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Colonization by the endophyte *Piriformospora indica* leads to early flowering in *Arabidopsis thaliana* likely by triggering gibberellin biosynthesis

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A B S T R A C T

Piriformospora indica is an endophytic fungus colonizing roots of a wide variety of plants. Previous studies showed that *P. indica* promotes early flowering and plant growth in the medicinal plant *Coleus forskohlii*. To determine the impact of *P. indica* on flowering time in *Arabidopsis*, we co-cultivated the plants with *P. indica* under long day condition. *P. indica* inoculated *Arabidopsis* plants displayed significant early flowering phenotype. qRT-PCR analysis of colonized plants revealed an up-regulation of flowering regulatory (*FLOWERING LOCUS T*, *LEAFY*, and *APETALA1*) and gibberellin biosynthetic (*Gibberellin 20-Oxidase2*, *Gibberellin 3-Oxidase1* and *Gibberellin requiring1*) genes, while the flowering-repressing gene *FLOWERING LOCUS C* was down regulated. Quantification of gibberellins content showed that the colonization with *P. indica* caused an increase in GA₄ content. Compared to wild-type plants, inoculation of the *Arabidopsis* *ga5* mutant affected in gibberellin biosynthetic gene led to less pronounced changes in the expression of genes regulating flowering and to a lower increase in GA₄ content. Taken together, our data indicate that *P. indica* promotes early flowering in *Arabidopsis* likely by increasing gibberellin content.

1. Introduction

Flowering is a key event in plant life cycle. Hence, the ability to adjust the timing of flowering under different environmental conditions is crucial for successful reproduction in plants [1]. The transition from vegetative growth to flowering is determined not only by environmental cues but also by endogenous signals, which interact when the time of the year and/or the growth conditions are favorable for sexual reproduction and seed maturation [2,3]. Genetic studies in *Arabidopsis* have suggested that flowering time is controlled by four major, interdependent genetic pathways:

photoperiod, vernalization, autonomous, and gibberellin (GA) pathways [3–5].

Photoperiod promotes flowering by integrating inputs through the circadian clock and light receptors, and represents the major environmental signal regulating flowering [6,7]. Under long-day (LD) conditions, genes that act in the photoperiod pathway play a major role in controlling flowering. In some plant species, vernalization, a prolonged exposure to low temperature, is required for floral induction [8]. In *Arabidopsis*, the major effect of vernalization is the down-regulation of the expression of the flowering repressor *FLOWERING LOCUS C (FLC)* [9]. Flowering is also promoted by the autonomous pathway, which involves genes encoding components of RNA processing or histone modification complexes [4]. The GA-signaling pathway requires genes for GA biosynthesis, such as *gibberellin requiring1 (GA1)*, and genes involved in GA-signaling, such as *gibberellic acid insensitive (GAI)* to promote flowering [10,11].

GAs are a class of phytohormones, which plays a pivotal role in controlling flowering in several plant species. In *Arabidopsis*, severe reduction of endogenous GAs has been shown to delay

flowering in long days and prevent flowering in short days, as shown with different GA biosynthesis and signaling mutants [12]. For example, the *ga1-3* mutant, in which GA levels are severely reduced, is unable to flower in short days [13]. The reduction of GA levels in this mutant is caused by a deletion in the gene encoding *ent-copalyl diphosphate synthase*, which controls a key step in GA biosynthesis [14,15].

Piriformospora indica is an endopytic fungus of the *Sebacinaceae* family, which colonizes the roots of many plant species including *Arabidopsis* [16,17]. *P. indica* acts as a bioprotector against pathogens [18], alters plant secondary metabolites, increases nutrient uptake and promotes plant growth [19,20], confers drought tolerance to *Arabidopsis* and barley [21,22], and alleviates salt stress in barley and rice by increasing the activity of detoxifying enzymes and the content of photosynthetic pigments in colonized plants [16,23]. Recently, *P. indica* has been shown to promote inflorescence development leading to early flowering in the medicinal plant *Coleus forskohilli* [18]. However, the mechanism by which *P. indica* influences flowering time is unknown.

