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Cross-Talk Between Calcium–Calmodulin and Brassinolide for Fungal Endophyte-Induced Volatile Oil Accumulation of *Atractylodes lancea* Plantlets

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Abstract Using pharmacological and biochemical approaches, the signalling pathways between calcium (Ca^{2+})–calmodulin (CaM), brassinolide (BL), and nitric oxide (NO) for fungal endophyte-induced volatile oil accumulation were investigated in *Atractylodes lancea* plantlets. *Gilmaniella* sp. AL12 inoculation elevated the concentrations of BL, CaM, and $[Ca^{2+}]_{cyt}$, expression of the calmodulin 1 (*CaM1*) gene, and the levels of volatile oils. Treatment with AL12 or exogenous BL led to significant increases in the levels of cytosolic Ca^{2+} and CaM and *CaM1* expression in plantlets. However, the upregulation of BL was almost completely blocked by pretreatments with CaM antagonists and Ca^{2+} channel blockers. Pretreatment with a BL inhibitor, brassinazole (BRz), did not influence the increase in levels of CaM induced by the endophyte. $CaCl_2$-induced increases in NO generation, CaM antagonists, and Ca^{2+} channel blockers were able to suppress NO production, and the NO-specific scavenger was not able to suppress the generation of $[Ca^{2+}]_{cyt}$ in plantlets. Exogenous BL was not able to induce NO generation, and BRz had no effect on NO generation. Our results suggest that Ca^{2+}–CaM induced by this endophyte mediates NO generation and BL concentration, and also functions downstream of BL signalling, resulting in the upregulation of volatile oil accumulation in *A. lancea* plantlets.

Keywords *Atractylodes lancea* · Endophytic fungi · Calcium–calmodulin · Brassinolide · Medicinal herb

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Introduction

Atractylodes lancea, a traditional Chinese medicinal herb, is a member of the Compositae family, also known as sword-like *Atractylodes* (Duan and others 2008; Yuan and others 2009). It is believed to act primarily on the digestive system. Volatile oils from *A. lancea* also show antimicrobial activities and include the characteristic atractylone, β-eudesmol, hinesol, and atractylodin (Wang and others 2009). Endophytes can coexist with their hosts for part of their life cycle without causing obvious symptoms of infection, have great potential to affect the metabolism of hosts (Suryanarayanana and others 2009), promote plant growth (Lewis 2004), and indirectly increase plant resistance to protect plants against environmental stress (Vega and others 2008; Hao and others 2010). Their effects on plant accumulation of medicinal components have received much attention recently (Saunders and Kohn 2009; Wang and others 2012). Unlike pathogens, endophytic fungi do not cause strong hypersensitive reactions in the host. However, long-term colonization can induce the accumulation of various kinds of metabolites in hosts (Li and Tao 2009; Saunders and Kohn 2009). How endophytic fungus–host interactions affect the accumulation of plant secondary metabolites is an intriguing issue.

More and more evidence indicates that nitric oxide (NO), identified as primarily a diffusible signal molecule in animals, plays roles in various physiological processes in plants, such as regulating plant growth, development, and defense responses (Neill and others 2003; Wendehenne and others 2004; Lamotte and others 2005). Gao and others (2012) reported that NO is a signalling molecule of the fungal endophyte *Fusarium* sp. E5 elicitor-induced isoeuphpekinensin accumulation in *Euphorbia pekinensis* suspension cells. NO also has been shown to be a key

messenger in endophyte-induced volatile oil accumulation in *A. lancea* plantlets (Wang and others 2011). There are several potential sources of NO in plants, including the NOS-like enzyme nitrate reductase and nonenzymatic sources (Neill and others 2003; del Río and others 2004; Wendehenne and others 2004; Lamotte and others 2005). NO activity has been biochemically characterized in pea leaf peroxisomes; it is strictly dependent on CaM and requires Ca^{2+} (Corpas and others 2004). However, whether Ca^{2+}–CaM is required for NO-induced secondary metabolism in plants, and, if so, what the regulatory relationship is remain to be determined.

Calcium is a universal second messenger in the responses of plant cells to biotic and abiotic stresses (Snedden and Fromm 2001; Zhang and Lu 2003; Bouche and others 2005). Increases in transient $[Ca^{2+}]_{cyt}$ are sensed by several Ca^{2+} sensors such as calmodulin (CaM), calcium-dependent protein kinase, and calcineurin B-like protein (Snedden and Fromm 2001; Bouche and others 2005). As one of the most conserved Ca^{2+} receptors, CaM activates numerous downstream target proteins by binding Ca^{2+}. It has been shown that Ca^{2+}–CaM is involved in plant responses to environmental stimuli such as osmotic stress, temperature stress, hormones, and related microbes (Snedden and Fromm 2001; Yang and Poovaiah 2003). The activated Ca^{2+}–CaM complex binds to target proteins and modulates their activities. Du and Poovaiah (2005) found that two brassinosteroid (BR) biosynthesis-related enzymes, DWF4 and CPD, were Ca^{2+}–CaM binding proteins, and that binding regulates the physiological effects of BR in a wide-ranging manner. However, there is only limited evidence to date of these interactions between Ca^{2+}–CaM and BR.

Using pharmacological and biochemical approaches, we investigated the signalling pathways between Ca^{2+}–CaM, BR, and NO in endophyte-induced volatile oil accumulation in *A. lancea* plantlets. We found that Ca^{2+}–CaM induced by the endophyte *Gilmaniella* sp. mediates NO generation and BL concentration and also functions downstream of BL signalling, resulting in the upregulation of volatile oil accumulation in *A. lancea* plantlets.

Materials and Methods

Plant Materials and Treatments

Meristem cultures of *A. lancea* (collected at Mao Mountain, Jiangsu Province, China) were established following the methods of Wang and others (2011). The explants were surface sterilized and grown in Murashige and Skoog medium (MS) (Murashige and Skoog 1962) supplemented with 0.3 mg/L naphthalene acetic acid (NAA), 2.0 mg/L 6-benzyladenine, 30 g/L sucrose, and 10 % agar. The rooting medium (1/2 MS) contained 0.25 mg/L NAA, 30 g/L sucrose, and 10 % agar. Explants were kept at 25/18 °C day/night, with a light intensity of 3,400 lm/m^2 and a photoperiod of 12 h, and subcultured every 4 weeks. Thirty-day-old rooted plantlets were used for investigations.

