Effects of Root and Foliar Applications of 24-Epibrassinolide on Fusarium Wilt and Antioxidant Metabolism in Cucumber Roots

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Abstract. Root and foliar applications of 24-epibrassinolide (EBL), an immobile phytohormone with antistress activity, were evaluated for their effects on reducing fusarium wilt and their influence on antioxidant and phenolic metabolism in roots of cucumber plants (Cucumis sativus L. cv. Jinyan No. 4). EBL pretreatment significantly reduced disease severity together with improved plant growth and reduced losses in biomass regardless of application methods. EBL treatments significantly reduced pathogen-induced accumulation of reactive oxygen species (ROS), flavonoids, and phenolic compounds, activities of defense-related and ROS-scavenging enzymes. The enzymes included superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase, catalase as well as phenylalanine ammonia-lyase and polyphenoloxidase. There was no apparent difference between two application methods used. EBL applications triggered a slight increase in H2O2 concentration followed by increases in the transcript levels of WRKY transcription factor and defense-related genes. This study demonstrated that EBL enhanced resistance to fusarium wilt by a novel mechanism that was not related to its active transport or increase in antioxidant system.

Cucumber (Cucumis sativus L.) is one of the major greenhouse vegetables in the world and is very vulnerable to fusarium wilt caused by Fusarium oxysporum (FO) (Ahn et al., 1997; Ye et al., 2004). Fusarium pathogen infects the roots and then moves into the stems resulting in rapid wilting and death of shoots. Disease control is normally dependent on the use of resistant varieties. Chemical control with fungicides is not very effective.

Brassinosteroids (BRs) are a new group of phytohormones that are structurally similar to animal and insect steroid hormones. They control a broad range of processes, including seed germination, stem elongation, cell division and expansion, xylem differentiation, plant growth, and apical dominance (Azpiroz et al., 1998; Clouse and Sasse, 1998; Li et al., 1996; Sasse, 2003; Szekeres et al., 1996). In addition to its roles in plant growth and development, there is considerable evidence that BRs exert antistress effects on plants such as those caused by heat, cold, drought, and salt (Anuradha and Rao, 2001; Dhaubhadel et al., 1999, 2002; Kagale et al., 2007; Ogweno et al., 2008). The potential role of BRs in pathogen defense has also been the topic of recent studies. Potato plants sprayed with BRs had a lower incidence of infection by Phytophthora infestans (Khiripach et al., 1996). BR-induced disease resistance was also noted in barley, potato tubers, and cucumber plants (Khiripach et al., 2000). Recently, BRs have been shown to induce disease resistance in tobacco and rice against a broad range of pathogens (Nakashita et al., 2003). However, most of the early evidence about the protective activities of BRs against plant diseases is based on field observations but not field experiments (Khiripach et al., 2000).

Plants have a natural array of defense mechanisms to protect themselves from pathogenic organisms. Recently, the generation and scavenging system of reactive oxygen species (ROS) has attracted increasing attention with regard to their roles in the defense of plants against biotic stresses. It is known that low-dose ROS could function as a signal for inducing stress tolerance, whereas high-dose ROS has been implicated in the oxidative damage and the hypersensitive response (HR) (Apel and Hirt, 2004; Laloi et al., 2004). Evidence for the role of ROS in HR has mainly been obtained from observations on localized infections of foliar tissues by obligate biotrophic or necrotrophic pathogens and relatively little is known about oxidative metabolism in plant resistance to pathogens that invade plant vascular system (Garcia-Limones et al., 2002; Mehdy et al., 1996). Although BRs are known as a kind of immobile hormones, it has been shown to enhance transport of auxin (Symons and Reid, 2004; Symons et al., 2008). It is interesting to investigate whether BRs are able to induce resistance in plant parts that have not received BRs directly. Furthermore, little is known about the mechanism by which BRs induce resistance. The increased resistance to spraying and diseases in BR-treated potato tubers were found to be associated with enhancement of the synthesis of abscisic acid (ABA) as well as phenolic and terpenoid substances (Khiripach et al., 2000). In cucumber plants, increased activities of peroxidase (POD) and polyphenoloxidase (PPO) enzymes, which are involved in the metabolism of polyphenols, have been suggested as a factor contributing to BR-induced disease resistance (Khiripach et al., 2000). BR-induced resistance in tobacco was not associated with an increase in salicylic acid levels or induction in pathogenesis-related gene (PR) expression, suggesting that the mechanism of BR-induced resistance is distinct from systemic acquired resistance (SAR) and wound-inducible resistance (Nakashita et al., 2003). Most recently, we have found that BR-induced tolerance to a broad range of stresses was dependent on H2O2 accumulation generated by NADPH oxidase (Xia et al., 2009).