In this study, we examined the impact of *P. indica* colonization on flowering in *Arabidopsis* and investigated the role of GA in this process. Our data show that *P. indica* colonization leads to early flowering in *Arabidopsis*, likely due to an enhancement of the endogenous GA production.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana Columbia ecotype (Col-0) was used for the flowering experiment in soil and *Landsberg erecta* (Ler) ecotype was

used for *ga5* and *ft-1* mutants flowering experiment. *ga5* (*ga20ox1*) and *ft-1* mutants were obtained from European Arabidopsis Stock Centre (<http://www.arabidopsis.info>). Seeds on agar plates were cold-treated at 4 °C for 3 days in the dark and then transferred to growth chamber at 22 °C with a 16-h-light and 8-h-dark photoperiod (long day condition). After 7 days, seedlings were transferred to soil and co-cultivated with *P. indica* [24].

2.2. *P. indica* growth condition and co-cultivation with *Arabidopsis*

P. indica was propagated on KM medium at 28 °C in the dark for 2 weeks [25]. For liquid culture, 1000 ml Erlenmeyer flasks were filled with 250 ml liquid KM medium and supplied by 5 fungal plugs and incubated for 10 days at 28 °C at 150 rpm on a rotary shaker. For soil experiment, *P. indica* was cultured in KM liquid medium for 10 days as described by Johnson et al. (2011) [24]. Seven-day-old *Arabidopsis* seedlings were transferred to soil and allowed to adapt for 3 days. After 3 days, 3 ml of *P. indica* suspension were injected into the soil containing the 10-day-old *Arabidopsis* seedlings. The suspension was applied two more times at 3 day interval. For the plate experiment, 7 days old germinated seedlings were placed on modified PNM medium. Two weeks old *P. indica* plugs were placed 1–1.5 cm away from each seedling root [24].

2.3. Measurement of flowering time

To measure the flowering time, *Arabidopsis* seedlings were grown in the soil under long day condition (16 h light - 8 h dark), 22 °C and inoculated with *P. indica*. Flowering time was measured by counting the number of rosette and cauline leaves at the first flower blooming [26]. The experiment was repeated three times

