Phosphate suppression of arbuscular mycorrhizal symbiosis involves gibberellic acid signalling

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Abstract

Most land plants entertain a mutualistic symbiosis known as arbuscular mycorrhiza with fungi (*Glomeromycota*) that provide them with essential mineral nutrients, in particular phosphate (P$_i$), and protect them from biotic and abiotic stress. Arbuscular mycorrhizal (AM) symbiosis increases plant productivity and biodiversity, and is therefore relevant for both, natural plant communities and crop production. However, AM fungal populations suffer from intense farming practices in agricultural soils, in particular P$_i$ fertilization. The dilemma between natural fertilization from AM symbiosis and chemical fertilization has raised major concern and emphasizes the need to better understand the mechanisms by which P$_i$ suppresses AM symbiosis. Here, we test the hypothesis that P$_i$ may interfere with AM symbiosis via the phytohormone gibberellic acid (GA) in the Solanaceous model systems *Petunia hybrida* and *Nicotiana tabacum*. Indeed, we find that GA is inhibitory to AM symbiosis, and that P$_i$ may cause GA levels to increase in mycorrhizal roots. Consistent with a role of endogenous GA as an inhibitor of AM development, GA-defective *N. tabacum* lines expressing a GA-metabolizing enzyme (GA methyltransferase; GAMT), are colonized more quickly by the AM fungus *Rhizoglomus irregularare*, and exogenous P$_i$ is less effective in inhibiting AM colonization in these lines. Systematic gene expression analysis of GA-related genes reveals a complex picture, in which GA degradation by GA2 oxidase plays a prominent role. These findings reveal potential targets for crop breeding that could reduce P$_i$ suppression of AM symbiosis, thereby reconciling the advantages of P$_i$ fertilization with the diverse benefits of AM symbiosis.
Introduction

Arbuscular mycorrhizal (AM) fungi provide multiple benefits to their host (Chen et al. 2018), in particular increased supply with macronutrient elements such as phosphorus (Karandashov and Bucher 2005), nitrogen (Govindarajulu et al. 2005), and sulfur (Allen and Shachar-Hill 2009), and with the microelements copper (Lehmann and Rillig 2015) and zinc (Cavagnaro 2008). In addition, AM fungi increase the resistance of their hosts against biotic and abiotic stresses (Abdel-Latef and Miransari 2014; Pozo and Azcon-Aguilar 2007), they prevent soil erosion by wind and water (Chaudhary et al. 2009), and they reduce nutrient leaching from the soil (Cavagnaro et al. 2015). These combined benefits promote plant productivity and biodiversity (van der Heijden et al. 1998) and are considered to be of central importance for future strategies towards more sustainable agriculture systems (Gianinazzi et al. 2010; Solaiman et al. 2014).

However, AM fungal diversity and inoculum potential is often decreased in agricultural soils, an effect that is attributed primarily to intensive farming practices, in particular plowing and fertilization (Douds and Millner 1999; Oehl et al. 2004; van Geel et al. 2015; Verbruggen and Kiers 2010). The major inhibitory component in fertilizers is inorganic phosphate (P$_i$) (Balzergue et al. 2011; Breuillin et al. 2010; Nouri et al. 2014). Split-root experiments have shown that P$_i$ does not act directly on AM fungi but through the increased P-status of the host plant (Balzergue et al. 2011; Breuillin et al. 2010). P$_i$ is known to decrease host secretion of the AM fungal stimulant strigolactone (SL) (Balzergue et al. 2011; Yoneyama et al. 2007a; Yoneyama et al. 2007b), however, this effect cannot be the main reason for the inhibitory effect of P$_i$, since the application of exogenous SL cannot rescue AM symbiosis at high P$_i$ levels (Breuillin et al. 2010). Alternative potential mechanisms,
including the induction of defense against the fungal partner, have not received experimental support in a transcriptomic study on *P. hybrida* (Breuillin et al. 2010). Hence, the mechanism of action of P<sub>i</sub> in the inhibition of AM symbiosis has remained elusive.

Here, we have tested the hypothesis that the inhibitory effect of P<sub>i</sub> on AM symbiosis may involve hormonal pathways, which are known to regulate various aspects of AM symbiosis (Gutjahr 2014). Gibberellic acid (GA) has been identified as a negative regulator of AM symbiosis, whose application can reduce AM colonization and arbuscule abundance, although insufficient GA signaling has also been shown to inhibit AM development (El Ghachtouli et al. 1996; Floss et al. 2013; Foo et al. 2013; Takeda et al. 2015a; Takeda et al. 2015b; Yu et al. 2014). Based on these findings, we tested whether the inhibition of AM symbiosis by P<sub>i</sub> may involve GA signaling. Our results show that P<sub>i</sub> fertilization tends to increase GA levels in roots, and that transgenic lines with reduced GA signaling exhibit increased mycorrhizal colonization levels, both, in the presence and in the absence of high P<sub>i</sub>. In addition, we show that P<sub>i</sub> strongly affects the expression levels of several genes involved in GA biosynthesis or signaling. Taken together, these data indicate that endogenous GA has a role in controlling AM colonization at low P<sub>i</sub> levels, as well as under high inhibitory P<sub>i</sub> conditions.

**Results**

**Assessing hormonal influence in AM of petunia**

In order to assess the potential of GA to interfere with AM in petunia (*Petunia hybrida*), plantlets were inoculated with *Rhizoglomus irregulare* (MUCL 43204) in the presence of various GA<sub>3</sub> concentrations. GA<sub>3</sub> concentrations of 1 μM or more
significantly reduced AM development, whereby 3 µM GA₃ resulted in 63% inhibition of root colonization (from 51.3% to 19%) (Fig. 1A). For comparison, the hormones abscisic acid (ABA), ethylene (applied as its precursor 1-aminocyclopropane-1-carboxylic acid (ACC)), and auxin (in the form of indole-3-acetic acid (IAA)) had only minor effects and only at elevated concentrations (Fig. S1). Notably, the stress and defense hormones jasmonic acid (JA; applied as its methyl ester) and salicylic acid (SA), and elicitors of defense responses (flg22, chitin oligosaccharides, and yeast extract) had only weak effects on AM symbiosis (Fig. S1). Hence, GA₃ had the strongest negative impact on AM symbiosis among all tested hormones and elicitors, since it inhibited AM development at the lowest concentrations (1 µM), and it reduced colonization more than any other treatment (compare Fig. 1A and Fig. S1). By comparison, even higher concentrations of the other hormones did not cause comparable negative effects (compare Fig. 1A and Fig. S1), with the strongest inhibition observed with 100 µM ACC, which reduced colonization by only 40% (from 70% to 41%). ACC is commonly used as a reliable proxy for ethylene in symbiosis research (Okazaki et al. 2004), although ACC can also exert hormonal function on its own (Polko and Kieber 2019). In general, it should be noted that endogenous hormone concentrations may not always be proportional to exogenously applied hormone concentrations, since uptake rates at the root surface may vary between hormones.