The aim of the present study was to evaluate the effect of 24-epibrassinolide (EBL; Fig. 1) application to roots or shoots on the development of fusarium wilt and changes in metabolism of antioxidant and phenolic compounds in roots of cucumber plants. Subsequently, we determined to what extent these changes could be associated with EBL-induced resistance.

Materials and Methods

Greenhouse experiments. Cucumber (Cucumis sativus L.) cv. Jinyan No. 4 was used because of its known susceptibility to fusarium wilt [Fusarium oxysporum (Schlectend.Fr) f. sp. cucumerinum (Owen) Snyder & Hansen] (FO) (Ye et al., 2004). Seeds were sterilized in 1% (w/v) NaClO and germinated in perlite. After emergence, batches of eight seedlings were grown hydroponically in a plastic tank (13 L) filled with 10 L half-strength Enshi nutrient solution
Spraying with a concentration of 0.2 mg L\textsuperscript{-1} induced phytotoxicity (Yu et al., 1994). Samples of 0.5 g of root material, which was ∼10 cm from the root tip, were homogenized. The homogenate was centrifuged at 3000 × g for 10 min, and the absorbance of the supernatant was read at 440, 532, and 600 nm. MDA equivalents were calculated according to the method of Hodges et al. (1999).

**Total RNA extraction and gene expression analysis.** To determine the changes in the transcript of defense-related genes, roots of cucumber plants were sampled at Day 2 after EBL spray. Total RNA was extracted from roots using Trizol according to the supplier’s recommendation. Residual DNA was removed with purifying column. One microgram total RNA was reverse-transcribed using 0.5 μg of Oligo (dT) 12-18 (Invitrogen) and 200 units of Superscript II (Invitrogen) following the supplier’s recommendation. On the basis of EST sequences, the gene-specific primers were designed (Table 1) and used for amplification.

Quantitative real-time polymerase chain reaction (PCR) was performed using the iCycler™ Real-time PCR Detection System (Bio-Rad, Hercules, CA). PCRs were performed using the SYBR Green PCR Master Mix (Applied Biosystems). The PCR conditions consisted of denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified using software provided with the iCycler™. Real-time PCR Detection System. The identity of the PCR products was verified by single-strand sequencing using MegaBACE 1000 DNA analysis system (Amersham Biosciences). To minimize sample variations, mRNA expression of the target gene was normalized relative to the expression of the housekeeping gene actin. All experiments were repeated.

Table 1. Primers used for real-time polymerase chain reaction assays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pairs</th>
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<tbody>
<tr>
<td><strong>WRK30</strong></td>
<td>F: CATCTGACCGTCCTTCTCAT</td>
</tr>
<tr>
<td><strong>PR-1</strong></td>
<td>F: AACTCTGGGCGACCTTAC</td>
</tr>
<tr>
<td><strong>PAL</strong></td>
<td>F: AGGTTGTGGCCTTACTA</td>
</tr>
<tr>
<td><strong>cAPX</strong></td>
<td>F: ATGGTGAGATGCTACCCCTT</td>
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**Fig. 1.** The chemical structure of 24-epibrassinolide.
Results

Untreated plants inoculated with pathogenic FO started to wilt 2 d after inoculation and by 12 d had reached a wilting percentage of 75.4 ± 7.5% (Figs. 2 and 3A). Plants treated with EBL applied either to roots or shoots showed a delay in the onset of wilting and after 12 d wilting percentage decreased to 39.4% ± 4.2% and 30.9% ± 11.7%, respectively. The roots of FO plants showed a vascular bundle browning index of 2.9 ± 0.2 at Day 12 (Fig. 3B). The corresponding values for EBL-treated plants were 1.6 ± 0.1 by root application and 1.4 ± 0.1 by shoot application. FO inoculation caused a reduction in dry weight of 67.1% in roots and 51.7% in shoots (Table 2). This reduction was significantly alleviated by EBL applications, in which corresponding reductions in dry weight were 34.3% and 17.9% when it was applied to the roots and 28.6% and 15.4% when it was applied to the shoots, respectively.