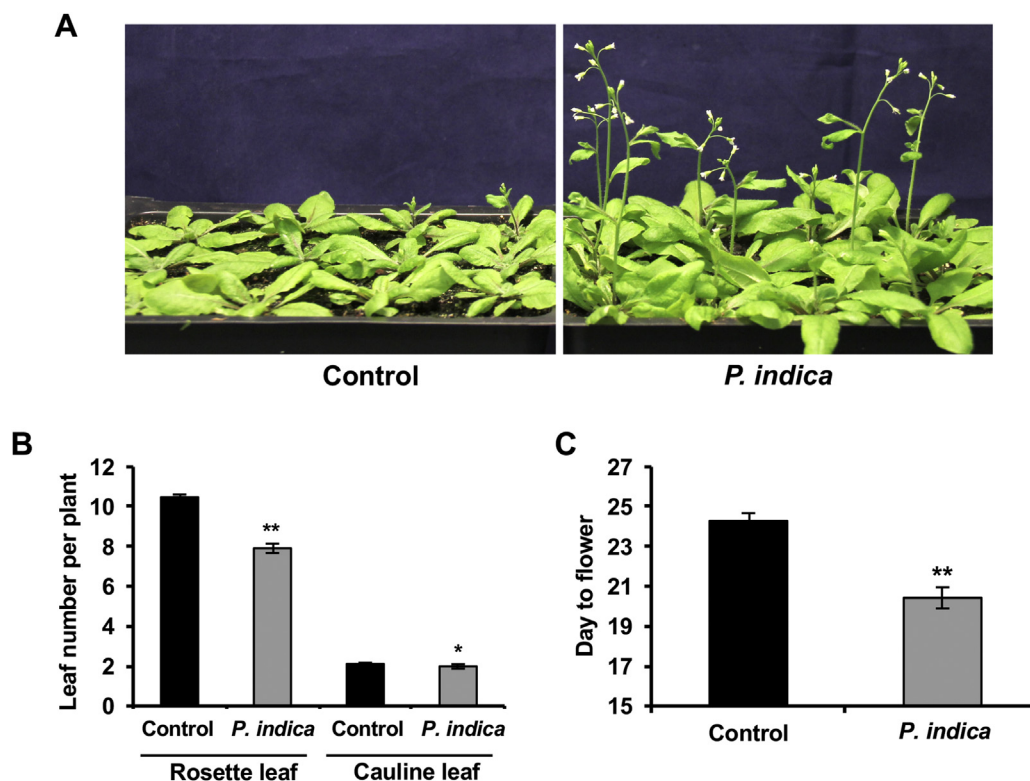


Fig. 1. Represents *Arabidopsis thaliana* (Col-0) under long day condition, 3 weeks after co-cultivation with *P. indica*. (A) Flowering phenotype of *P. indica* colonized and non-colonized *Arabidopsis*. Flowering time of *P. indica* colonized *Arabidopsis* plants compared with non-colonized ones. (B) Time to flowering was determined as the total number of Rosette leaves and cauline leaves in the primary shoot. (C) Mean of flowering day of *P. indica* colonized *Arabidopsis* and compared with non-colonized plants. Data are means $\pm$ SE ($n \geq 100$). Significant differences as determined by Student's *t*-test (** $P < 0.01$, * $P < 0.05$).