In order to test whether GA may act directly on the AM fungus or indirectly through physiological changes in the plant host, we applied GA₃ (10 µM) by spraying it to the leaves of inoculated plants. This resulted in an even stronger reduction of colonization (Fig. 1B), showing that GA₃ acts in the host plant and not directly on the AM fungus.
Closer microscopical inspection revealed that GA$_3$ had a pronounced effect on the formation of arbuscules (Fig. 2), fungal structures with which AM fungi supply their hosts with P$_i$ (Harrison 2012), and which are thought to serve for the uptake of lipids from the host (Brands et al. 2018; Bravo et al. 2017; Jiang et al. 2017; Keymer et al. 2017; Rich et al. 2017b; Roth and Paszkowski 2017). Most arbuscules were malformed and exhibited considerably fewer fine branches than in control roots (Fig. 2), suggesting that they could be impaired in their function as an interface for nutrient exchange. Besides the effects on arbuscule formation, GA$_3$ also inhibited the formation of storage vesicles, consistent with the assumption that malformed arbuscules result in reduced carbon transfer to the fungus.

Confocal microscopic analysis revealed additional subcellular detail, showing that GA$_3$ caused progressive inhibition of arbuscule differentiation over 14 days, with a lesser effect at the infection stage (Fig. 3B,E,H). This effect resembled the inhibitory effects of P$_i$ on AM (Breuillin et al. 2010), which was also particularly pronounced for arbuscule formation (Fig. 3C,F,G). While the effects of P$_i$ and GA$_3$ on AM symbiosis were qualitatively similar, the extent of inhibition of arbuscule formation tended to be more extreme with GA$_3$, in particular at the 14d time point (Fig. 3H). The fact that the formation of hyphopodia (Fig. 2), and infection of hypodermal cells with hyphal coils (Fig. 3A-C) were not affected indicates that P$_i$ and GA$_3$ act at the level of arbuscule formation rather than at the stage of root infection.

**Expression of GA-related genes in mycorrhizal roots and under high P$_i$ conditions**

To test whether P$_i$ impinges on GA, we next explored how P$_i$ affects the expression of GA-related genes, since GA biosynthesis and catabolism are regulated
primarily at the transcriptional level (Hedden and Thomas 2012). First, the homologs of GA biosynthetic and metabolizing genes were identified in the genome sequence of the parent of *P. hybrida, Petunia axillaris*, which is 98.5% identical at the DNA level with *P. hybrida* (Bombarely et al. 2016). The respective amino acid sequences encoded by GA-related genes from *Arabidopsis thaliana* and pea (*Pisum sativum*) were used as queries for searches of the predicted *P. axillaris* transcriptome by tblastn. Most enzymes in GA biosynthesis and components in GA signaling were found to be encoded in *P. axillaris* by gene families with multiple members like in *A. thaliana* and other species (Hedden and Thomas 2012; Sun 2008).

Among a total of 47 predicted GA-related petunia genes, 11 were found to be expressed in control roots or mycorrhizal roots, or in roots treated with 5 mM KH$_2$PO$_4$, a P$_i$ concentration that has been shown to result in efficient inhibition of AM (Breuillin et al. 2010; Nouri et al. 2014) (Table S1). In order to test whether P$_i$ influences the expression of these GA-related genes, and to see whether they respond to exogenous GA, plants were inoculated and allowed to be colonized by the AM fungus for 4 weeks, followed by a treatment with 5 mM KH$_2$PO$_4$ or with 10 µM GA$_3$, for 3 or 7 days. Non-mycorrhizal plants were treated in the same way. Established mycorrhizal marker genes (*RAM1, RAM2, STR, PT4*; Table S1) (Rich et al. 2017a) served as markers for AM colonization and for the influence of the treatments on AM symbiosis. This alternative experimental design was chosen because long-term treatments reduce colonization (Breuillin et al., 2010; Fig. 1), hence differences in gene expression could be due directly to the treatment or, indirectly, to the reduced colonization level. Exogenous treatment of mycorrhizal plants changes colonization levels only after >7d of treatment (Breuillin et al., 2010), hence, in such a scenario, effects on gene expression can be assigned to the treatment with confidence.
Altogether, the expression pattern showed a conspicuous trend. Several GA biosynthetic genes were induced in mycorrhizal roots versus controls (Table 1; AM/c), indicating that GA production may be increased during symbiosis. However, even stronger was the induction of the GA-degrading enzyme GA2-oxidase (GA2ox), represented by the genes GA2ox1 and GA2ox3 (Table 1). These results suggest that GA biosynthesis is induced in mycorrhizal roots, but that at the same time, GA levels are attenuated by GA catabolism. In contrast, P_i repressed most GA-related genes, both, in non-mycorrhizal, as well as in mycorrhizal roots (Table 1; Pi/c and Pi-AM/AM, respectively). In addition, P_i repressed the AM marker genes in mycorrhizal roots.

GA had a similar inhibitory effect as P_i on GA biosynthetic genes, in particular in mycorrhizal roots at the earlier time point (GA-AM/AM; 3d), consistent with negative feedback regulation (Hedden and Thomas 2012) (Table 1; compare GA-AM/AM and Pi-AM/AM). In contrast, the GA-metabolizing genes GA2ox1 and GA2ox3 tended to be induced by GA, indicative of feed-forward regulation to stimulate removal of GA (Hedden and Thomas 2012). While the effects of P_i and GA were similar on GA biosynthetic genes, they had opposite effects on AM-related genes: GA strongly induced the AM-related genes (Table 1; GA/c), whereas P_i strongly repressed them in mycorrhizal roots (Table 1; P_i-AM/AM), as it was shown in a previous report (Breuillin et al. 2010). Comparison of gene expression in P_i- and GA-treated mycorrhizal roots vs. non-treated controls (Table S2; Pi-AM/c and GA-AM/c) showed that several GA-related, and all AM-related genes, where still induced in the mycorrhizal roots but to a lesser extent than in non-treated mycorrhizal roots (AM/c). The unexpected stimulatory effect of GA on AM-related marker genes was confirmed in a second independent experiment (Table S3), although in this case, the
induction of RAM1 by GA was not significant. As in the first experiment, the induction was stronger after 3d, compared to 7d after treatment (Table S3, GA/c), indicating that GA has a transient effect. On the other hand, a third time point for the P₁ treatment (14d) showed that repression of AM-related marker genes became increasingly strong, and was not transient, consistent with earlier findings (Breuillin et al. 2010), and with the strong long-term effect of P₁ treatments on AM symbiosis.

