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<tr>
<td>OPDA</td>
<td>4495 ± 473</td>
</tr>
<tr>
<td>JA</td>
<td>157 ± 37</td>
</tr>
<tr>
<td>SA</td>
<td>2375 ± 165</td>
</tr>
<tr>
<td>ABA</td>
<td>2515 ± 79</td>
</tr>
</tbody>
</table>

Levels of oxo-phytodienoic acid (OPDA), jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) were measured by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) in leaves of the Slccd8 mutant and corresponding wild-type (WT). Data represent the means of five independent replicates ± standard error (SE). Asterisks (*) indicate statistically significant differences according to Fisher’s least-significant difference (LSD) test (*P < 0.05 and **P < 0.01).

No effect was detected when using higher concentrations (10−4 and 10−5 M) of GR24 (Fig. S2, see Supporting Information). These results suggest that there is no direct effect of SLs on either spore germination or the development of this pathogenic fungus.

Plant resistance/susceptibility to pathogens is generally associated with changes in hormonal defence pathways (Robert-Seilaniantz et al., 2011). To determine whether the increased susceptibility of Slccd8 to B. cinerea was related to alterations in the hormonal profiles, the contents of the defence-related phytohormones JA, SA, ABA and the JA precursor oxo-phytodienoic acid (OPDA), also biologically active (Wasternack, 2007), were analysed in the leaves of Slccd8 and compared with those of the corresponding wild-type. The samples were analysed by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS), as described by Flors et al. (2008). Analysis by HPLC-MS/MS allowed the simultaneous quantification of all four compounds from each sample. A 100-mg aliquot of dried leaf tissue was used per sample, and five independent replicates per genotype were employed. Quantifications were carried out with MassLynx 4.1 software (Waters, Yvelines Cedex, France) using internal standards as a reference for extraction recovery and the standard curves as quantifiers. Hormone analysis was conducted at the Scientific Instrumentation Service at the Estación Experimental del Zaidín-CSIC (Granada, Spain). As for the pathogenicity bioassay, hormonal data were subjected to one-way ANOVA, and Fisher’s LSD test was applied to determine the statistical significance.

The levels of JA in Slccd8 were less than 30% (about 75% reduction) of those in the corresponding wild-type Craigella (Table 1, P < 0.01). A similar trend was observed for OPDA, although the reduction here was not significantly different. A significant reduction of about 50% was also observed in the levels of SA in the mutant leaves (Table 1, P < 0.05). As in the case of JA and SA, the ABA content was also reduced (about 35%) in the mutant compared with the wild-type (Table 1, P < 0.05), confirm-
ing an altered hormonal balance in the mutant. In addition to the hormonal profiles by HPLC-MS/MS, the expression of marker genes for the different signalling pathways regulated by these hormones was analysed in the leaves of Slccd8 and the wild-type from noninoculated plants by real-time quantitative polymerase chain reaction (qPCR). qPCR was conducted using the iCycler iQ5 system (Bio-Rad, Hercules, CA, USA) and specific primers for each gene (Table 2). As JA marker, we used the responsive gene PinII, encoding for the proteinase inhibitor II involved in plant defence against insects and B. cinerea (El Oirdi et al., 2011; Wasternack, 2007). The genes PR1a and Le4 were used as markers for SA- and ABA-regulated responses, respectively (López-Ráez et al., 2010b). PR1a encodes a pathogenesis-related protein which is inducible by SA, and Le4 encodes for an ABA-inducible dehydrin. Total RNA from leaves was extracted using Tri-Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions. The RNA was treated with RQ1 DNase (Promega, Madison, WI, USA) purified through a silica column using the NucleoSpin RBA Clean-up Kit (Macherey-Nagel, Düren, Germany) and stored at −80 °C until use. The first-strand cDNA was synthesized with 1 μg of purified total RNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions. Six independent biological replicates were analysed per genotype. Relative quantification of specific mRNA levels was performed using the comparative 2−ΔΔCt method (Livak and Schmittgen, 2001). Expression values were normalized using the housekeeping gene SIEF, which encodes for the tomato elongation factor-1α. Interestingly, the expression of the SA and ABA response marker genes, PR1a and Le4, respectively, was not altered significantly in Slccd8 relative to the wild-type. However, the expression of the JA marker PinII was reduced by more than four-fold in the mutant (Fig. 3).