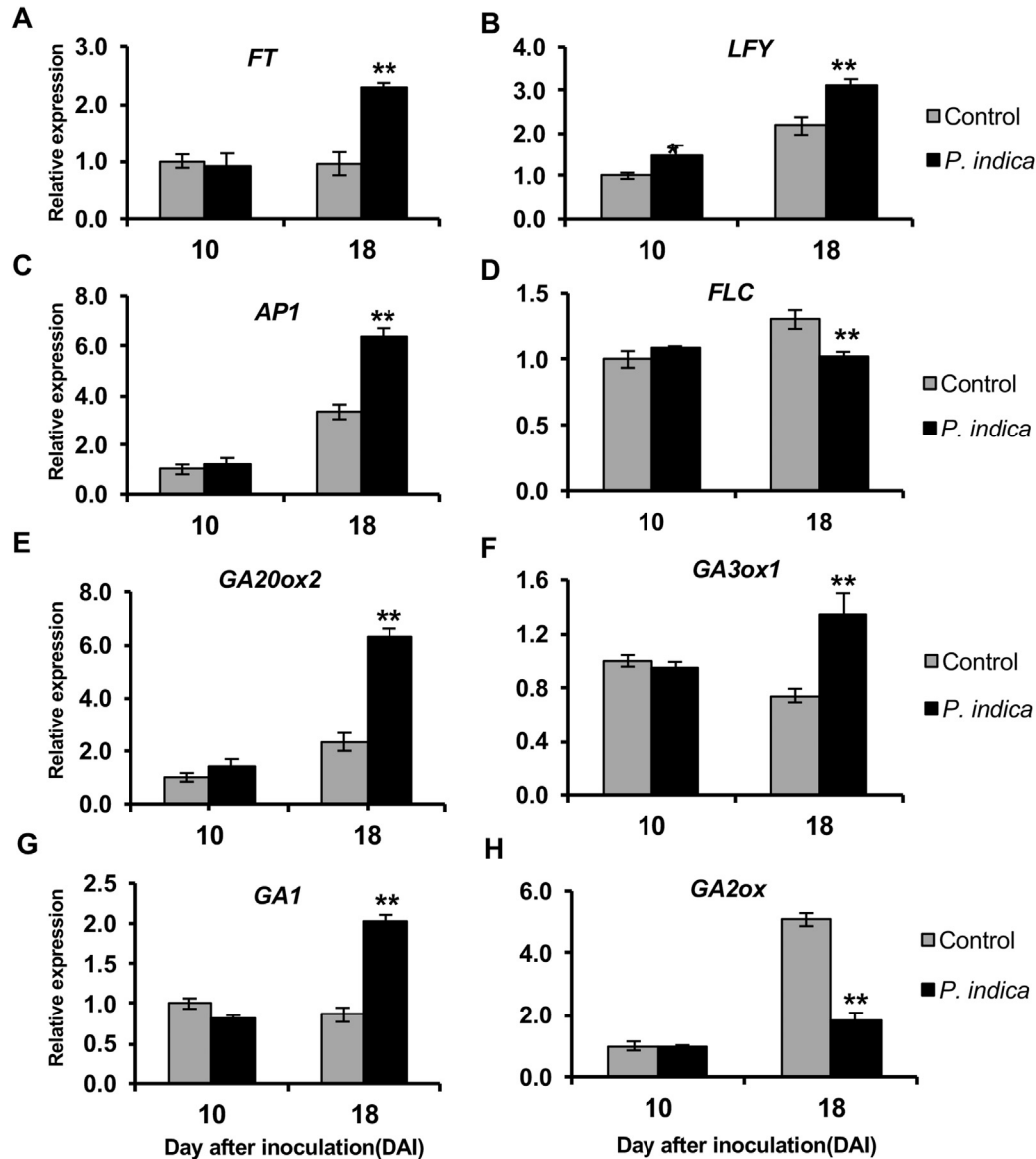


Fig. 2. *P. indica* regulates the expression of flowering-regulatory and gibberellin (GA) biosynthesis genes. Total RNA was extracted from *P. indica* inoculated plants and non-inoculated plants at the indicated time points. Gene expression was analyzed by qRT-PCR. The constitutively expressed *CACS* gene was used for normalization. Expression of the (A) *FT*, (B) *LFY*, (C) *AP1* and (D) *FLC*, (E) *GA20ox2*, (F) *GA3ox1*, (G) *GA1* and (H) *GA2ox* gene in *P. indica* inoculated plants and non-inoculated plants. Data are mean fold changes $\pm$ SE of results from three independent experiments each containing three repeats. Asterisks indicate significance at **P < 0.01 and * P < 0.05 by student t-test.

and data obtained from 100 plants were statistically analyzed.

2.4. RNA preparation and analysis of relative gene expression by qRT-PCR

Total RNA was extracted using 50 mg of colonized and non-colonized *Arabidopsis* plants with a Qiagen Plant RNeasy Kit. One microgram of total RNA was used for cDNA synthesis with Super Script III reverse transcriptase (Invitrogen, Paisley, UK, Cat. No#18080-051) according to the manufacturer's protocol. Quantitative Real-time PCR was performed using Step One Plus Bio-Rad Thermocycler (Bio-Rad; www.biorad.com). For amplification, Absolute SYBR Green Master Mix (Applied biosystems; www.lifetechnologies.com) was used to quantify the expression of the genes. The $2^{-\Delta Ct}$ methods were used to calculate the relative gene expression [27]. qRT-PCR primers are listed in Table S1.

2.5. Quantification of plant hormones

Aliquots (about 500 mg fresh weight) of frozen material were extracted with 80% methanol, 1% acetic acid, and the extracts were passed consecutively through hydrophilic-lipophilic balance, mixed-mode cation exchange, and mixed-mode anion exchange columns (Oasis 30 mg, Waters) to purify the GA₄. The final residue was dissolved in aqueous 5% (v/v) acetonitrile, 1% (v/v) acetic acid. Analysis of plant hormones were performed by comparing retention time and mass transitions with the standards using an Agilent 1200 HPLC coupled with an AB SCIEX Q-TRAP 5500 MS with an electrospray source. Chromatographic separation was carried out on a Phenomenex Gemini C18 (150 $\times$ 2.0 mm, 5 μ m) column, at 35 $^{\circ}$ C. The mobile phase A and B was 0.1% formic acid, 95% ACN, 5% water and 0.1% formic acid, 95% water, 5% ACN, respectively. The gradient used was 0–20 min, 0% – 100% A; 20–25 min, 100% A; 25–26 min, 100% – 0% A; 26 – 36 min, 0% A. The flow rate was 200 μ L/