**Role of GA in phosphate suppression of AM symbiosis**

To test whether P₁ could potentially act through GA, we measured the levels of GA in the roots of *P. hybrida* in mycorrhizal and control roots treated with 5 mM KH₂PO₄. Interestingly, the levels of both, GA₁ and GA₃, tended to be increased upon treatment with exogenous P₁ in both, mycorrhizal roots (inoculated with *R. irregulare*), as well as in non-mycorrhizal controls (Fig. 4A,B), but in the case of the mycorrhizal roots, the differences were not statistically significant. Overall, two-way ANOVA revealed a significant interaction of P₁ treatment with time. However, AM had no significant effect on GA levels (Table S6), although in general, mycorrhizal roots tended to contain lower levels of GA than non-mycorrhizal controls (Fig. 4A,B). To test whether P₁ may also act by enhancing the general defense status, we measured the levels of the defense marker salicylic acid (SA) (Fu and Dong 2013). All samples contained between 40-60 µg/g SA (Fig. 4C), irrespective of the mycorrhizal status or P₁ levels. This is in the range of non-challenged tissues in various plant species (Gao et al. 2015), and therefore indicates that P₁ does not act by increasing SA-dependent defense mechanisms in petunia roots. In order to test for other stress- and defense-related pathways, we also quantified the levels of the stress hormones ABA and JA after P₁ treatments (Fig. S2). In both cases, the levels were very low (approximately 1
ng g⁻¹ FW), and were not affected by Pᵢ treatment in a consistent fashion (Fig. S2).

Finally, the potential of Pᵢ to induce the stress and defense hormone ethylene was assessed, however, as in a previous study on petunia roots (Chen et al. 2021), ethylene levels were consistently around the detection limit for all treatments. Furthermore, ethylene induction caused by the experimental procedure (root excision and ethylene accumulation in gas-tight glass vials) dominated the very low natural ethylene production rates, hence precluding consistent analyses (data not shown).

Based on the finding that Pᵢ tended to induce GA levels, we next wanted to test whether GA may be causally related to the inhibitory effect of Pᵢ on AM. To this end, we used transgenic lines that overexpress a GA-metabolizing enzyme, GA methyltransferase (GAMT) from *Arabidopsis thaliana*. GAMT esterifies the carboxyl group of GA, thereby inactivating it (Varbanova et al. 2007). We used two lines, GAMT1 and GAMT2, that had previously been characterized at the molecular level (Varbanova et al. 2007). Transgenic petunia plants expressing these constructs were infertile and did not survive (Varbanova et al. 2007), hence, transgenic tobacco (*Nicotiana tabacum*) expressing GAMT1 and GAMT2 (Varbanova et al. 2007) were used in our experiments. Firstly, the effect of Pᵢ and GA on AM development in tobacco was investigated to see whether this species can serve as an experimental model system to investigate the relationship between Pᵢ and GA in AM symbiosis. We quantified AM colonisation in response to Pᵢ and GA in tobacco, and found that, as in petunia, overall colonization was reduced by both 5 mM Pᵢ, as well as 10μM GA (Fig. S3, compare with Fig. 1-3). Ethanol treatment (0.04%) served as a control for GA, because this hormone can only be applied from a stock solution in ethanol. Arbuscule formation was particularly sensitive to GA and Pᵢ, and the latter had the stronger effect, by almost completely suppressing AM, while GA caused a reduction to
approximately half the AM levels in controls (compare with Fig. 1A). Taken together, these results show that AM colonization in tobacco is affected by P$_i$ and GA in a similar fashion as in petunia.

Next, we performed a time course experiment to investigate the dynamics of AM development in GAMT lines. Both, GAMT1 and GAMT2 plants, were colonized more quickly than the wild type (Fig. 5), suggesting that colonization of the wild type is attenuated by endogenous GA. Hence GA appears to be involved in the regulation of fungal proliferation under conditions that favor AM (low P$_i$). Next, we explored whether the action of P$_i$ on AM symbiosis is influenced by GAMT activity, hence, plants were treated with 5 mM KH$_2$PO$_4$. In the wild type, this treatment resulted in a 7.5-fold reduction of AM colonization relative to the controls (Fig. 6, left; compare with Fig. S3). In contrast, both transgenic lines were less affected by P$_i$. GAMT1 showed a reduction of only 2.1-fold (Fig. 6, middle), and reached levels of AM colonization in the range of wild type plants grown at low P$_i$ levels. In GAMT2, AM colonization was reduced 3.9-fold, significantly less than in the wild type (Fig. 6, right). Hence, both GAMT lines exhibited a weaker reaction to P$_i$ than the wild type. The weaker effect of GAMT2 on P$_i$-dependent inhibition of AM (relative to GAMT1) correlates with higher residual GA levels in GAMT2 over-expressor lines compared to the respective GAMT1 lines (Varbanova et al. 2007). Taken together, these results suggest that at least partially, the inhibition of AM by high P$_i$ levels involves GA signaling.

**Discussion**

Exogenous nutrients have a tremendous impact on the development of the root system in plants (Shahzad and Amtmann 2017), and they influence the interaction
with AM fungi (Carbonnel and Gutjahr 2014). AM symbiosis is particularly sensitive to P\(_i\), which is the central nutrient provided by AM fungi (Balzergue et al. 2011; Breuillin et al. 2010; Nouri et al. 2014). This provides a strong negative feedback mechanism that allows plants to reduce the costs of AM fungal colonization in case of optimal nutrient supply. Under these conditions, the plant can reach its maximal growth potential without the fungus, thus fungal colonization would not confer any benefit, and could even reduce plant growth (Smith et al. 2009), since mycorrhizal roots consume 4%-20% more photosynthates than control roots (Bago et al. 2000). Therefore, AM colonization can result in growth suppression, also known as negative mycorrhizal growth response under conditions that allow non-symbiotic plants to reach their full growth potential. This effect creates a strong selection pressure towards reduction of AM fungal colonization under conditions that are not beneficial for the host.