Reagents used as specific scavengers or inhibitors, including 10 mM ethylene glycol tetraacetic acid (EGTA), 5 mM $LaCl_3$, 100 μM trifluoperazine (TFP), 100 μM W7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide hydrochloride], 1.25 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO), and 50 μM verapamil, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Brassinazole (BRz) (100 μM) was purchased from TCI (Tokyo Kasei Kogyo Co., Ltd., Japan). Exogenous signalling molecules were 1–10 mM $CaCl_2$ and 10 nM brassinolide (TCI). All exogenous signalling molecules and inhibitors were filtered before use through 0.22-μm-diameter microporous membranes. Unless otherwise stated, inhibitors were applied 1 day before the application of signalling molecules or fungal inoculation and sprayed directly on plant leaves and roots.

Fungal Culture and In Vitro Inoculation

The endophytic fungus AL12 (*Gilmaniella* sp.) was isolated from *A. lancea*, cultured on potato dextrose agar, and incubated at 28 °C for 5 days. Young plantlets were inoculated with 5-mm AL12 mycelial disks. All treatments were conducted in a sterile environment and performed in triplicate to examine the reproducibility.

Measurement of $[Ca^{2+}]_{cyt}$ and CaM Levels

Atractylodes lancea mesophyll protoplasts were isolated using the method described by Shang and others (2005). Leaf sections were digested in enzyme solution containing 1 % cellulose R-10 (Yakult Honsha, Tokyo, Japan) and 0.4 % macerozyme R-10 (Yakult Honsha, Tokyo, Japan). The isolated protoplasts were washed twice with washing and incubation solution (0.6 M mannitol, 4 mM MES, and 20 mM KCl, pH 5.7). The mesophyll protoplasts were incubated with Fluo-3/AM ester (Molecular Probes, Eugene, OR, USA) at 4 °C for 1 h and then incubated at 25 °C for 1 h in the dark. The incubation solution contained 10 μM Fluo-3/AM ester, 0.4 M mannitol, 5 mM MES (pH 5.7), and 20 mM KCl. Pictures were taken by scanning three times every 30 s using confocal laser scanning microscopy, excited with a 488-nm laser, and fluorescent emissions were filtered by a 515-nm filter to eliminate the autofluorescence of chlorophyll. The fluorescence intensities of these pictures were measured by fluorescence microscopy after establishing a stable baseline.

For isolation of CaM, the *A. lancea* plantlets were ground in liquid N_2 and then homogenized in buffer solution (1:1 w/v): 50 mM Tris–HCl (pH 8.0), 1 mM EGTA, 0.5 mM PMSF, 20 mM $NaHSO_4$, and 0.15 M NaCl. The homogenates were sonicated for 2 min, incubated at 90 °C for 2 min, and then centrifuged at 10,000×*g* and 4 °C for 30 min. Supernatants were used to measure protein levels and CaM concentration. CaM concentration was determined by enzyme-linked immunosorbent assay (ELISA) following Sun and others (1995).

Determination of Brassinolide Levels

The endogenous brassinolide of *A. lancea* was extracted following Jana and others (2007). Freeze-dried plant tissues were ground to a fine powder under liquid nitrogen and extracted twice in ice-cold 80 % (v/v) methanol in an ultrasonic bath, 30 min each time (10 mL/g FW). The mixture was centrifuged at 14,000×*g* and 4 °C for 10 min, then the supernatant was evaporated to 200 µl under a vacuum and stored at −20 °C for analysis. The generation of brassinolide was monitored using a Plant Brassinolide ELISA Kit (Sunred Biological Technology, Shanghai, China) following the manufacturer's instructions. No fewer than 15 plantlets were used at each time point. All treatments were performed in triplicate.

Real-Time Quantitative PCR Analysis

Total RNA was extracted from plantlet leaves as described by Dong and Beer (2000). First-strand cDNA was synthesized from 1 µg of total RNA (PrimeScript RT Reagent Kit, Takara, Dalian, China). Real-time qPCR was performed using the DNA Engine Opticon 2 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) and SYBR® Green probe (SYBR Premix Ex Taq system, Takara). The constitutively expressed gene *EF1α* was used as an internal positive control. The gene-specific primers used to amplify *EF1α* were 5′-CAGGCTGATTGTGCTGTTCTTA-3′ and 5′-TGGTGGC ATCCATCTTGT-3′ (241-bp product), and the primers for *CaM1* genes were 5′-ACTTCTTCATCCGTCAGC-3′ and 5′-GGAATGGGACTATTGATTT-3′ (141 bp). The GenBank accession numbers of the *CaM1* and *EF1α* genes are EF090602.1 and GR724649.1, respectively. The thermocycler program was as follows: 90 s at 95 °C; 40 cycles of 30 s at 95 °C, 30 s at 57 °C, and 30 s at 72 °C; and 5 min at 72 °C. To standardize the data, the ratio of the absolute transcript level of *CaM1* genes to the absolute transcript level of *EF1α* was calculated for each sample of each treatment.

Measurement of NO

The generation of NO was monitored using a NO detection kit (Nanjing Jiancheng Bioengineering Inst., Nanjing, China) following the manufacturer's instructions. Leaf samples (1 g) were ground with 5 mL of 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2) and the homogenate was centrifuged at 14,000×*g* for 10 min. The supernatant was used for the NO assays. One unit of NO was defined as the absorbance variation caused by the internal standard of 1 µM NO per gram fresh weight. At least 15 plantlets were assayed for each time point, and all treatments were performed in triplicate.