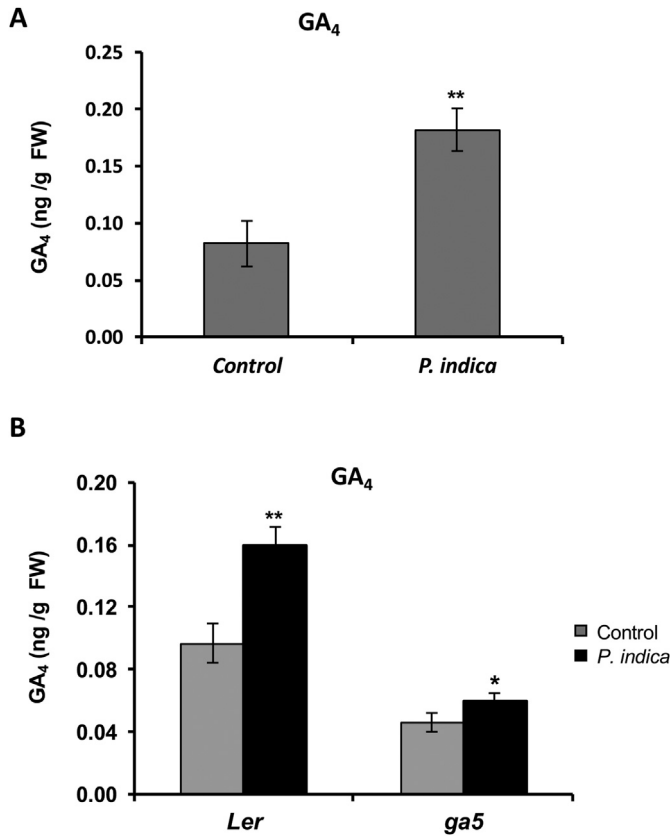


Fig. 3. Endogenous GA₄ levels in *P. indica* colonized and non-colonized plants. (A) Levels of endogenous GA₄ in shoots of Arabidopsis Col-0 wild-type at 18 DAI grown in soil. (B) Levels of endogenous GA₄ in *Ler* wild type and *ga5* (*ga20ox1*) plants. Taken sample from the 12-day old plants grown on modified PNM media co-cultivated with or without *P. indica*. The quantities were calculated on the basis of peak area ratio with the standard GA₄ using LC/MS. Each value is the mean $\pm$ SE of three replicates. Asterisks indicate significance at ***P* < 0.01 and * *P* < 0.05 by student *t*-test.

min. Analysis of the plant hormones was based on appropriate Multiple Reaction Monitoring (MRM) of ion pairs for labeled and endogenous GA₄ using the following mass transitions: ²H₂GA₄ 333 > 215, GA₄ 331 > 213. The MS was operated in negative ionization mode for the GA₄. The conditions were as follows: Temperature 500 °C, Ion source gas1 50 psi, Ion source gas2 60 psi, Ion Spray Voltage -4500 V, curtain gas 40 psi, Collision Gas Medium; DP (-25 V), EP (-9) and CXP (-2) were held constant for all transitions. Collision energies (CE) and dwell times (DT) were specific for each compound/internal standard pair, the parameters used were GA₄ CE (-50), DT (250). Data were acquired and analyzed using Analyst 1.4 software (Applied Biosystems).

3. Result

3.1. Flowering time and root colonization

To examine the effect of *P. indica* on flowering time in Arabidopsis, one-week old seedlings were transferred to soil and co-cultivated with fresh mycelium of *P. indica*. *P. indica* colonized Arabidopsis plants displayed an early flowering phenotype, compared to non-colonized plants (Fig. 1A). On average, colonized plants flowered 4 day earlier, and had significantly fewer rosette and cauline leaves than non-colonized plants (Fig. 1B and C). The number of the inflorescence and length of the main shoot were also higher in the colonized plants than the non-colonized plants

(Fig. 1A). To visualize fungal structures in plant root, segments of 7 day-old colonized Arabidopsis roots were stained with WGA-AF 488 and florescence image was recorded with confocal microscope. Florescent images showed spread of hyphae in the roots of colonized plants (Supplementary Fig. 1A). Meanwhile, we analyzed the development of fungal colonization over time by PCR using the marker gene *Pi ITS* at 10, 15 and 21 days after inoculation (DAI). Clear signals were detected in the colonized roots, with an increase in the intensity from day 10–21 days after inoculation (Supplementary Fig. S1B), while no PCR-products were detected in non-colonized plants. These results indicate that *P. indica* promotes flowering after inoculation of Arabidopsis plants.

3.2. *P. indica* controls the expression of flowering-regulatory genes

To shed light on the factors leading to the early flowering phenotype in *P. indica* colonized plants, we checked the expression of flowering-regulatory genes *FLOWERING LOCUS T* (*FT*), *LEAFY* (*LFY*), *APETALA1* (*AP1*) and *FLC* at 10 and 18 DAI (Fig. 2A–D). *FT*, *LFY* and *AP1* are flowering activators while *FLC* is a flowering repressor [2,3,7]. At 10 DAI, there was no obvious difference between colonized and control plants in the transcript levels of *FT*, *AP1* and *FLC*, except elevated expression of *LFY*. Eight days later, there was a significant increase in the expression of flowering activators *FT* (2.3 fold), *LFY* (1.0 fold) and *AP1* (3.0 fold), and a simultaneous reduction in the expression of *FLC* in colonized plants compared to non-colonized plants (Fig. 2A–D). These results are in line with the flowering time measurements in which colonized plants started flowering 20 DAI and suggests that *P. indica* is a positive regulator of *FT*, *LFY* and *AP1* expression, while is a negative regulator of *FLC*.

