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Ethylene alleviates the suppressive effect of phosphate on arbuscular mycorrhiza formation

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Short Title: Ethylene/Pi regulation of arbuscular mycorrhiza

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Abstract

Phosphorus is one of the most important macronutrients required for plant growth. Plants have evolved many strategies for inorganic phosphorus (Pi) acquisition, including the symbiotic pathways, involving the formation of mycorrhiza. With regard to arbuscular mycorrhiza (AM), high Pi availability has long been known to negatively affect this association, although the underlying mechanism is unknown. In the present study, the interactive role played by ethylene and Pi in AM regulation was investigated in the tomato-*Rhizophagus irregularis* symbiosis. AM colonization was analysed in *epi*, *rin* and NRO ethylene responsive mutants under different Pi availability conditions, with a focus on the late stages of the interaction. Although Pi inhibited mycorrhizal parameters in the ethylene-insensitive *rin* mutant and wild-type cultivars, it did not alter the mycorrhization of the *epi* tomato mutant, which exhibits a constitutive ethylene-induced response. As with the colonization parameters, root ethylene content and the expression of AM-related and ethylene receptor 6 (ETR6) genes were inhibited by Pi in wild-type cultivars and *rin* mutants but were unaffected or slightly activated in *epi* plants. The application of ethephon offset the negative impact on the mycorrhizal development caused by the application of Pi. This compensation effect is dose-dependent and was ineffective in the NRO mutant, which is more insensitive to the action of ethylene. Our results provide evidence that ethylene signalling negatively affects the suppressive effect of Pi on AM formation and suggests an overlap between this suppressive effect and the regulatory mechanism of Pi starvation response pathway in plants mediated by ethylene.

Key words: Tomato; Arbuscular mycorrhiza; Ethylene; Phosphate

Introduction

Among the different types of mycorrhiza, arbuscular mycorrhiza (AM) is the most prevalent symbioses on earth (Smith and Read 2008). This root endosymbiosis is characterized by the formation of specialized structures called arbuscules where bidirectional exchange of nutrients between plant and fungi partners occurs. The fungus develops an extensive network of hypha in the rhizosphere that provides the plant with water and nutrients, mainly Pi, from soil areas that would otherwise be inaccessible to the root (Smith and Read 2008).

Under conditions of elevated exogenous phosphate availability, AM are suppressed as part of a regulatory saving mechanism by the plant host which limits carbohydrate allocation and fungal growth under conditions of optimal Pi supply. Certain mechanisms involved in the negative regulation of AM by Pi, albeit unknown, have been suggested (Nagy and others 2009; Fracetto and others 2013). It is clear that multiple layers of control exist and that the mycorrhizal Pi uptake pathway is controlled, at least partially, by the plant host (Nagy and others 2009; Balzerge and others 2011), with this control overlapping with the regulatory mechanism of the Pi starvation response pathway in plants. In this regard, some studies have examined the role of phytohormones in mediating plant responses to Pi starvation, with special attention being paid to ethylene.

Ethylene is involved in Pi starvation responses in plants, whereby ethylene mediates the inhibition of primary root growth and root hair formation in the adaptive response to Pi deficiency (Borch and others 1999; Zhang and others 2003). Ethylene signalling is an important component involved in enabling plants to adapt more efficiently to low-Pi stress, and certain unknown factors may act as a point of convergence between ethylene and a low-Pi signal in the regulation of plant responses to Pi starvation (Lei and others 2011).

Ethylene also contributes to regulating AM development. However, no specific role has been demonstrated for ethylene during AM symbiosis, although some findings suggest that it is involved in regulating AM. Thus, ethylene-related genes and genes involved in ethylene metabolism are highly up-

regulated in AM roots (Liu and others 2007; Fiorilli and others 2009). Furthermore, it has been observed that exogenous ethylene may either inhibit or stimulate AM formation, depending on concentration levels (Ishii and others 1996; Mukherjee and Ané 2011), while the specific stages of AM development were altered differentially in ethylene plant mutants. In this regard, the *epi* tomato mutant, which constitutively exhibits an ethylene-induced response (Barry and others 2001), and *rin* (ripening inhibitor), a mutant fruit that is unable to produce high levels of ethylene or to ripen in response to exogenous ethylene (Vrebalov and others 2002), displayed inhibition or promotion, respectively, of mycorrhiza parameters in the late stages of AM development (Torres de Los Santos and others 2011).

In this study, given that AM formation is regulated by Pi and ethylene and that ethylene is involved in Pi starvation responses, we explore the putative interactive role played by ethylene and Pi in AM regulation. We used the previously characterized *epi*, *rin* and NRO mutants to carry out an extensive comparative study of AM development under different conditions of Pi availability and ethylene signalling alterations, with a particular focus on the late stages of the interaction. This has enabled us to correlate the alterations in both AM colonization parameters and transcripts of AM marker genes with Pi concentration levels in soil and to assess the interference of ethylene in this relationship. Our results provide evidence that ethylene signalling negatively affects the suppressive impact of Pi on AM formation.