Extraction and Determination of Volatile Oils

Volatile oils were extracted from whole plantlets of *A. lancea*, including leaves and rhizomes (0.8–1.6 % oil content in leaves, 2.2–3.4 % in rhizomes) following Zhang and others (2009). The volatile oils were dried with anhydrous sodium sulfate and stored in dark glass bottles at 4 °C for gas chromatography (GC) analysis. GC determination was carried out using a 1890 series GC (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector. A DB-5 ms (30 m × 0.25 mm × 0.25 µm) column (Agilent, Santa Clara, CA, USA) was used with the following temperature program: column held at 60 °C for 1 min after injection, increased by 10 °C/min to 190 °C, held for 2 min, increased by 5 °C/min to 210 °C, held for 2 min, increased by 10 °C/min to 220 °C, and held for 8 min. Nitrogen was used as carrier and the flow rate was 4 mL/min. Four main components of the volatile oils, atractylone, hinesol, β-eudesmol, and atractylodin, were quantitatively analyzed following the method of Fang and others (2009); their retention times were 14.57, 15.24, 16.21, and 22.18 min, respectively.

Statistical Analyses

Data were compiled using Microsoft Excel (Redmond, WA, USA). Values are presented as mean ± SD (standard deviation) of three replicates for each treatment. One-way ANOVA and Duncan's multiple-range test were used to identify significant differences (SPSS ver. 13.0, SPSS Inc., Chicago, IL, USA).

Results

Ca^{2+}–CaM is Required for Fungus-Induced Volatile Oil Production

To investigate the possible role of Ca^{2+}–CaM in fungal endophyte-induced volatile oil accumulation, *A. lancea* plantlets were inoculated with *Gilmaniella* sp. AL12. When protoplasts in the mesophyll cells from *A. lancea* leaves were isolated and loaded with Fluo-3/AM. The

relative fluorescence intensity increased significantly 4 days after endophyte inoculation (Fig. 1a) , indicating that the fungus may elevate $[Ca^{2+}]_{cyt}$ concentration in *A. lancea* cells. Concurrently, the levels of CaM also began increasing significantly 4 days after fungus inoculation (Fig. 1c). To investigate whether Ca^{2+}–CaM was involved in the fungus-induced volatile oil accumulation, the Ca^{2+} chelator EGTA, channel blockers La^{3+} and verapamil, and CaM antagonists W_7 and TFP were applied individually or in combination. Compared with the fungus inoculation group, application of La^{3+} and verapamil and EGTA significantly reduced the fungus-induced $[Ca^{2+}]_{cyt}$ concentration and the fungus-triggered volatile oil production, but none of the La^{3+}, verapamil, or EGTA alone could affect the volatile oil production without fungus inoculation (Fig. 1b). Compared with the fungus inoculation group, W_7 and TFP significantly suppressed the fungus-induced CaM level and the fungus-triggered volatile oil production, but it could not affect the volatile oil production when they were applied alone (Fig. 1d). These results suggested that Ca^{2+}–CaM is important for fungus-induced volatile-oil synthesis in *A. lancea* plantlets.

Brassinolide is Required for Fungus-Induced Volatile Oil Accumulation

To investigate whether brassinolide was involved in fungus-induced volatile oil accumulation, *A. lancea* plantlets were inoculated with *Gilmaniella* sp. AL12. Compared with the control, brassinolide levels significantly fluctuated in the plantlets inoculated with the fungus (Fig. 2a). The brassinolide content in the plantlets began to increase 6 days after