\(P_i\) supply has long been known to be negatively correlated with SL secretion (Balzergue et al. 2011; Yoneyama et al. 2007a; Yoneyama et al. 2007b), such that high phosphate can potentially interfere with early signaling in AM symbiosis. However, this effect cannot explain the strong inhibitory effect of \(P_i\) on AM, since SL application cannot restore AM at high \(P_i\) levels (Balzergue et al. 2011; Breuillin et al. 2010). Although it has been shown that \(P_i\) acts not directly on the fungus, but indirectly by increasing the P-status of the plant (Balzergue et al. 2011; Breuillin et al. 2010), it has long remained unclear how the P-status interferes with AM fungal colonization. GA has been identified as a strong negative regulator of AM in species such as pea (\textit{Pisum sativum}), \textit{Medicago truncatula}, rice (\textit{Oryza sativa}), and \textit{Lotus japonicus} (El Ghachtouli et al. 1996; Floss et al. 2013; Foo et al. 2013; Takeda et al. 2015b; Yu et al. 2014). This raises the question whether \(P_i\) may act through induction
of GA levels or GA signaling. In *P. hybrida*, GA is the strongest inhibitor of AM among a range of hormones, including the stress and defense hormones ethylene, SA, and JA, and established defense signals such as chitin oligosaccharides, flagellin peptide (flg22), and yeast extract, all potent elicitors of a robust defense response (*Fig. 1; Fig. S1*). In addition, exogenous P$_i$ induced GA levels (*Fig. 4*), consistent with a potential role of GA in the repression of AM by P$_i$. However, quantitative real time RT-PCR (qPCR) indicated that the role of GA in AM may be more complex. Exogenous P$_i$ and GA both tended to repress GA biosynthetic genes (*Table 1*), which is not in line with the induction of GA levels by P$_i$, but with negative feedback regulation of GA homeostasis by GA (Sun 2008). However, GA levels are not only regulated by GA biosynthesis, but also by GA catabolism, initiated mainly by GA2ox (Hedden and Thomas 2012; Sun 2008). GA2ox was induced by GA and in mycorrhizal roots, while it was repressed by P$_i$ (*Table 1*).

A more striking difference between P$_i$ and GA was the effect on AM-related genes such as *RAM1, RAM2, STR* and *PT4*. These genes, that are normally expressed only in mycorrhizal roots, were strongly and rapidly induced by exogenous GA$_3$ (at 3d), whereas they were repressed in mycorrhizal roots by P$_i$ (*Table 1, Table S2, S3*; see also Breuillin et al., 2010). Taken together, gene expression analysis reveals that GA$_3$ treatments have overlapping effects with both, P$_i$ and AM, respectively, and suggests that GA plays a complex role in AM. The fact that GAMT lines exhibited elevated levels of colonization at low P$_i$ levels (*Fig. 5*) indicates that GA attenuates colonization even under AM-promoting conditions. In addition, the observation that GAMT lines tolerated higher levels of AM colonization at high P$_i$ levels (relative to the wild type) (*Fig. 6*), suggests that GA signaling may contribute to the inhibitory effect of P$_i$ on AM.
How can the promotive effect of GA on AM-related genes, and the induction of GA biosynthetic genes in mycorrhizal roots be reconciled with the well-documented inhibitory role of GA in AM? GA is required for proper AM development (Takeda et al. 2015a; Takeda et al. 2015b), and the fact that gain-of-function mutants in the AM-related signaling component CCaMK induced GA biosynthetic genes (Takeda et al. 2015b) indicates that GA is an essential component of AM development. In light of the strong inhibitory effects of GA on AM, it follows that GA homeostasis (biosynthesis and degradation), and signaling, must be under tight control to allow for a balance between promotion and repression of AM. Interestingly, GA has been proposed to be involved in a feedback mechanism by which mycorrhizal plants attenuate AM colonization in response to the increased $P_i$ levels resulting from symbiosis (Floss et al. 2013).

Taken together, these results suggest a mechanism in which mycorrhizal roots exhibit an increased flux through the GA biosynthetic pathway, while steady state GA levels remain relatively low due to high activity of the GA-degrading enzyme GA2ox (Fig. 7). Feedback regulation is known to regulate GA biosynthesis at multiple levels, thereby keeping GA levels in the tissues in narrow limits (Hedden and Thomas 2012). Due to GA inactivation by GA2ox, DELLA proteins in mycorrhizal roots are protected from degradation, allowing for the induction of $RAM1$ and other AM-related marker genes (Pimprikar et al. 2016; Rich et al. 2015), and resulting in AM development (Floss et al. 2013).

At elevated $P_i$ levels, GA biosynthetic genes exhibited low expression, but GA-degradation was repressed as well, potentially resulting in elevated GA levels (Fig. 4), degradation of DELLA, and hence a lack of induction of AM-related genes, therefore inhibition of AM (Fig. 7). The similar expression pattern of $GA2ox1$ and $GA2ox3$
relative to the AM-related marker genes (*RAM1, RAM2, STR, PT4*) (Table 1), indicates that they may be under control of the same regulatory mechanism. Taken together, we suggest that P<sub>i</sub> suppression of AM symbiosis is at least partly regulated by GA signaling. However, we cannot exclude that P<sub>i</sub> and GA act in parallel independent pathways that converge on AM symbiosis. Furthermore, the role of GA in AM symbiosis is complex (Floss et al. 2013; Takeda et al. 2015a; Takeda et al. 2015b), and requires further investigation, in particular the transient induction of AM-related genes by GA<sub>3</sub> (Table 1, Table S3, Fig. 7).

GA alone cannot account for the entire effect of phosphate on AM, since the overall effect of P<sub>i</sub> on AM is stronger than that of GA, and the GAMT lines still showed an effect of P<sub>i</sub>, although the extent of inhibition was weaker, and higher levels of colonization were tolerated in these lines after P<sub>i</sub> addition. These results are compatible with the view that P<sub>i</sub> triggers a multi-factorial syndrome that results in the robust inhibition of AM (Breuillin et al. 2010), of which GA is one component.