To determine whether *P. indica* promotes early flowering by triggering abiotic stress response, we checked the expression levels of three stress responsive genes, *WRKY family transcription factor 22* (*WRKY22*), *Ethylene response factor 1* (*ERF1*) and *Salicylic acid induction deficient 2* (*SID2*) at 5 and 7 days after inoculation (Supplementary Fig. S2). Compared to the control plants, *P. indica* colonization significantly induced the expression of these genes 5 days after inoculation. However, two days later, transcript levels of these genes in colonized plants were similar to those in the control. These results suggest that the early flowering phenotype in colonized Arabidopsis was not just the result of stress response.

3.3. *P. indica* modulates the expression of GA biosynthesis genes

GAs are involved in flower initiation in plants and have specifically been shown to promote flowering in Arabidopsis. Therefore, we checked the effect of *P. indica* on the expression of the GA biosynthesis genes *GA20ox2*, *GA3ox1* and *GA1* (positive regulators) and *GA2ox* (a negative regulator) at 10 and 18 DAI. Consistent with the expression of flowering regulatory genes, there were no differences between the colonized and non-colonized plants in the expression of these genes at 10 DAI (Fig. 2E–H). However, at 18 DAI, there was a four-fold decrease of *GA2ox* expression whereas the expression of *GA20ox2*, *GA3ox1* and *GA1* increased in colonized plants compared to non-colonized plants (Fig. 2E–H). These results suggest that GA biosynthesis is promoted by *P. indica* colonization and that *P. indica* is a positive regulator of *GA20ox2*, *GA3ox1* and *GA1* expression, but a negative regulator of *GA2ox* expression. In general, these results along with the expression of flowering regulatory genes suggest that *P. indica* probably regulates early flowering by promoting GA biosynthesis in Arabidopsis.