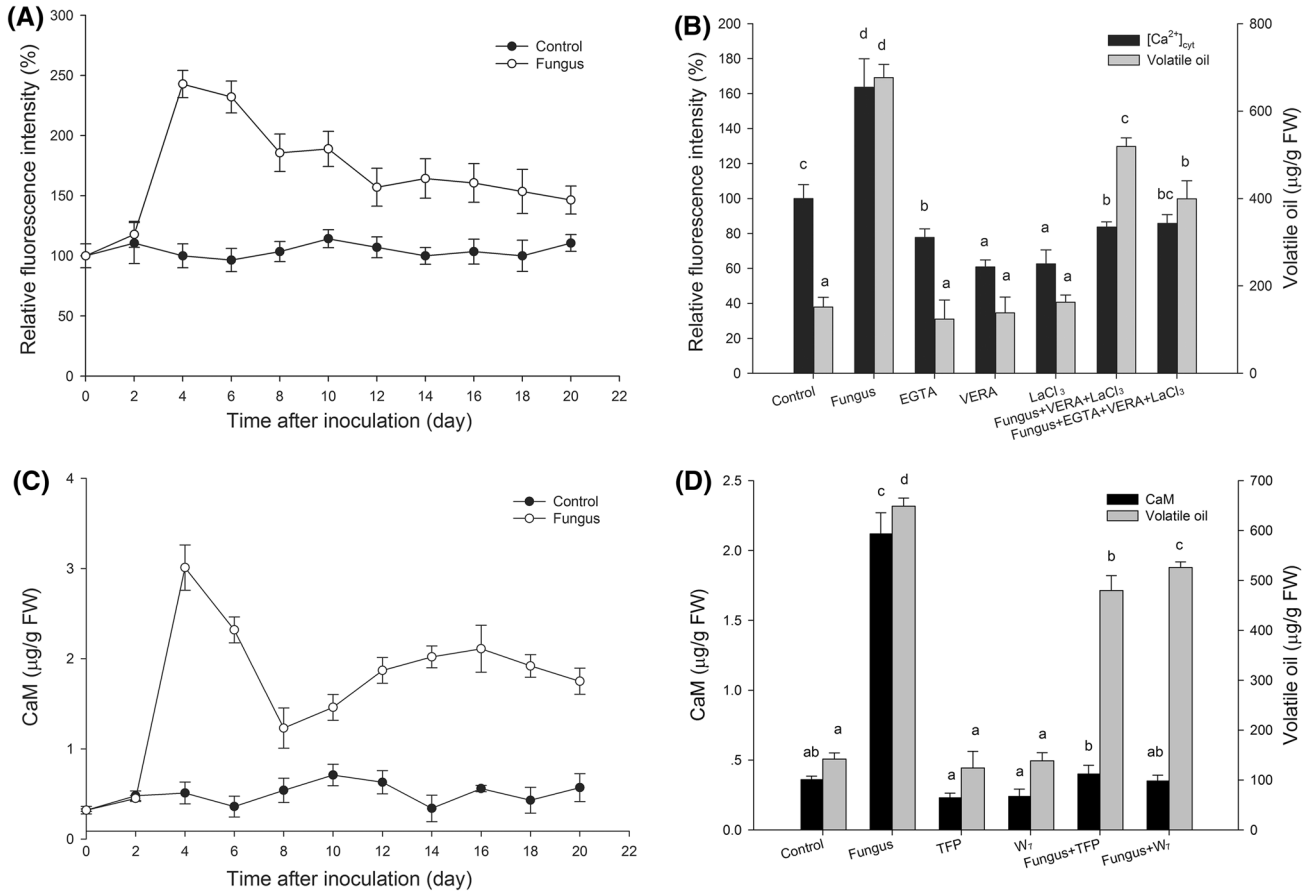

Fig. 1 The influence of $[Ca^{2+}]_{cyt}$ and CaM on endophytic fungus-induced volatile oil accumulation of *A. lancea*. **a** Changes in relative fluorescence intensity of $[Ca^{2+}]_{cyt}$. The mesophyll protoplasts loaded with Fluo-3/AM were observed by LSCM. The fluorescence intensity was measured by fluorescence microscopy and calculated as the average value of those obtained by scanning over 100 protoplasts from five different experiments. **b** Effects of the Ca^{2+} chelator and channel blockers on volatile oil accumulation after 18 days. The Ca^{2+} chelator EGTA (10 mM) and channel blockers (1 mM $LaCl_3$, 50 μM verapamil) were added 1 day before fungal inoculation. **c** Changes in the content of CaM. **d** Effects of CaM antagonists on volatile oil accumulation after 18 days. CaM antagonists W_7 100 μM and TFP 100 μM were added 1 day before fungal inoculation. Thirty-day-old plantlets of *Atractylodes lancea* were incubated with 5-mm mycelial disks or PDA disks (control). Values are the mean of three independent experiments. *Bars* with different lower-case letters were significantly different (one-way ANOVA, Duncan's multiple range test, $P < 0.05$)

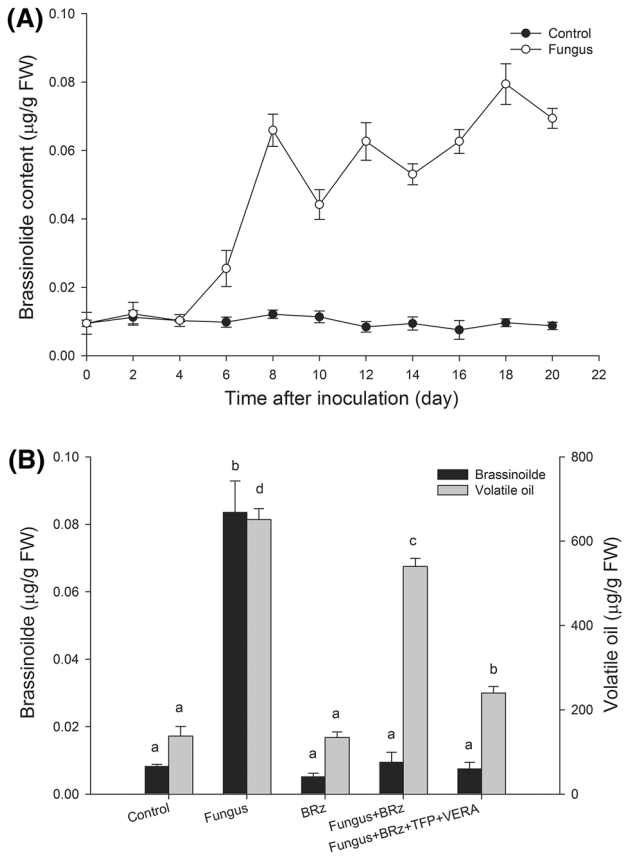

Fig. 2 The influence of brassinolide on endophytic fungus-induced volatile oil accumulation of *A. lancea*. **a** Brassinolide content at 2-day intervals. **b** Effects of brassinazole (BRz) volatile oil accumulation after 18 days. An inhibitor (100 μM BRz) was added 1 day before fungal inoculation. Thirty-day-old plantlets of *Atractylodes lancea* were incubated with 5-mm mycelial disks or PDA disks (control). Values are the mean of three independent experiments. *Bars* with different *lower-case letters* were significantly different (one-way ANOVA, Duncan's multiple range test, $P < 0.05$)