Material and Methods

Plant material, AM inoculation and chemical treatments

Solanum lycopersicum L. (Mill.) Ailsa Craig, VFN8 and Floradade wild-type cultivars as well as *rin*, *epi* and NRO mutants were grown in a growth chamber (day/night cycle: 16 h, 24° C/8 h, 19° C; relative humidity: 50 %). Inoculation with *Rhizophagus irregularis* (DAOM 197198) and growth conditions were previously described (Martín-Rodríguez and others 2011). One week after planting in pots, and weekly thereafter, 20 mL of a modified Long Ashton nutrient solution containing 15 ppm of the P concentration was added in soil (Hewitt 1966). After twenty days of growth, the plants were treated once with 100 ppm P and harvested after fifteen days of Pi applications. The root system was washed and rinsed several times with

sterilized distilled water. The root system was weighed and used for the different measurements according to the nature of the experiments. In each experiment, five independent plants were analysed per treatment.

For ethylene treatments, tomato plants were treated in soil with ethephon (Sigma). The solutions were prepared by dilution from a stock. 20 mL of the corresponding diluted solution was applied to each 200 mL pot containing one tomato plant.

Estimation of mycorrhizal root colonization

A trypan blue staining procedure and microscopic observation of stained roots were carried out as described in Martín Rodríguez and others (2011). The parameters measured were the percentage of mycorrhizal root length colonization, colonization intensity (M %) and arbuscular abundance (A %) in mycorrhizal plant roots. Three microscope slides were analysed per plant root system, and each slide contained thirty 1-cm root pieces.

RNA isolation and gene expression analysis by Real-Time Quantitative PCR

For the quantitative reverse transcription PCR (qRT-PCR) experiments, total RNA was isolated from a 0.5 g sample taken from the root, which was a representative part of the root system for each plant and was treated as a biological replicate. Total RNA was isolated from the roots stored at -80°C using the RNeasy Plant Mini Kit (Qiagen, MD, USA) and cDNAs were obtained from 1 μg of total DNase-treated RNA in a 20 μL reaction volume using the iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). qRT-PCR was carried out using an iCycler apparatus (Bio-Rad) according to the method described in Martín-Rodríguez and others (2011). The experiments were carried out on three biological replicates, and the threshold cycle (Ct) was determined in triplicate. The Ct values of all genes were normalized to the Ct value of the EF α -1 housekeeping gene from tomato. All primer names and corresponding sequences of the genes analysed were listed previously (Martín-Rodríguez and others 2011).

Ethylene quantification

Ethylene content in roots was measured in a gas chromatograph (Hewlett Packard 5890) as previously described (Martín-Rodríguez and others 2011).

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA). The mean values of five biological replication samples for mycorrhization parameters and three replications for qRT-PCR experiments were compared using Duncan's multiple range test ($P=0.05$).

Results and discussion

To examine a possible interaction of ethylene and Pi in AM regulation, we determined different mycorrhizal parameters and transcript accumulation rates of AM and ethylene-responsive genes in an experiment involving the mycorrhization of *rin* and *epi* mutants which are insensitive and more sensitive to ethylene, respectively. To eliminate pre-symbiotic effects of high Pi level on the early stages of AM formation, twenty days old colonized plants growing with 15 ppm Pi were treated once with 100 ppm and harvested after fifteen days of Pi applications.

Under control conditions, *rin* plants were positively affected with respect to all the mycorrhizal colonization parameters measured in relation to their isogenic Ailsa Craig line (Fig.1A). Nevertheless, as it has been already published (Torres de Los Santos and others 2011), the *epi* plants recorded a significant reduction in arbuscular abundance (%A) and mycorrhizal intensity (%M) with regard to the VFN8 cultivar (Fig. 1B). The application of 100 ppm Pi caused a similar reduction (approximately 50%) in these parameters in wild-type cultivars Ailsa Craig and VFN8 as well as in *rin* mutant plants. However, *epi* plants did not show any reduction in their mycorrhizal parameters due to the application of Pi (Fig. 1B). As for mycorrhization, the application of Pi caused a decline in ethylene levels in the roots of Ailsa Craig and VFN8 cultivars as well as *rin* mutant plants, which was, however, insignificant in *epi* mutant roots (Fig. 1A, B). In this regard, phosphorus-deficient roots have been shown to produce twice as much ethylene as

phosphorus-sufficient bean roots (Borch and others 1999), while phosphate deficiency in maize plants increased the sensitivity of roots to ethylene (He and others 1992).

The expression of AM markers genes *GinEF* and *LePT4* and the *LeETR6* ethylene receptor gene, used as molecular marker of ethylene pathway activation, was highly down-regulated by the application of Pi in wild-type plants and *rin* mutants, thus bearing out histochemical assessments (Fig. 2). An opposite trend can be observed for these genes under high Pi conditions in the *epi* mutant, in which expression levels slight increased when Pi was applied (Fig. 2).