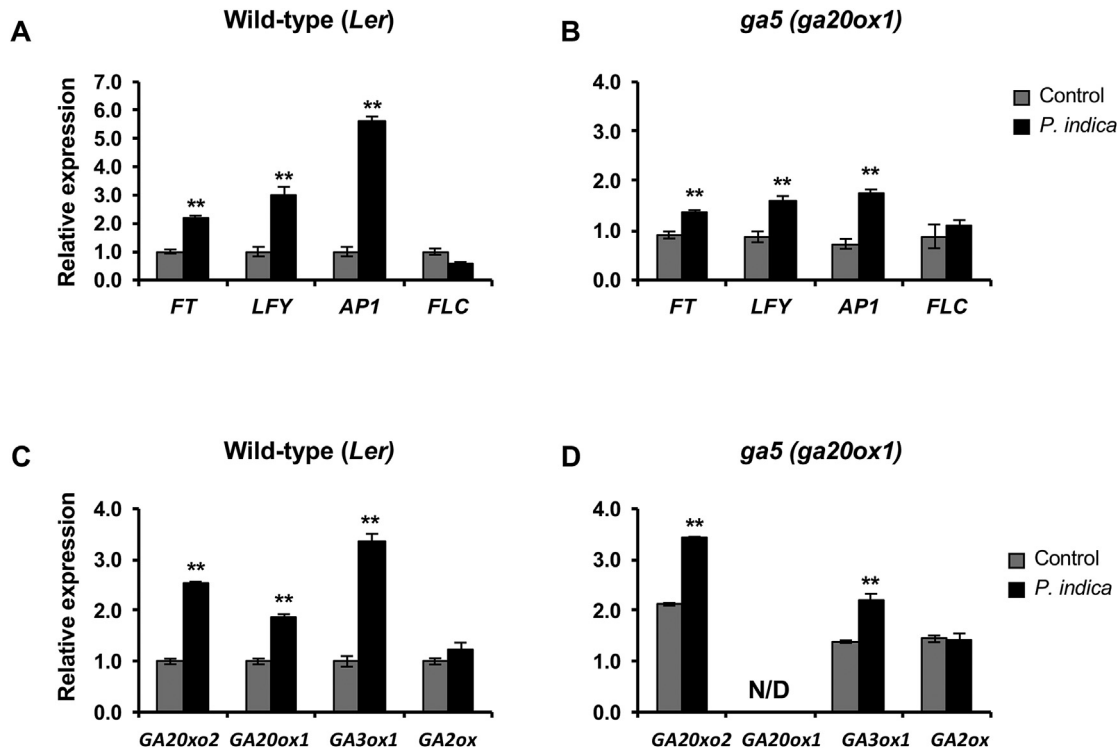


Fig. 4. The expression of flowering-regulatory genes and GA biosynthetic genes in Ler wild-type and *ga5 (ga20ox1)* plants in colonized and non-colonized *P. indica* plants. Relative mRNA levels were determined using qRT-PCR with RNA extraction from the 12-day old plants grown on modified PNM media co-cultivated with or without *P. indica*. Relative gene expression level in (A, C) wild-type (Ler) and (B, D) *ga5 (ga20ox1)* mutant. Data are mean fold changes $\pm$ SE of results from three independent experiments each containing three repeats. Asterisks indicate significance at $^{***}P < 0.01$ by student *t*-test. N/D is non-detected.

3.4. Analysis of endogenous GA₄ levels in Arabidopsis Col-0 wild-type and *ga5* mutant

To check whether changes in the expression levels of GA biosynthetic genes are mirrored by an increase in the level of this hormone, we measured the content of GA₄ HPLC/MS. The results showed that GA₄ content significantly increased in *P. indica* colonized plants compared to non-colonized control (Fig. 3A), which is consistent with the expression pattern of the GA biosynthetic genes.

To further explore the role of GAs in *P. indica* induced early flowering, we measured the transcript levels of flowering regulatory and GA biosynthetic genes in *ga5 (ga20ox1)* mutant. *GA20ox1* is a key GA biosynthetic gene, and deficiency of *GA20ox1* results in obvious decrease of GA content [28]. As shown in Fig. 4, transcript levels of the flowering-regulatory *FT*, *LFY*, *AP1*, and the GAs biosynthetic genes *GA20ox2*, *GA20ox1*, *GA3* were less induced in *ga5* mutant upon colonization, compared to wild type (Fig. 4). Consistently, the content of GA₄ in *ga5* mutant was also less increased compared to wild type after *P. indica* colonization (Fig. 3B). These results suggest *P. indica* promotes early flowering mainly through up-regulation of GA biosynthesis genes and GA₄ content in Arabidopsis.

4. Discussion

To check if *P. indica* influences flowering time, we co-cultivated Arabidopsis plants with the fungus under long-day conditions. We observed that *P. indica* colonization resulted in early flowering and an increase the transcript levels of flowering regulatory and GA biosynthetic genes as well as in the GA₄ content.

In Arabidopsis, flowering time is regulated by four pathways, the photoperiod pathway, which induces flowering in response to seasonal changes in day length; the GA pathway, which promotes flowering in non-inductive conditions; the vernalization pathway, which stimulates flowering after prolonged exposure to low temperatures; and the autonomous pathway that constitutively repress flowering [29]. In this work, *P. indica* colonized plants flowered earlier than wild-type plants (Fig. 1) and this correlates under long-day conditions with reduced expression of *FLC*, a repressor of flowering, and increased expression of the floral integrators such as *FT*, *LFY* and *AP1*, suggesting that *P. indica* is involved in flowering initiation.

GAs have been reported to promote flower production. GA 20-oxidase activity is the major determinant of GA production [28], and GA₄ is the GA responsible for promoting flower initiation in Arabidopsis [30]. In this study, qRT-PCR data analysis indicates that *P. indica* caused increased expression of *GA20ox2*, which was accompanied by elevated levels of endogenous GA₄ in colonized plants. These data suggest that *P. indica* dependent early flowering is likely caused by the positive effect of this fungus on GA biosynthesis in Arabidopsis.

To confirm the role of GAs in the *P. indica*-dependent early flowering, we tested the GA biosynthetic mutant *ga5 (ga20ox1)*. Here, we observed less pronounced effects of *P. indica* colonization on the transcript levels of *GA20ox2*, *GA20ox1* and *GA3* and *FT*, *LFY* and *AP1*, in *ga5* mutant compared with the corresponding wild-type (*Ler* ecotype) (Fig. 4).

Taken together, our data demonstrate that *P. indica* triggers early flowering in Arabidopsis and strongly indicate that this effect is mediated by an induction of GA biosynthesis at transcript level.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2017.06.169>.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.bbrc.2017.06.169>.

Conflict of interest

The authors declare that they have no conflict of interest.

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