Overall, the reduced levels in Slccd8 of SA, ABA and, more markedly, JA, together with the lack of a direct effect of SLs on B. cinerea observed in the in vitro assay with GR24, indicate an indirect involvement of SLs in the regulation of plant defence responses. The results suggest a cross-talk between SLs and some of the signalling pathways related to defence. An interaction between SLs and other phytohormones has been shown for their other physiological functions described so far (reviewed in Kohlen et al., 2011). For instance, a cross-talk between SLs, auxin and cytokinins has been proposed to control shoot branching (Domagalska and Leyser, 2011). Similarly, an interaction of SLs with auxin and ET seems to regulate root system architecture and root hair formation (Kapulnik et al., 2011b; Ruyter-Spira et al., 2011). A regulatory role for ABA in SL biosynthesis has been proposed as tomato ABA-deficient mutants and tomato plants treated with the ABA inhibitor abamine-SG show a reduced capacity to produce SLs (López-Ráez et al., 2010a). Here, we show that SLs may also alter ABA levels. ABA has emerged as an important regulator of biotic defence responses (Ton et al., 2009). Therefore, it seems likely that the effect of SLs on ABA content may impact on the plant’s ability to cope with stresses. In this sense, it has been

### Table 2

<table>
<thead>
<tr>
<th>ID</th>
<th>Gene</th>
<th>Pathway</th>
<th>Primers (5'–3')</th>
<th>Reference</th>
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<tr>
<td>K03291</td>
<td>Proteinase inhibitor II (PinII)</td>
<td>JA</td>
<td>GAAATCGTIAATTATCTCCAC ACATACAAACCTTCATCTTT</td>
<td>Uppalapati et al. (2005)</td>
</tr>
<tr>
<td>M69247</td>
<td>Pathogenesis-related protein PR1a</td>
<td>SA</td>
<td>ATGTTGTTGGTTGGGTTGT ACTTGGGCATCCAAGACG</td>
<td>López-Ráez et al. (2010b)</td>
</tr>
<tr>
<td>X51904</td>
<td>Le4</td>
<td>ABA</td>
<td>ACTCAAGGGCATGGTAAGTG CCTTCTTCTCCTCCACCT</td>
<td>López-Ráez et al. (2010b)</td>
</tr>
<tr>
<td>X14449</td>
<td>Elongation factor-1 (SIEF)</td>
<td>Housekeeping</td>
<td>GATTGGTGGTATTGGAACTGTC AGCTCGTGGTGGCAYCTC</td>
<td>Rothenberg et al. (2006)</td>
</tr>
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ABA, abscisic acid; JA, jasmonic acid; SA, salicylic acid.

**Fig. 3** Expression levels in the tomato mutant Slccd8 compared with the corresponding wild-type (WT) Craigella. Gene expression analysis by real-time quantitative polymerase chain reaction (qPCR) of molecular markers for different defence signalling pathways. M values (log2 ratio) are represented. The M value is zero if there is no change, and ‘+1’ and ‘−1’ indicate a two-fold induction and reduction, respectively. PinII, PR1a and Le4 genes were the markers for the jasmonic acid (JA)-, salicylic acid (SA)- and abscisic acid (ABA)-dependent pathways, respectively. Bars represent the means of six independent replicates [±standard error (SE)]. Asterisk (*) indicates significantly different (P < 0.01) expression levels according to Fisher’s least-significant difference (LSD) test.
shown that the tomato ABA-deficient mutant sitiens is more resistant than wild-type plants to B. cinerea (Audenaert et al., 2002; Curvers et al., 2010), suggesting a role of ABA in tomato susceptibility to this pathogen. Here, we show that, despite a moderate reduction in ABA content, the SL-deficient line Sccoli was more susceptible to B. cinerea. Although a positive effect of ABA in early defence responses against B. cinerea has also been reported (Abuqamar et al., 2009), our data do not support a major contribution of the changes in ABA levels to the enhanced susceptibility of Sccoli to B. cinerea and A. alternata.

