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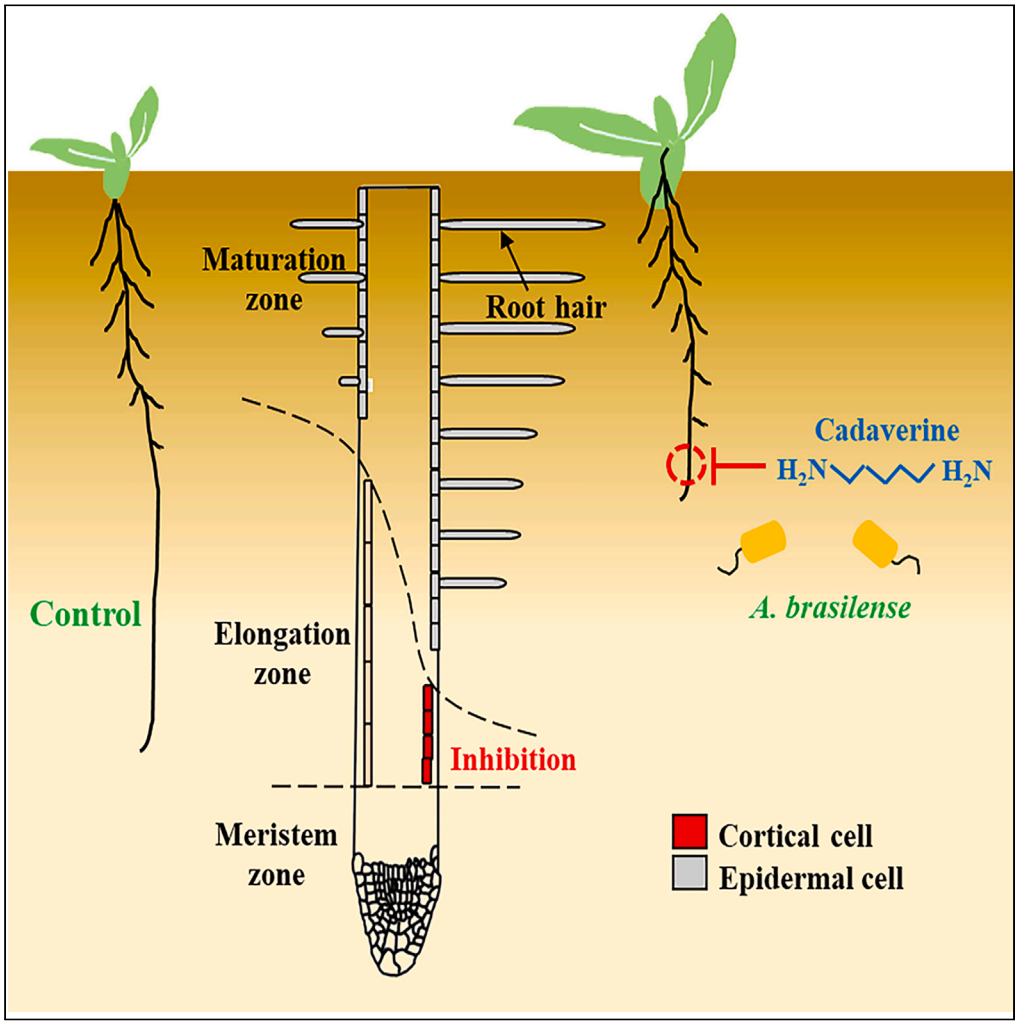

Hongcheng Zhao, Nan Sun, Lin Huang, Ruyi Qian, Xianyong Lin, Chengliang Sun, Yongguan Zhu

clsun@zju.edu.cn

Highlights

A. brasilense enhances carbon and nitrogen metabolism, and promoted plant growth

A. brasilense inhibited root elongation is because of inhibitory effects on cell expansion

Non-volatile compounds inhibit cell elongation by POD-mediated cell wall modification

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Azospirillum brasilense activates peroxidase-mediated cell wall modification to inhibit root cell elongation

Hongcheng Zhao,[1] Nan Sun,[1] Lin Huang,[1] Ruyi Qian,[1] Xianyong Lin,[1] Chengliang Sun,[1,3,*] and Yongguan Zhu[1,2]