The data clearly show a Pi/ethylene interaction during AM depending on plant ethylene responsiveness. The negative regulation of mycorrhization by Pi is accompanied by a down-regulation of the signalling pathway of ethylene. *Epi* mutation leads to a constitutive ethylene response, meaning that the down-regulation of the ethylene pathway by Pi fails and that negative regulation of mycorrhization was ineffective. This suggests that ethylene, which is an important component of the Pi starvation response in plants (Lei and others 2011), also interferes in the suppressive effect of Pi on AM formation.

To corroborate the antagonistic Pi/ethylene interaction regulating AM, we conducted an experiment to determine whether ethylene pathway activation interferes with the negative effect of Pi applications on mycorrhization. We studied the impact of ethephon applications on wild-type tomato cultivars and NRO tomato mutants under conditions of higher Pi levels. NRO plants overexpress the gene encoding the *LeETR3* ethylene receptor (Ciardi and others 2000), thus demonstrating their insensitivity to the action of ethylene. The plants were inoculated with *R. irregularis* and supplemented on a weekly basis with a Long Ashton nutrient solution containing 15 ppm of Pi. After twenty days of colonization, the plants were treated once with 100 ppm Pi supplemented, or not, with different ethephon doses and harvested fifteen days later.

Floradade wild-type cultivar and NRO mutants showed similar mycorrhization levels under control conditions (Fig. 3). Equally, the application of 100 ppm Pi led to a reduction in wild-type and NRO plants with respect to all the mycorrhization parameters determined. Nevertheless, NRO showed less affectation

than Floradade (65% reduction in wild-type cultivars and a reduction of 50% in NRO), suggesting again an interactive role played by ethylene and Pi in AM regulation. When ethephon was applied in combination with 100 ppm Pi, a dose-dependent response was observed in Floradade line. Thus, Floradade plants treated with Pi and ethephon were found to be less affected in relation to the mycorrhization parameters assessed. The level of interference in the negative effect of Pi was higher at a dose of 40 μ M, and doses of 5 μ M and 70 μ M also triggered recovery, albeit to a lesser extent. NRO plants, with reduced ethylene perception and thus lower sensitivity to ethylene, were unaffected in their response to Pi by ethephon applications with respect to the percentage of root length colonization, arbuscular abundance and mycorrhizal intensity (Fig. 3).

Our results show that ethylene negatively interferes with the suppressive effect of Pi on AM formation and that interference is dose-dependent. In the NRO mutant, which is more insensitive to the action of ethylene, the application of Pi reduced mycorrhization in all cases, while the addition of ethephon did not alter the inhibitory effect of phosphorus; moreover, the application of Pi with different combinations of ethephon resulted in a sharper decline than Pi applied on its own. This confirms the hypothesis of ethylene interference in the negative regulation of mycorrhization due to the application of Pi.

In conclusion, in this study, we show that ethylene signalling negatively interferes with the suppressive effect of Pi on AM. Since ethylene is involved in the Pi starvation response in plants, our results suggest overlaps between the suppressive effect of Pi on AM and the regulatory mechanism of the Pi starvation response pathway in plants mediated by ethylene. Although no specific role has been demonstrated for ethylene during AM symbiosis, both positive and negative roles have been proposed. In our study, we show that ethylene plays a positive role in alleviating the suppressive effect of Pi.

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Figure legends

Fig. 1. Mycorrhization parameters and ethylene content in roots. Ailsa Craig wild-type and *rin* mutant plants (A) or VFN8 wild-type and *epi* mutant plants (B) were inoculated with *R. irregularis* and treated weekly with 15 ppm of Pi. After twenty days of colonization, the plants were treated once with 15 ppm or 100 ppm Pi and harvested fifteen days later. Values correspond to means $\pm$ SE of five independent biological replicates and bars with similar letters do not significantly differ ($p = 0.05$) according to Duncan's multiple range test.

Fig. 2. Expression analysis of *GinEF* (A), *LePT4* (B) or *LeETR6* (C) genes. Ailsa Craig wild-type and *rin* mutant plants or VFN8 wild-type and *epi* mutant plants were inoculated with *R. irregularis* and treated weekly with 15 ppm of Pi. After twenty days of colonization, the plants were treated once with 15 ppm or 100 ppm Pi and harvested fifteen days later. Values represent mean values $\pm$ SE of three independent biological replicates and bars with similar letters do not significantly differ ($p = 0.05$) according to Duncan's multiple range test.

Fig. 3. Mycorrhization parameters in the roots of Floradade wild-type and NRO mutant plants. Plants were inoculated with *R. irregularis* and treated weekly with 15 ppm of Pi. After twenty days of colonization, the plants were treated once with 15 ppm Pi or 100 ppm Pi alone and in combination with 5, 40 or 70 μ M of ethephon and harvested fifteen days later. Values correspond to means $\pm$ SE of five independent biological replicates and bars with similar letters do not significantly differ ($p = 0.05$) according to Duncan's multiple range test.

Figure 1

A

B

Figure 2

A

B

C

Figure 3