Hence, future breeding programs should seek to generate crops that exhibit weaker P<sub>i</sub>-related inhibition of AM, in order to reconcile the multiple benefits of AM symbiosis with the advantages of fertilization. Such crops may be characterized by attenuated GA signaling in the roots, analogous to the green-revolution crops that exhibit weaker GA signaling in the shoots (Hedden 2003).

**Materials and Methods**

**Plant material, growth conditions, and hormone treatments**

Seeds of petunia (*Petunia hybrida*, line W115), or of tobacco (*Nicotiana tabacum*) transgenic for GAMT1 or GAMT2 (Varbanova et al. 2007), as well as wild type tobacco (*Xanthi*) were germinated on seedling substrate (Klasmann, http://www.klasmann-deilmann.com). After 4 weeks, plantlets were transferred to
small pots (volume: 20 ml) with a sterilized mixture of 75% sand with 25% unfertilized soil (v/v; further referred to as “substrate”) for another two weeks. Subsequently, plantlets were transferred to larger pots (volume: 150 ml) with the same substrate and inoculated with one teaspoon (ca. 10 g) of mycorrhizal inoculum per plant directly to the root system. Plants were further cultured as described (Nouri et al. 2014). Plants were weekly fertilized with a nutrient solution containing the following mineral nutrients (Strullu and Romand 1987): 3 mM MgSO$_4$, 0.75 mM KNO$_3$, 0.87 mM KCl, 0.2 mM KH$_2$PO$_4$, 1.52 mM Ca(NO$_3$)$_2$, 20 µM NaFeEDTA, 11 µM MnSO$_4$, 1µM ZnSO$_4$, 30µM H$_3$BO$_3$, 0.96 µM CuSO$_4$, 0.03 µM (NH$_4$)$_6$Mo$_7$O$_24$ and 0.01 µM Na$_2$MoO$_4$. For hormonal treatments and P$_i$ or elicitor treatments (Figs. 1, 2, 3, 5, 6, S1, S2, S3), plants were watered twice per week with 25 ml of nutrient solution containing the indicated concentrations of GA$_3$ (Sigma-Aldrich; product No. G7645), IAA (Sigma-Aldrich; product No. I2886), ABA (Sigma-Aldrich; product No. 862169), methyl-JA (Sigma-Aldrich; product No. 392707), SA (Sigma-Aldrich; product No. S5922), ACC (Sigma-Aldrich; product No. A3903), flg22 (Meindl et al. 2000), chitin oligosaccharides (Thuerig et al. 2005), or yeast extract (Alfa Aesar, product No. J23547), at indicated concentrations. For ethylene treatments, the precursor ACC was employed, because prolonged treatments during AM development with the gaseous hormone in closed air-tight vessels would cause multiple pleiotropic effects not (or only indirectly) related to ethylene. Foliar GA treatment was performed by spraying the leaves twice per week with 10 µm GA$_3$, while the soil was covered by aluminum foil to avoid contact with the root system. For gene expression and metabolite analysis, plants were first inoculated and grown for 4 weeks in order to reach a colonization rate of approximately 50%, and then plants were watered twice per week with 25 ml 5 mM KH$_2$PO$_4$ or with GA$_3$ as indicated for 3d, 7d, or 14d. For all GA$_3$ treatments, the respective control plants were treated with 0.04% ethanol.

**AM fungal inoculation**

Inoculum of *R. irregularare* (MUCL 43204) was produced in chive pot cultures. The inoculum consisted of a mixture of soil and roots, and was tested for the presence of spores before use. Root staining and quantification of mycorrhizal colonization were carried out as described (Sekhara Reddy et al. 2007).
Microscopy and quantification of root colonization

Roots were harvested, washed and stored overnight in 10% KOH (w/v) in glass tubes. Then, they were cleared for 30 min at 95°C, washed twice with water, stained for 10 min with Trypan Blue (TB) staining solution at 95°C, and rinsed with 10% lactic acid (v/v). TB staining solution consisted of 10% glycerol (w/v), 10% lactic acid (v/v) and 0.01% Trypan Blue (w/v). Microscopic inspection and quantification of root colonization was carried out as described with a modified grid intersection method (Sekhara Reddy et al. 2007).

Mycorrhizal roots were stained with wheat germ agglutinin coupled to Alexa488 (WGA-Alexa488; Invitrogen, catalogue No. W11261) in Soerensen's phosphate buffer (0.133 M, pH=7.2), followed by counterstaining with 0.2% basic fuchsin (Sigma, 857343). For microscopy, the roots were immersed in a modified version of ClearSee (Kurihara et al. 2015) containing 10% (w/v) xylitol, 25% (w/v) urea, and 2% (w/v) SDS. Images were acquired on a Leica SP5 confocal microscope.

Identification of GA-related genes

GA-related genes were identified by searching the Petunia axillaris predicted transcriptome at the SolGenomics database (https://solgenomics.net) using established GA-related genes from Arabidopsis thaliana (Hedden and Thomas 2012) by tblastx. Primers for quantitative real-time reverse-transcriptase PCR (qRT-PCR) were designed by the primer3 tool (https://bioinfo.ut.ee/primer3-0.4.0/) (see Table S1). Preliminary analysis of gene expression was performed in mycorrhizal and non-mycorrhizal roots to identify genes that are expressed in any of the tested conditions, and these were used for further qRT-PCR analysis.

RNA extraction and quantitative real-time RT-PCR

Frozen petunia roots were placed in a 2 ml Eppendorf tube containing a glass bead and were ground using a ball mill. Total RNA was extracted from the powdered roots according to the protocol of the Direct-zol RNA miniprep kit from Zymo Research, using trizol solution for lysis (38% v/v), saturated phenol (pH 8), 0.8 M guanidine thiocynate, 0.4 M ammonium thiocynate, 0.1 M Na-acetate pH 5, and 5% (v/v) glycerol). The Direct-zol RNA kit involves a DNAse step to remove genomic DNA
during RNA extraction. RNA was used for reverse transcription according to the protocol of the SensiFAST™ cDNA synthesis kit from Bioline. PCR reactions were carried out with 5 μl of 100x diluted cDNA solution, 1 μl of 10 μM forward and reverse primer, 12.5 μl of SensiMix SYBR Hi-ROX (BIO-RAD) and DNAse/RNAse free water up to 15 μl. The reaction cycle was 95°C for 10 min followed by 45 amplification cycles (95°C for 20s, 64°C for 20s, 72°C for 20s). All samples were analysed in technical duplicates from seven independant replicate plants. Actin and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as reference genes. Relative expression values were calculated using the delta-delta-Ct (ΔΔCt) method (Pfaffl 2001). Gene expression data are shown in Table 1, Table S2 and Table S3 as -fold changes relative to gene expression in the respective control treatment (treatment/control). Averaged expression values of individual treatments, normalized to the two reference genes (actin and GAPDH) are shown in Tables S4 and S5.