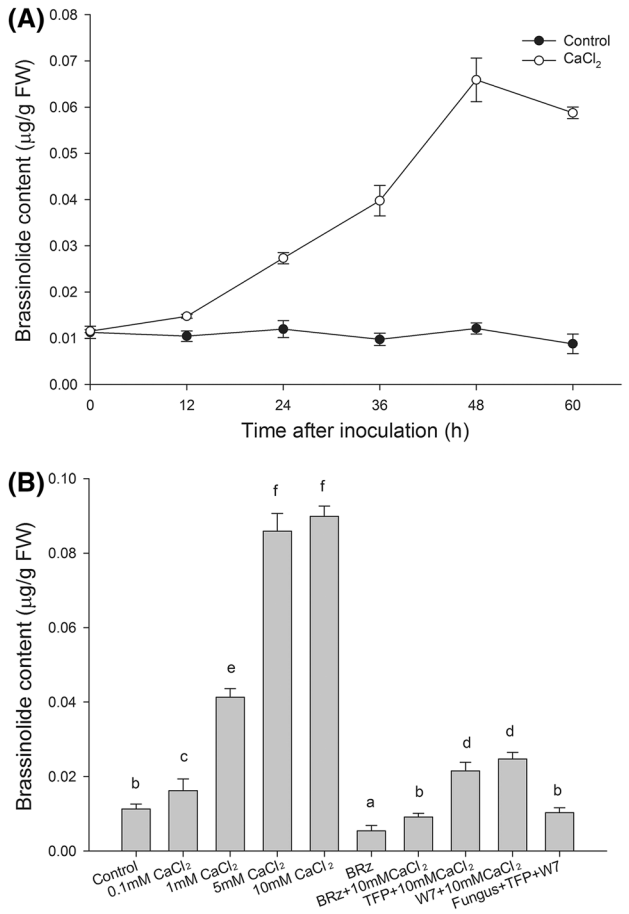

Fig. 3 Brassinolide production in response to $CaCl_2$ treatment in plantlets of *A. lancea*. **a** Time course for $CaCl_2$-induced brassinolide production in *A. lancea*. The plantlets were treated with 10 mM $CaCl_2$ for various times. **b** Dose dependence for $CaCl_2$-induced brassinolide production and effects of pretreatments with CaM antagonists on fungus-induced brassinolide production. Inhibitors (100 μM BRz, 100 μM W_7, 100 μM TFP) were added 1 day before fungal inoculation or $CaCl_2$ treatment. Brassinolide content was detected 18 days after various treatments. Values are the mean of three independent experiments. *Bars* with different *lower-case letters* were significantly different (one-way ANOVA, Duncan's multiple range test, $P < 0.05$)

fungus infection. The maximum concentration of brassinolide in plant tissues induced by the fungus was approximately eight times that in the control. When pretreated with the brassinosteroid biosynthesis-specific inhibitor brassinazole, the volatile oil accumulation induced by fungus was significantly suppressed, to 74.48 % of that in the fungus treatment, whereas brassinolide generation was completely blocked (Fig. 2b). Jana and others (2007) successfully applied the ELISA method to the measurement of BR; the highest cross-reactivity with nontarget BR analogs is 1.3 %. Therefore, the above data indicate that brassinolide may be involved in fungus-induced volatile oil accumulation.

Ca^{2+}–CaM Functions Both Upstream and Downstream of Brassinolide Production Induced by Fungus

Because both Ca^{2+}–CaM and brassinolide signalling may be involved in volatile oil accumulation induced by the endophyte, interactions between Ca^{2+}–CaM and brassinolide were further investigated. When treated with 10 mM $CaCl_2$, brassinolide levels increased steadily in plantlets and peaked at 48 h (Fig. 3a. The $CaCl_2$-induced increase was also dose-dependent in the concentration range of 1–10 mM $CaCl_2$ compared with the control (Fig. 3b). Pretreatments with the CaM antagonists W_7 and TFP substantially reduced the brassinolide accumulation induced by $CaCl_2$ treatment (Fig. 3b). These results clearly indicate that Ca^{2+} can induce increased production of brassinolide in *A. lancea*. To investigate whether brassinolide also affects the levels of Ca^{2+}–CaM, exogenous brassinolide was used. Compared with the control, we observed an increase in the Ca^{2+}-sensitive fluorescence of the protoplasts, which were isolated