FO inoculation resulted in an overall increase in the activities of antioxidant enzymes (Fig. 4). GPX and APX activities peaked at Day 4, whereas SOD and CAT activities peaked at Day 8 after FO inoculation. EBL application to roots or shoots brought about a decrease in the activities of these antioxidant enzymes. The activities of these antioxidant enzymes in EBL-treated plants were slightly higher than those of control plants in most cases. There were no apparent differences in the activities of SOD, GPX, APX, and CAT whether EBL was applied to roots or shoots.

There was a gradual increase of PPO and PAL activities in roots after exposure to FO (Fig. 5A–B). PPO activity increased by 16.6%, 46.3%, and 45.1%, whereas PAL activity increased by 17.6%, 24.3%, and 25.1%, respectively, at 4, 8, and 12 d after inoculation. A pretreatment of roots or shoots with EBL significantly attenuated this increase with EBL application to shoots having more significant effects than application to roots.

Accompanied with the change in PPO and PAL activities, FO inoculation also resulted in a gradual increase in flavonoids and total phenolics in roots (Fig. 5C–D). At Day 12, flavonoids and total phenolics increased by 343.0% and 63.4%, respectively, EBL applications to roots and shoots both significantly attenuated this increase. In comparison with the FO treatment without EBL, EBL application to shoots decreased flavonoids by 64.3%, 50.6%, and 57.3%, respectively, at Days 4, 8, and 12 after FO inoculation. A similar trend was also observed in total phenolics. It was interesting to note that flavonoid content for EBL spray treatment was not significantly different from that of control plants.

Changes in antioxidant enzymes were accompanied by changes in H$_2$O$_2$ and MDA contents. EBL applications resulted in a slight increase in H$_2$O$_2$ content in roots regardless of whether it was supplied to roots or shoots (Fig. 6A). H$_2$O$_2$ content in roots significantly increased after FO inoculation and reached a value 1.5 times higher than that of the control at 12 d. This increase, however, was significantly attenuated by both EBL treatments. Similarly, MDA content also significantly increased with time (Fig. 6B). FO-induced increase in MDA, however, was significantly reduced by both EBL treatments, especially by the foliar spray. At Day 12, MDA content in roots of FO, rEBL + FO, and sEBL + FO increased by 173.8%, 152.3%, and 87.4%, respectively, compared with control plants.

To further study the role of H$_2$O$_2$ in BR-induced stress tolerance, we examined expression of genes encoding proteins involved in H$_2$O$_2$ signaling and antioxidative/defense responses in roots of BR-treated plants. The transcript levels of WRKY30 (transcription factor), PR-1 (pathogenesis-related proteins), PAL (phenylalanine ammonia-lyase), and cAPX (cytosolic ascorbate peroxidase) were all upregulated in roots for plants at 3 d after EBL application to shoots (Fig. 7). However, no differences were found at 5 d after EBL treatment (data not shown).
Fig. 4. Effects of 24-epibrassinolide (EBL) application on the activities of superoxide dismutases (SOD, A), ascorbate peroxidase (APX, B), glutathione peroxidase (GPX, C), and catalase (CAT, D) in roots of cucumber plants with or without inoculation of *Fusarium oxysporum* (FO). Data are the means of three replications within an experiment with SEs. Different letters are significantly different between the treatments at 5% level according the Tukey tests.

Fig. 5. Effects of 24-epibrassinolide (EBL) application on the activities of polyphenoloxidase (A), phenylalanine ammonia-lyase (B), flavonoids content (C), and total phenolics content (D) in roots of cucumber plants with or without inoculation of *Fusarium oxysporum* (FO). Data are the means of three replications within an experiment with SEs. Different letters are significantly different between the treatments at 5% level according the Tukey tests.