SUMMARY

The molecular mechanism of beneficial bacterium *Azospirillum brasilense*-mediated root developmental remain elusive. *A. brasilense* elicited extensively transcriptional changes but inhibited primary root elongation in *Arabidopsis*. By analyzing root cell type-specific developmental markers, we demonstrated that *A. brasilense* affected neither overall organization nor cell division of primary root meristem. The cessation of primary root resulted from reduction of cell elongation, which is probably because of bacterially activated peroxidase that will lead to cell wall cross-linking at consuming of H_2O_2. The activated peroxidase combined with downregulated cell wall loosening enzymes consequently led to cell wall thickness, whereas inhibiting peroxidase restored root growth under *A. brasilense* inoculation. We further showed that peroxidase activity was probably promoted by cadaverine secreted by *A. brasilense*. These results suggest that *A. brasilense* inhibits root elongation by activating peroxidase and inducing cell wall modification in *Arabidopsis*, in which cadaverine released by *A. brasilense* is a potential signal compound.

INTRODUCTION

The plant rhizosphere harbor an immense number of microbial communities, including some microorganism that are capable of promoting plant growth and development, such as plant growth-promoting rhizobacteria (PGPR).[1–3] These bacteria can interact with a variety of plants, and enhance host nutrition, protect plants from numerous forms of abiotic stress and soil-borne diseases.[4,5] In this context, several PGPR strains belonging to the genus *Pseudomonas*, *Azospirillum*, and *Rhizobium* have been applied as biofertilizers on a variety of economical crop plants.[6,7] Despite strong evidence that PGPR exert beneficial effects and improve plant production, most previous studies focused on evaluating plant growth-promoting effects on above-ground biomass[8,9] and have paid little attention to the molecular alterations in roots, which would reflect the local primary response of plants to rhizobacteria.

It has been well known that many PGPRs cause alterations of the root system architecture, usually promoting the formation of secondary roots and ultimately improving root's exploratory capacity.[3,10–13] Different root architectural traits reprogrammed by PGPR would confer plant tolerance to drought and salt stress, and also improve capturing capacity of the root system for water and nutrients such as phosphorus and nitrogen. For example, deep rooting is advantageous for accessing subsoil water and facilitating nitrogen capture, whereas a dense shallow root system increases topsoil foraging and improves the uptake of nutrient of low mobility such as phosphorus.[14–16] Dicots such as *Arabidopsis* mainly form a taproot system architecture, and primary root may act as a significant determinant of root depths. Considering the proliferation of lateral roots and root hairs by PGPR under most circumstances,[3,10–12,17,18] alterations of primary root growth mediated by rhizobacteria are important characteristics to be evaluated when developing inoculant consortia for different soil constraints arising from future climatic conditions.

Current evidence demonstrates that primary root development in response to PGPR is dependent on bacterial strains and inoculation methods. For example, *Burkholderia phytofirmans* PsJN significantly enhanced primary root growth in *Arabidopsis* plants.[19] Similarly, promotion of *Arabidopsis* root was observed after *Trichoderma guizhouense* NJAU4742 inoculation.[11] In other studies, however, it has been reported that certain PGPR strains significantly inhibited primary root growth with promoting lateral

[1]MOE Key Laboratory of Environment Remediation and Ecological Health, College of Natural Resource & Environmental Sciences, Zhejiang University, Hangzhou 310058, China