**Extraction and quantification of GAs from roots**

GAs were collected from 5-week-old petunia plants in 2 replicates with each replicate consisting of 6 plants. For root extracts, 200 to 250 mg fresh weight of ground root tissue was extracted by 1 ml of methanol : Milli-Q®-water (80:20) containing 2.5 mM diethyldithiocarbamic acid (as antioxidant) and 1 ml of a mixture of internal standards (IS) at a concentration of 0.02 nmol/ml. After vortexing, samples were shaken at 4 °C overnight. Then the extraction was repeated with 2 ml of methanol : Milli-Q®-water (80:20) without IS. The two solvent fractions were pooled in the same 4 ml glass vials for each sample after centrifugation (10 min at 600 g) and then dried under nitrogen flow. A third step of extraction was performed with 2 ml of ethyl acetate (EtOAc) at 4 °C for 30 min. Meanwhile, the pooled fractions from methanol extraction were evaporated to near dryness, and subsequently combined with the EtOAc fraction in the same 4ml vials for each sample. Afterwards, each vial was evaporated to a volume of approximately 200 ul, taken up in 1 ml Milli-Q®-water, loaded on pre-equilibrated (with 3 ml methanol and 6 ml Milli-Q®-water) Grace Pure 500 mg C18 columns for purification. The columns were washed with 1ml Milli-Q®-water, eluted with 4ml acetone, evaporated, and the residue was re-dissolved in 200 μl of 25% (vol/vol)
acetonitrile in water and filtered through RC4 Minisart 0.2-μm filters before further analysis.

Gibberellins were detected and quantified by Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) using a Waters Xevo TQ mass spectrometer equipped with an electrospray-ionization source and coupled to a Waters Acquity ultraperformance LC system. A water/acetonitrile gradient was applied to an Acquity UPLC BEH C18 column (100 mm, 2.1 mm, 1.7 mm; Waters), starting from 5% (vol/vol) of acetonitrile for 1 min and rising to 50% (vol/vol) of acetonitrile in 6.67 min, followed by a 0.66-min gradient to 90% (vol/vol) acetonitrile, which was maintained for 0.67 min before going back to 5% (vol/vol) acetonitrile using a 0.13 min gradient, before the next run. Between two measurements, the column was equilibrated for 1.87 min with 5% (vol/vol) acetonitrile. The total run length was 10 min. The column was operated at 50°C with a flow rate of 0.5 ml·min\(^{-1}\). Sample injection volume was 20 μl, and the sample temperature was 10°C. The mass spectrometer was operated in positive electrospray ionization mode for GA1, 4, 5, 8, 9, 19, 20, and in negative mode for GA3 and 7. Cone and desolvation gas flows were set to 50 and 1000 l·h\(^{-1}\), respectively. The capillary voltage was set at 3 kV, the source temperature at 150 °C and the desolvation temperature at 550 °C. Argon was used for fragmentation by collision-induced dissociation. Multiple reaction monitoring (MRM) was used for GA identification and quantification. Parent–daughter transitions for the standards, GA1, 4, 5, 8, 9, 19, 20, and D2-GA1, 4, 20, 3, 7 (used as internal standards), were set by using the IntelliStart MS Console. MRM transitions selected for identification of GAs in petunia were as follows:

In positive mode, for GA9, cone voltage (CV) was set to 14eV, mass-to-charge ratio (m/z) 317.22 > 225.24 at a collision energy of 30 eV, and 317.22 > 271.24 at 16 eV; for GA5, CV 12eV, m/z 331.22 > 267.22 at 20eV, and 331.22 > 285.18 at 10 eV; for GA20, CV 18eV, m/z 333.22 > 269.22 at 18 eV and 333.22 > 287.24 at 12 eV; for GA4 CV 12eV, m/z 333.29 > 269.16 at 18eV and 333.29 > 315.16 at 8eV; for D2-GA4 CV 16eV, m/z 335.22 > 271.23 at 14eV, and 335.22 > 317.23 at 10 eV; for GA44 CV 22eV, m/z 347.29 > 255.25 at 22eV, and 347.29 > 301.25 at 14eV; for GA1 CV 20eV, m/z 349.16 > 285.17 CV at 16eV, and 349.16 > 331.16 at 10eV; for D2-GA1 CV 10eV, m/z 351.23 > 287.24 at 16 eV; for GA19 CV 14eV, m/z 363.22 >
299.26 at 12eV, and 363.22 > 317.27 at 12eV; for GA8 CV 10eV, m/z 365.22 > 301.18 at 14eV, and 365.22 > 347.17 at 8eV.

In the negative mode: for GA7 CV 26eV, m/z 329.16 > 211.13 at 30eV, and 329.16 > 223.18 at 14eV; for D2-GA7 CV 26eV, m/z 331.22 > 213.15 at 26eV, and 331.22 > 225.19 at 24eV; for GA3 m/z CV 28eV, 345.16 > 143.04 at 22eV, and 345.16 > 239.21 at 14eV; for D2-GA3 CV 30eV, m/z 347.22 > 143.12 at 24eV, and 347.22 > 241.20 at 14eV; for GA53 CV 48eV, m/z 347.22 > 303.20 at 28eV, and 347.22 > 329.16 at 22eV.

GAs were quantified by using a calibration curve with known amount of standards and based on the ratio of the peak areas of the MRM chromatogram for GA standards to the MRM chromatogram for each D2-GA as internal standards. GA5, 8, 9, 19 and 53 were quantified by using a calibration curve with known amount of standards (no internal standards were available). Data acquisition and analysis were performed by using MassLynx 4.1 (TargetLynx) software (Waters).