Discussion

Although BRs have been implicated in a broad range of stress responses in plants, relatively little is known about their role in pathogen defense. In our study, EBL application to either roots or shoots significantly increased the resistance to fusarium pathogens as evidenced by a decreased severity of fusarium wilt. This result confirmed the role of BRs in plant response to pathogen attack. BRs have been found to induce resistance in tobacco plants against tobacco mosaic virus, the bacterial pathogen *Pseudomonas syringae* and the fungal pathogen *Oidium* sp. In rice, BRs induced resistance to *Magnaporthe grisea* and *Xanthomonas oryzae*, which cause rice blast and bacterial blight, respectively (Nakashita et al., 2003). Early evidence for BR-induced resistance to diseases was, however, obtained by local application to the foliage for protection against aerial diseases or to the roots for protection against root diseases (Khripach et al., 2000). In this regard, our finding that EBL application to shoots suppressed the severity of a disease originating from root infection is interesting because BRs are immobile in the plant with little transport from shoots to roots (Symons and Reid, 2004; Symons et al., 2008). It is likely that EBL application to shoots might generate a secondary signal, which was transmitted from shoots to roots. This opens new possibilities for its application in agricultural production because foliar spraying is more practical than drenching soils.

Protective activities of BRs against plant diseases have been indicated based on evaluations from field trials and greenhouse experiments, but its mechanism at the molecular level remains to be clarified. Increases in the activities of POD and PPO enzymes were suggested as a factor contributing to EBL-induced disease resistance (Khripach et al., 2000). It has been shown that BR-induced resistance in tobacco is not correlated with increases in salicylic acid levels or induction of pathogenesis-related gene (PR) expression, suggesting that the mechanism of BR-induced resistance is distinct from SAR and wound-inducible resistance (Nakashita et al., 2003). To date, there is little evidence about the interaction of BRs with other hormones in the pathogenesis. Recently, evidence is emerging that BRs may affect the long-distance transport of auxin (IAA) (Jager et al., 2007). IAA has been found to reduce diseases caused by *Pythium ultimum* on tomato plants and *Phytophthora infestans* on potato plants (Gravel et al., 2007; Noel et al., 2001; Terrile et al., 2006). It is, therefore, possible that EBL application to shoots attenuated the severity of fusarium wilt partly by the promotion of IAA transport from shoots to roots.

An increased rate in ROS-scavenging metabolism was frequently observed in localized infection of foliar tissues by obligate biotrophic or necrotrophic pathogens associated with a rapid HR (Mehdy et al., 1996; Ye et al., 2006). In this study, we found that the significant increases in activities of ROS-scavenging enzymes SOD, APX, GPX, and CAT, together with increased levels of H$_2$O$_2$ and MDA after FO infection were greatly attenuated by EBL pretreatment, suggesting that less peroxidative stress occurred in the EBL-treated plants. It needs to be noted that high levels of ROS cause cell death; low levels of ROS, however, have regulatory roles in plant stress responses. Application of ABA and SA as well as exposure to low temperature all resulted in a transient elevation of H$_2$O$_2$ and oxidative stress (Dat et al., 1998; Prasad et al., 1994; Zhang et al., 2001). It has been proposed that ROS plays a critical role in induced tolerance by activating or inducing stress response-related factors such as MAP kinases, transcription factors, antioxidant enzymes, dehydrins,
In agreement with the changes in disease severity and the ROS metabolism, FO-induced increases in activities of PPO and PAL, and associated accumulation of flavonoids and phenolics were significantly attenuated by the EBL pretreatment either to roots or shoots. All these results indicated that BR-induced resistance to FO is not attributed to changes in phenolic metabolism. However, phenolic metabolism may play an important role in the defense against pathogen attack. In summary, we have found that the application of EBL either to shoots or to roots alleviated the symptoms of fusarium infection and reduced pathogen-induced oxidative stress, flavonoids, and phenolic compounds. Foliar EBL application triggered a slight increase in H2O2 concentration followed by increases in the transcript levels of defense-related genes in roots. This study provided evidence that BRs could induce resistance to the pathogen by a novel long-distance signal relay mechanism. This finding is of importance not only for basic understanding of the role of hormone, but also for potential use of such compounds in agriculture and horticulture.

Literature Cited


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