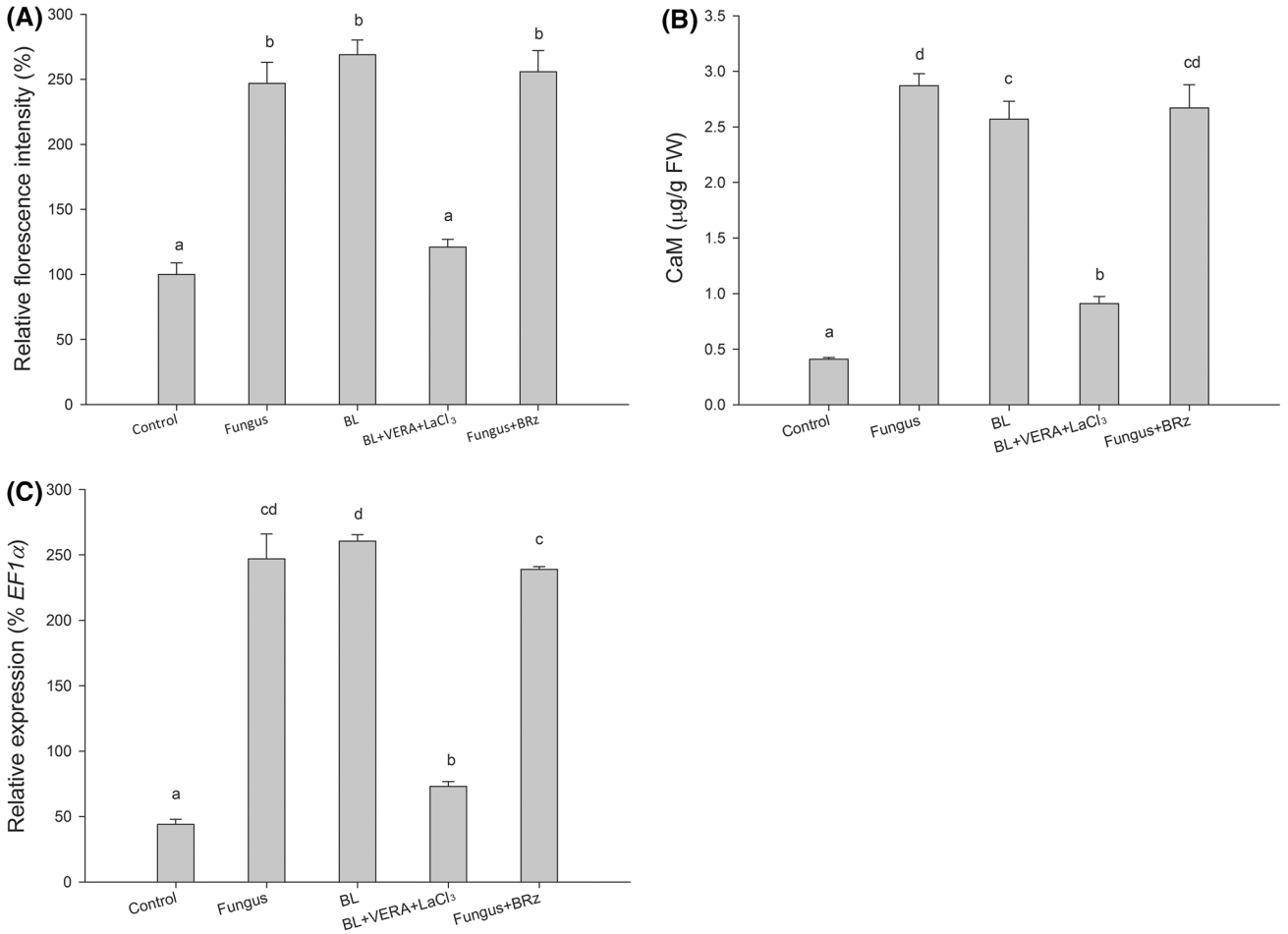

Fig. 4 Effects of pretreatments with brassinolide and brassinolide inhibitor on Ca^{2+}, CaM, and *CaM1* gene expression in *A. lancea* plantlets inoculated with endophytic fungus. **a** Changes in $[Ca^{2+}]_{cyt}$ concentration. The mesophyll protoplasts loaded with Fluo-3/AM were observed by LSCM. **b** Changes in CaM content. **c** Expression levels of *CaM1* genes by real-time PCR analysis. Leaves were used for RNA isolation. Relative expression is based on the expression of *EF1α*. Changes in $[Ca^{2+}]_{cyt}$, CaM, and *CaM1* gene expression were investigated 4 days after various treatments. Values are the mean of three independent experiments. *Bars* with different *lower-case letters* were significantly different (one-way ANOVA, Duncan's multiple range test, $P < 0.05$)

from *A. lancea* leaves, treated with brassinolide, and loaded with Fluo-3/AM, a Ca^{2+}-sensitive fluorescent probe (Zhang and others 1998), and the fluorescence intensity was similar to fungus inoculation (Fig. 4a). In addition, pretreatments with BRz were not able to reduce the increase in fluorescence intensity induced by fungus treatment (Fig. 4a). Changes in CaM content and *CaM1* gene expression under the same conditions are consistent with $[Ca^{2+}]_{cyt}$ (Fig. 4b, c). These results suggest that Ca^{2+}–CaM functions both upstream and downstream of brassinolide production in *A. lancea* plantlets induced by the endophyte.

NO is Involved in Ca^{2+}–CaM Pathway and has no Interaction with the BL Pathway

Previous work has shown that NO is a key upstream messenger in the signalling pathway regulating endophyte-induced volatile oil accumulation in *A. lancea* plantlets (Wang and others 2011; Ren and Dai 2012). To investigate whether NO is involved in the Ca^{2+}–CaM and brassinolide interaction, NO generation was measured in *A. lancea* plantlets pretreated with $CaCl_2$, exogenous brassinolide, Ca^{2+} channel blockers ($LaCl_3$ and verapamil), CaM antagonists (W_7 and TFP), and a brassinolide biosynthesis inhibitor (BRz). NO production was continually enhanced by $CaCl_2$ within 12 days and maintained at a concentration higher than that in the control (Fig. 5a). The Ca^{2+} channel blockers were able to inhibit NO production in inoculated plantlets, but the NO scavenger cPTIO was not able to inhibit Ca^{2+} production in inoculated plantlets (Fig. 5b), showing that NO may act as a downstream signal of Ca^{2+}. In addition, the brassinolide inhibitor BRz was not able to inhibit NO production, and cPTIO was also unable to inhibit brassinolide production with fungus inoculation (Fig. 5c). Although NO levels could be enhanced by exogenous brassinolide treatment, the enhancement was