**Extraction and quantification of SA, JA, and ABA**

Salicylic acid was quantified as described (Fragniere et al. 2011) with minor modifications. Root samples of approximately 1 g were extracted with 2 ml 70 % ethanol and 200 μl of the internal standard ortho-anisic acid (1 ng μl⁻¹) by blending for 30 s with a polytron (Kinematica, New York, USA). After centrifugation (10 min at 1200 g), the supernatant was transferred to a fresh tube. The pellet was extracted with 2 ml methanol (90% v/v), centrifuged as before, and the two supernatants were combined. After evaporation of the solvents under vacuum at 30°C during 40 min, ca. 400 μl of aqueous solution was left. 500 μl trichloroacetate (5% v/v) were added and the mixture was centrifuged at 5500 g for 10 min at room temperature. The supernatant was combined with 500 μl 1:1 ethyl-acetate:cyclohexane and mixed thoroughly by vortexing at maximum speed. After centrifugation (2 min at 11'000 g), the upper phase was transferred to a fresh Eppendorf tube and the lower phase was re-extracted with 500 μl 1:1 ethylacetate:cyclohexane. The combined extracts were dried in a speedvac and dissolved in 200 μl methanol for analysis with a reverse phase HPLC column (25 cm x 4.6 mm, 5 μm Supelco discovery® C18, Bellefonte, PA, USA), and SA was quantified relative to the internal standard. JA and ABA were determined by ultra-high performance liquid chromatography-tandem mass
spectrometry (UHPLC-MS/MS) according to (Glauser et al., 2014).

**Statistical analysis**
All results were tested by one-way ANOVA or two-way ANOVA as indicated. All p-values are listed in Table S6.

**Data availability**
All gene sequences are available at the SolGenomics database (https://solgenomics.net), and can be retrieved using the gene identifiers (ID) listed in Table S1.

**Supplemental Information**
Figure S1. Effects of exogenous phytohormones on AM colonization in *P. hybrida*
Figure S2. Effect of Pi on JA and ABA levels in *P. hybrida*
Figure S3. Effect of Pi and GA on AM colonization pattern in *N. tabacum*
Table S1. Gene and primer list
Table S2. Complete list of gene expression ratios for GA- and AM-related genes (first gene expression analysis)
Table S3. Expression ratios for AM marker genes (second independent gene expression analysis)
Table S4. Expression values of GA- and AM-related genes in response to Pi and GA (first experiment)
Table S5. Expression values of AM-related genes in response to Pi and GA (second experiment)
Table S6. P-values and overview of statistical analysis

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Author contribution

E.N. and R.S. carried out most experiments, E.N., L.B., M.S., S.B., and M.C. performed gene expression analysis, E.N. carried out GA and SA measurements with the assistance of Y.Z., C.R.-S., and H.B.. G.G. performed JA and ABA analytics. E.N. and D.R. designed the experiments, and coordinated the project. All authors contributed to the writing of the manuscript.

Author information.

The authors declare they have no competing financial interest.

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**Table and Figure legends**

**Table 1. Regulation of GA-related genes and AM marker genes by phosphate and GA₃**

Gene expression of GA-related genes was determined for *ent*-Copalyl synthase (CPS), *ent*-Kaurene Synthase (KS), *ent*-Kaurene Oxidase (KO), *ent*-Kaurenoic acid Oxidase (KAO), GA20 Oxidase (GA20ox), and GA2 Oxidase (GA2ox). The genes Required for Arbuscular Mycorrhiza1 (*RAM1*), *RAM2*, Stunted Arbuscule (*STR*), and Phosphate Transporter4 (*PT4*) served as AM marker genes. Plants were inoculated for 4 weeks, and then treated with 5 mM KH₂PO₄ or 10 μm GA₃ for 3 or 7 days. qPCR was performed with actin7 and GAPDH as reference genes. Relative gene expression values were calculated by the delta-delta-Ct (ΔΔCt) method (Pfaffl 2001). Data are expressed as -fold changes relative to gene expression in the relative control (treatment/control). Significant differences (p-value < 0.05) are indicated by asterisks. Gene IDs are available in Table S1.
Figure 1. Effect of GA$_3$ on AM fungal development in *P. hybrida*

(A) Plants were inoculated with *R. irregularare* and watered with various concentrations of GA$_3$ from 0.1 µM to 10 µM. Total root colonization was determined after 5 weeks from inoculation. Bars represent the mean of six biological replicates + StDev.

(B) Plants were inoculated with *R. irregularare* and the leaves were sprayed with 10 µM GA$_3$. Roots were harvested after five weeks and root colonization was determined. Columns represent the mean of four biological replicates +StDev. Columns that do not share a letter are significantly different (p<0.05; one-way ANOVA).

Figure 2. GA$_3$ affects arbuscule formation

(A) Quantification of AM fungal structures in mycorrhizal roots after cultivation in the absence of GA (open bars), with 1 µM GA$_3$ (grey bars), or with 10 µM GA$_3$ (black bars) for 5 weeks. The occurrence of fungal structures was quantified for hyphopodia (Hyhop), normal arbuscules (Arbusc), abnormal arbuscules (Abnorm), and vesicles (Vesic). Columns represent the mean of six biological replicates +StDev. Columns that do not share a letter are significantly different (p<0.05; one-way ANOVA).

Figure 3. Confocal laser scanning analysis of AM colonization in *P. hybrida* in response to GA$_3$ and phosphate.

Plants were inoculated for 5 weeks with *R. irregularare* and subsequently treated with control solution (A,D,G), 10 µM GA$_3$ (B,E,H), or 5 mM KH$_2$PO$_4$ (C,F,I) for 7 days (A-F) or 14 days (G-I). Then root samples were cleared with KOH and stained with basic fuchsin and WGA-Alexa488 for confocal laser scanning microscopic analysis.
(A-C) hyphal coils in hypodermal cells after 7d of treatment

(D-F) colonization in the cortex after 7d of treatment

(G-I) colonization in the cortex after 14d of treatment

Asterisks denote arbuscules, arrows indicate infection hyphae in hypodermal cells, and arrowheads indicate defective fungal structures in the root cortex. All pictures show representative examples from at least ten assessed root fragments. Size bars, 25 μm.