Unlike ABA, as far as we know, no relationship between SLs and the SA- and JAs-related signalling pathways has been reported to date. Among the hormonal changes observed in the mutant Sccoli, those produced in the JA-dependent pathway were the most pronounced. Moreover, this was correlated with a strong reduction in the level of the JA-responsive gene PinII. Botrytis cinerea and A. alternata are necrotrophic fungi that colonize senescent or dead plant tissues (Dean et al., 2012). Necrotrophic pathogens are generally sensitive to defence responses regulated by JA (Pieterse et al., 2011). PinII has been proven to be important for tomato resistance to B. cinerea (El Oirdi et al., 2011). PinII is a protease inhibitor which accumulates on wounding and damage caused by herbivores and pathogens or by treatment with exogenous JA (Wasternack, 2007). El Oirdi et al. (2011) reported that tomato plants silenced in PinII were more susceptible to this pathogenic fungus, showing that this enzyme is required for resistance against B. cinerea in tomato. Therefore, the reduction in JA content and PinII basal levels could well explain the increased susceptibility of Sccoli to B. cinerea and A. alternata.

We can conclude that the SL-deficient mutant Sccoli is more susceptible to necrotrophic fungal pathogens. The reduced levels of the phytohormones JA, SA and ABA in Sccoli suggest a role of SLs in the regulation of plant defence responses through their interaction with other signalling pathways, especially with the JA-related pathway, rather than a direct effect on fungal development. The results provide insights into a possible new role for SLs in the regulatory network controlling plant responses to stresses. Unravelling the role of SLs in defence is definitely exciting and promising. However, further research is needed to define the mechanisms that may regulate this new function and to assess whether this effect can be extended to other pathosystems.

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El Oirdi, M., El Rahman, T.A., Bin-Del Zaidín-CSIC (Granada, Spain) for technical assistance in mechanical del Zaidín-CSIC (Granada, Spain) for technical assistance in shoot branching. (2012). Necrotrophic fungal pathogens are generally sensitive to defence responses regulated by JA (Pieterse et al., 2011). PinII has been proven to be important for tomato resistance to B. cinerea (El Oirdi et al., 2011). PinII is a protease inhibitor which accumulates on wounding and damage caused by herbivores and pathogens or by treatment with exogenous JA (Wasternack, 2007). El Oirdi et al. (2011) reported that tomato plants silenced in PinII were more susceptible to this pathogenic fungus, showing that this enzyme is required for resistance against B. cinerea in tomato. Therefore, the reduction in JA content and PinII basal levels could well explain the increased susceptibility of Sccoli to B. cinerea and A. alternata.


**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1** Effect of *Botrytis cinerea* inoculation on the tomato mutant *SccdB*. Evaluation of lesions in leaves of *SccdB* and corresponding wild-type (WT) 4 and 8 days after inoculation with 5-mm-diameter plugs containing actively growing hyphae. Disease rating is expressed as the percentage of leaves in disease classes. Asterisks (*) indicate statistically significant different contributions (P < 0.01) of the disease severity classes according to Fisher’s least-significant difference (LSD) test.

**Fig. S2** Effect of GR24 on fungal development of *Botrytis cinerea*. Radial growth of *B. cinerea in vitro* determined 5 days after application of 10 μL of different concentrations (10⁻⁴, 10⁻⁵ and 10⁻⁶ M) of the strigolactone (SL) analogue GR24 (A). As control, 10 μL of sterilized demineralized water were used. Data represent the means of 10 independent replicates ± standard error. Representative plate showing no effect of GR24 on *B. cinerea* growth (B).