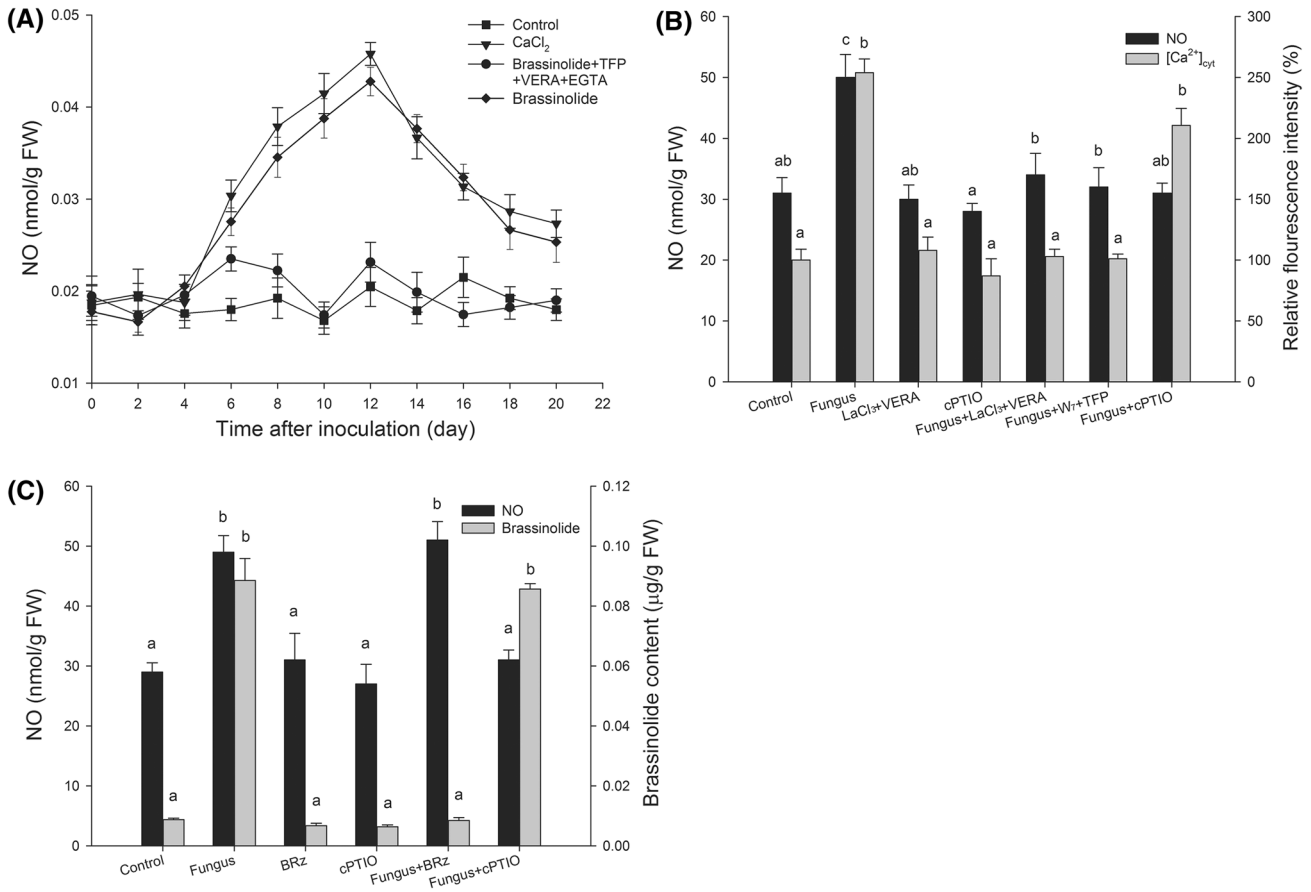

Fig. 5 Interactions between NO and Ca^{2+}–CaM or brassinolide signaling pathways induced by endophytic fungus. Endogenous NO, $[Ca^{2+}]_{cyt}$, and brassinolide were measured at 12, 4 and 18 days, respectively, after different treatments. **a, b** Interactions between NO and $[Ca^{2+}]_{cyt}$ pathways. Inhibitors were 1.25 mM cPTIO, 1 mM $LaCl_3$, and 50 μM verapamil. **c** Interactions between NO and brassinolide pathways. Inhibitor was 1.25 mM cPTIO or 100 μM BRz. All inhibitors were added 1 day before fungus inoculation. Values are means of three independent experiments. *Bars* with different *lower-case letters* were significantly different (one-way ANOVA, Duncan's multiple range test, $P < 0.05$)

blocked by the CaM antagonist TFP combined with the Ca^{2+} channel blockers verapamil and EGTA (Fig. 5a). These results imply that NO may act as a downstream signal of Ca^{2+}–CaM and may not direct regulation on the brassinolide pathway.

Discussion

Secondary metabolite accumulation is a common plant response to biotic or abiotic environmental stresses, and secondary messengers are widely employed to mediate the accumulation of plant secondary metabolites (Reymond and Farmer 1998; John and Jeffery 2000; Hahlbrock and others 2003; Mur and others 2006). Our study demonstrates that the fungus *Gilmaniella* sp. can induce calcium–calmodulin and brassinolide production (Figs. 1a and 2a), and promote the accumulation of volatile oils in host plantlets (Table 1). Acting as important signalling molecules, calcium–calmodulin and brassinolide play important roles in regulating the volatile oil production induced by the fungus. By using relevant scavengers and inhibitors, the links between signalling molecules are blocked and, in turn, the accumulation of related secondary metabolites is altered.

BRs are a class of plant steroid hormone involved in the regulation of growth, development, and various physiological responses (Bajguz 2007). In addition, BRs play important roles in inducing plant tolerance to various abiotic stresses (Kagale and others 2007; Xia and others 2009). Brz is a specific inhibitor for DWF4, a cytochrome P450 monooxygenase of the BR biosynthetic pathway (Asami and others 2001; Bajguz and Asami 2005), and 5-μM Brz treatment can alter the endogenous BR levels (Xia and others 2009). Pretreatment with Brz suppressed the endogenous level of brassinolide, and the degree of volatile oil accumulation induced by endophyte treatment (Fig. 2b). CaM (calmodulin) transduces cytosolic Ca^{2+} changes into cellular responses by changing its conformation in the

Table 1 Accumulation of volatile oils by *Atractylodes lancea* over time

Components	Treatment	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18
Atractylone (mg/g)	Control	3.45 ± 1.4a	4.75 ± 1.68ab	4.9 ± 1.23ab	3.17 ± 0.76a	5.44 ± 1.2ab	3.48 ± 0.96a	4.85 ± 0.41ab	4.02 ± 0.8ab	3.64 ± 1.14ab	5.92 ± 0.91b
	Fungus	3.45 ± 1.64a	4.43 ± 1.48ab	4.6 ± 0.81ab	5.3 ± 0.72ab	4.18 ± 0.63ab	4.11 ± 1.32ab	8.31 ± 1.17c	15.26 ± 1.84d	24.04 ± 1.62e	28.33 ± 1.87f
Hinesol (mg/g)	Control	31.79 ± 4.36a	35.21 ± 2.97a	32.23 ± 3.89ab	38.31 ± 4.95abc	35.37 ± 5.33ab	43.84 ± 5.29bcde	48.89 ± 1.49de	38.03 ± 6.92abc	47.75 ± 2.44de	45.1 ± 1.84cde
	Fungus	31.79 ± 4.36a	38.51 ± 3.18abc	43.01 ± 3.37bcd	43.28 ± 4.6bcde	48.96 ± 3.45de	58.49 ± 5.6fg	63.91 ± 6.05gh	70.5 ± 4.95h	104.3 ± 7.78i	127.3 ± 8.94j
b-Eudesmol (mg/g)	Control	85.52 ± 9.7abcd	82.15 ± 9.81abcd	77.39 ± 9.98a	81.11 ± 7.24ab	88.68 ± 1.92abcd	95.82 ± 6.27def	92.55 ± 4.12bcde	99.84 ± 3.44ef	93.62 ± 4.98cdef	83.72 ± 5.2abcd
	Fungus	85.52 ± 12.18abcd	81.32 ± 9.33ab	83.7 ± 2.73abcd	92.87 ± 5.53cedf	96.35 ± 4.07def	103.35 ± 4.92fg	104.37 ± 6.56fg	113.4 ± 3.4gh	119.51 ± 7.14h	121.1 ± 5.61h
Atractylodin (mg/g)	Control	90.4 ± 11.48a	99.93 ± 14.28ab	112.53 ± 13.37abc	111.86 ± 6.26abc	115.08 ± 5.65bcd	113.28 ± 7.13abcd	110.92 ± 13.44abc	112.54 ± 14.45abc	118.77 ± 11.2bcd	121.81 ± 13.46bcde
	Fungus	90.4 ± 9.35a	99.23 ± 7.51ab	108.76 ± 11.72abc	106.93 ± 15.05abc	115.83 ± 13.03bcd	128.21 ± 17.09cde	144 ± 12.87ef	136.62 ± 15.97def	155.33 ± 14.76f	178.14 ± 12.63g
Total (mg/g)	Control	202.47 ± 38.2a	222.1 ± 35.9ab	229.33 ± 25ab	235.5 ± 24.62abc	249.84 ± 19.27abc	264.93 ± 28.35bc	252.32 ± 24.06bc	251.97 ± 24.23bc	255.3 ± 22.38bc	257.67 ± 26.94bc
	Fungus	202.47 ± 21.92a	224.12 ± 22.81ab	238.91 ± 21.79abc	252.47 ± 24.9bc	265.83 ± 23.63bc	283.29 ± 23.21cd	316.05 ± 21.02de	345.82 ± 21.68e	393.6 ± 30.59f	451.08 ± 31.24g