Figure 4. GA levels in *P. hybrida* roots in response to AM and phosphate

Plants were inoculated for 5 weeks with *R. irregulare* and subsequently treated with 5 mM KH$_2$PO$_4$ for 3 days (3d) or 14 days (14d). Levels of GA$_1$ (A), GA$_3$ (B), and SA (C) were determined in non-mycorrhizal control plants (open bars), or inoculated plants (filled bars). A significant effect of P$_i$ on GA accumulation was revealed by two-way ANOVA with p=0.0006 for GA$_1$ (A) and p=0.0023 for GA$_3$ (B). No effect was observed on the levels of salicylic acid (SA). Columns represent the mean of six biological replicates ±StDev. Columns that do not share a letter are significantly different (p<0.05; one-way ANOVA).

Figure 5. *GAMT* lines exhibit accelerated AM colonization

Wild type tobacco (*N. tabacum*) (open bars), transgenic GAMT1 plants (grey bars), and GAMT2 plants (black bars) were inoculated with *R. irregulare* and grown with basic nutrient solution (low P$_i$) for the indicated times (3-5 weeks). Columns represent the mean of six biological replicates ±StDev. Columns that do not share a letter are significantly different (p<0.05; one-way ANOVA).
Figure 6. GAMT lines exhibit reduced inhibition of AM symbiosis by phosphate

Wild type tobacco (*N. tabacum*) and transgenic lines (*GAMT1* and *GAMT2*) were inoculated with *R. irregulare* and grown with and without 5 mM KH$_2$PO$_4$ for five weeks. Columns represent the mean of six biological replicates +StDev. Columns that do not share a letter are significantly different (p<0.05; one-way ANOVA). Reduction is indicated as -fold change for P$_i$-treated vs. the corresponding control (e.g. 7.5x for the wild type).

Figure 7. Model for the role of the GA pathway in regulation of AM symbiosis by phosphate

Schematic representation of GA function in AM symbiosis based on gene expression patterns and effects of GA$_3$ and P$_i$ on AM symbiosis. Promoting (arrows) and inhibiting (blocks) action of AMF, phosphate (P$_i$), and GA$_3$ is indicated on GA biosynthesis, GA inactivation by GA2ox, on the induction of AM-related genes (*RAM1*, *RAM2*, *STR*, *PT4*), and ultimately on functional symbiosis (represented by an arbuscule). GA acts by negative feedback onto GA biosynthesis, feed-forward activation of GA2ox, and by transient induction of AM-related genes (arrow with asterisk). Inhibitory action of GA onto AM symbiosis through the canonical DELLA pathway ultimately counteracts the transient induction of AM-related genes and leads to inhibition of AM symbiosis. Thick arrows exerted by AM fungi (AMF) indicate the strong induction of GA2ox genes and AM-related genes in the functional symbiosis (see Table 1). Dashed arrows represent multistep pathways.
Figure 5

![Figure 5 with AM colonization values for wt, GAMT1, and GAMT2 at 3w, 4w, and 5w showing statistical differences labeled with letters a to f.]

Figure 6

![Figure 6 with AM colonization values for cont, wt, GAMT1, and GAMT2 conditions showing reductions and statistical differences labeled with letters a to f.]

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Figure 7

GGPP

AMF

P_i

GA biosynthesis

* 

GA

GA2ox

P_i

AMF

AMF

DELLA

RAM1

RAM2, STR, PT4

Image of a cellular structure
Table 1

| Group     | Function | 3d | 7d | 3d | 7d | 3d | 7d | 3d | 7d | 3d | 7d |
|-----------|----------|----|----|----|----|----|----|----|----|----|----|
| GA biosynthesis |          |    |    |    |    |    |    |    |    |    |    |
| CPS1      |          | 6.46 | 0.58 | 0.0 | 0.0 | 1.9 | 0.2 | 0.0 | 0.0 | 0.2 | 0.2 |
| CPS3      |          | 2.83 | 0.66 | 0.2 | 0.3 | 3.3 | 0.9 | 0.8 | 0.8 | 1.2 | 1.9 |
| KS2       | 0.37     | 2.93 | 0.0* | 0.0* | 2.0 | 0.0* | 0.0* | 0.0* | 0.0* | 0.0* | 0.0* |
| KS5       |          | 2.34 | 2.33 | 0.5 | 2.0 | 0.6 | 1.2 | 0.3 | 0.3 | 0.3 | 0.9 |
| KO        | 2.51     | 2.24 | 0.1 | 0.1 | 0.4 | 0.1 | 0.4 | 0.1 | 0.2 | 0.2 | 0.4 |
| GA biosynthesis |          |    |    |    |    |    |    |    |    |    |    |
| KAO 1     |          | 1.34 | 3.50 | 0.1 | 0.5 | 0.7 | 1.5 | 0.2 | 0.4 | 1.1 | 0.7 |
| KAO 2     | 1.64     | 5.38 | 0.7 | 1.1 | 0.9 | 0.6 | 0.9 | 0.5 | 0.8 | 0.7 | 0.8 |
| GA2 ox1   | 1.20     | 4.46 | 0.6 | 1.0 | 0.2 | 0.0 | 0.4 | 0.5 | 2.0 | 0.1 | 4.0 |
| GA2 ox6   | 3.65     | 9.82 | 0.5 | 0.2 | 0.0 | 0.1 | 0.0 | 0.81 | 0.0 | 0.1 | 0.0 |
| GA degradation |          |    |    |    |    |    |    |    |    |    |    |
| GA2 ox1   |          | 33.3 | 26.4 | 0.2 | 3.5 | 4.0 | 6.5 | 0.3 | 0.0 | 0.7 | 1.7 |
| GA2 ox3   | 31.9     | 21.8 | 0.6 | 3.4 | 2.3 | 5.7 | 0.6 | 0.1 | 4.0 | 1.0 | 4.0 |
| AM-related genes |          |    |    |    |    |    |    |    |    |    |    |
| RAM 1     | 66.5     | 26.2 | 0.5 | 0.4 | 7.5 | 0.7 | 0.4 | 0.1 | 3.9 | 0.4 | 3.9 |
| RAM 2     | 143.0    | 94.3 | 1.0 | 2.0 | 9.0 | 1.5 | 0.1 | 0.0 | 0.4 | 1.7 | 0.4 |
| STR        | 201.0    | 533.7 | 0.9 | 1.1 | 1.0 | 1.6 | 0.2 | 0.0 | 16.0 | 1.2 | 16.0 |
| PT4        | 83.73    | 517.5 | 0.7 | 3.3 | 16.9 | 4.1 | 0.0 | 0.0 | 0.3 | 1.3 | 0.3 |

* Indicates significance.