Thirty-day-old plants were incubated with 5-mm mycelial disks or with an equal-sized potato dextrose agar disk (control). Data are presented as mean ± standard deviation (SDs) of triplicate samples. Within each row, values followed by different lower-case letters were significantly different (one-way ANOVA, Duncan's multiple-range test, $P < 0.05$)

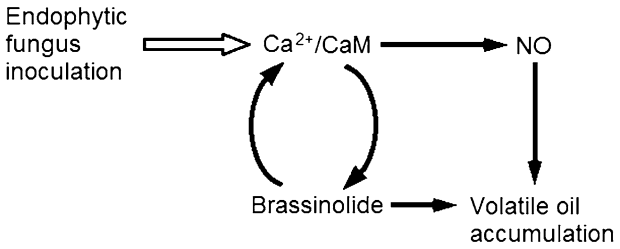

Fig. 6 Proposed cross-talk between signaling pathways for volatile oil accumulation induced by endophytic fungus in *A. lancea* plantlets

presence of Ca^{2+} and concomitantly binding and altering the activities of a series of target proteins (Bouche and others 2005; DeFalco and others 2009). The effects of CaM antagonists such as TFP and W_7 have suggested a role for the Ca^{2+}–CaM system in the responses to hormones (Schroeder and others 2001), light (Frohnmeyer and others 1999), abiotic stresses (Gong and others 1998), and microbial elicitors (Blume and others 2000). Pretreatment with TFP or W_7 suppressed endogenous brassinolide production induced by endophytic fungus inoculation, and 1–10 mM $CaCl_2$ was able to elevate endogenous brassinolide production (Fig. 3b). Plant defense reactions, including enzyme activation and the production of secondary metabolites, are not regulated by individual signalling pathways but by a collaborative regulation between multiple processes (Xu and Dong 2006). Exogenous brassinolide treatment was able to induce $[Ca^{2+}]_{cyt}$ concentration and *CaM1* gene expression and increase CaM levels. However, 100-mM Brz treatment, which was effective in suppressing endogenous brassinolide production, had almost no effect on Ca^{2+} concentration, *CaM1* gene expression, or CaM levels (Fig. 4). Moreover, combining Ca^{2+}–CaM pathway inhibitors with BRz was more effective in suppressing fungus-induced volatile oil accumulation than BRz treatment alone (Fig. 2b). These results indicate that Ca^{2+}–CaM induced by *Gilmaniella* sp. may mediate BL concentration and also functions downstream of BL signalling, resulting in upregulation of volatile oil accumulation in *A. lancea* plantlets.

Our previous work demonstrated that NO acts through SA- and H_2O_2-dependent pathways as an upstream signal in mediating volatile oil accumulation induced by the fungus in *A. lancea* plantlets (Ren and Dai 2012). In the study by Gao and others (2012), NO seems to function as an important signalling molecule of the endophyte *Fusarium* sp. E5 elicitor, which induces isoeuphpekinensin accumulation in *E. pekinensis* suspension cells. We thus used effective inhibitors of NO, Ca^{2+}–CaM, and brassinolide pathways to explore the possible relationships between NO, Ca^{2+}–CaM, and brassinolide. We found that BRz and cPTIO failed to suppress the production of other signalling molecules (Fig. 5c), which could indicate that the two pathways may

not be directly related. The enhancement of brassinolide on NO production was almost completely blocked by Ca^{2+}–CaM inhibitors (TFP+VERA+EGTA) (Fig. 5a), confirming that NO is regulated by Ca^{2+}, independent of the brassinolide pathway. Moreover, the enhancement between Ca^{2+}–CaM and brassinolide may indicate a regulation loop (Fig. 6).

The complex relationships between the diverse signal transduction pathways imply their key roles in regulating the accumulation of volatile oil induced by the fungus. However, it seemed that the stimulating effects on the synthesis of each component of volatile oil by the fungus were not of the same strength. The four detectable components, atractylone, β-eudesmol, hinesol, and atractylodin, were enhanced 3.78-fold, 0.44-fold, 1.82-fold, and 0.75-fold, respectively, in their maximum amount by the endophyte *Gilmaniella* sp. (Table 1). The selective initiation of specific secondary metabolite accumulation may be due to the unique signalling pathway launched by the endophyte or to the diversity of different secondary metabolites.

Volatile oil is the pharmaceutically active ingredient of *A. lancea*, and the low yield of active pharmaceutical ingredients is one of the greatest challenges for medicinal plant culture. Our study showed the potential signal transduction pathways in which the endophytic fungus *Gilmaniella* sp. affects the accumulation of volatile oil in plantlets of *A. lancea*. This information will aid in the understanding of the relationships between fungal endophytes and their host plants.

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