[2]Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, PR China

[3]Lead contact

*Correspondence: clsun@zju.edu.cn

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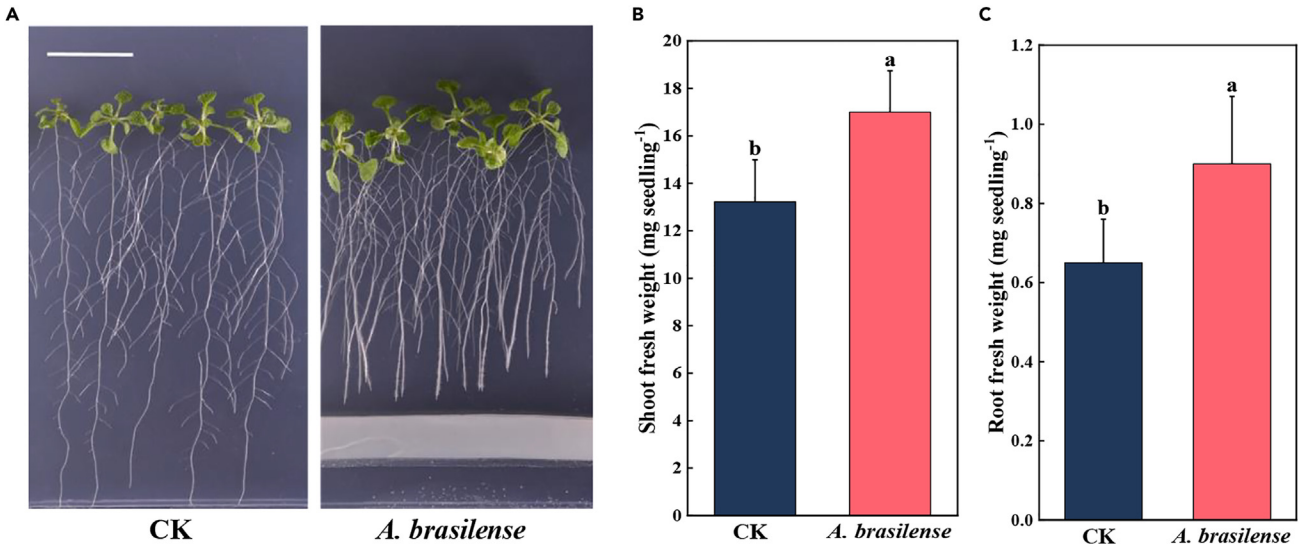

Figure 1. Effects of *A. brasilense* inoculation on plant growth

Four-day-old seedlings were treated with or without *A. brasilense* treatment for 12 days. *A. brasilense* colonized LB solid strips (1 cm) were applied at an 8-cm distance from the root tip of 4-day-old seedlings.

(A) The representative photos of *Arabidopsis* treated with or without *A. brasilense*. As the blank LB strip has no effects on plant growth, we removed the blank strip from the control treatment after 4 days to avoid obstacle sensed by roots after contact with the strip barrier. Bar, 2 cm.

(B) The variation in shoot fresh weight.

(C) The variation in root fresh weight. Values represent means ± SD (n = 10). Different letters indicate statistically significant differences.

root and root hair development. Such phenomenon has been observed in *Arabidopsis* after inoculation of *P.* spp WCS417 and *A. brasilense* Sp245.[10,20] Our recent study reported that *Azospirillum brasilense*-modified primary root growth and root hair development, forming a dense shallow root system and facilitating phosphorus acquisition.[21] Evidence has demonstrated that the bacteria-mediated root morphological alterations are intimately correlated with improved plant growth.[6,21] Despite considerable progress has been made understanding the bacterially mediated proliferation of lateral roots and root hairs,[17,20] the mode of action and mechanisms controlling primary root development largely remain elusive.

In the present study, we first investigated the primary response of *Arabidopsis* roots to *A. brasilense*, which is mainly isolated from grasses and cereals and has been commercially applied on a wide range of crop plants.[22–24] By analyzing the phenotypical changes and transcriptional responses of roots, we further explored the molecular mechanisms governing *A. brasilense*-promoted root biomass production. Finally, we focused on the mechanistic basis of *A. brasilense*-mediated cessation of primary root, which will foster versatile and valuable utilization of such beneficial bacteria to certain soil constraints where crops need a dense shallow root system.

RESULTS

A. brasilense promotes growth and development of *Arabidopsis* seedlings

To study *A. brasilense*-mediated phenotypic changes in *Arabidopsis* plants, bacterial colonized LB solid strips (1 cm) were applied at an 8-cm distance from the root tip of 4-day-old seedlings. *A. brasilense* has a remarkably growth-promoting effect on *Arabidopsis* plants and leads to significantly changes in root architecture (Figure 1A). After 12 days of inoculation, our results showed that *A. brasilense* treatment increased shoot and root biomass by 40.0% and 38.5% of *Arabidopsis* seedlings, respectively (Figures 1B and 1C). It is noteworthy that the positive effects of *A. brasilense* on root fresh weight may probably result from increased root diameter and lateral root length. These results suggest that *A. brasilense* can stimulate *Arabidopsis* growth and trigger root developmental alterations.

A. brasilense induces major transcriptional alterations in carbon and nitrogen metabolism

Since *Arabidopsis* root is the primary site interacting with rhizobacteria and could reflect direct plant responses to *A. brasilense*, we focused our attention solely on root tissues. Compared to control treatment,

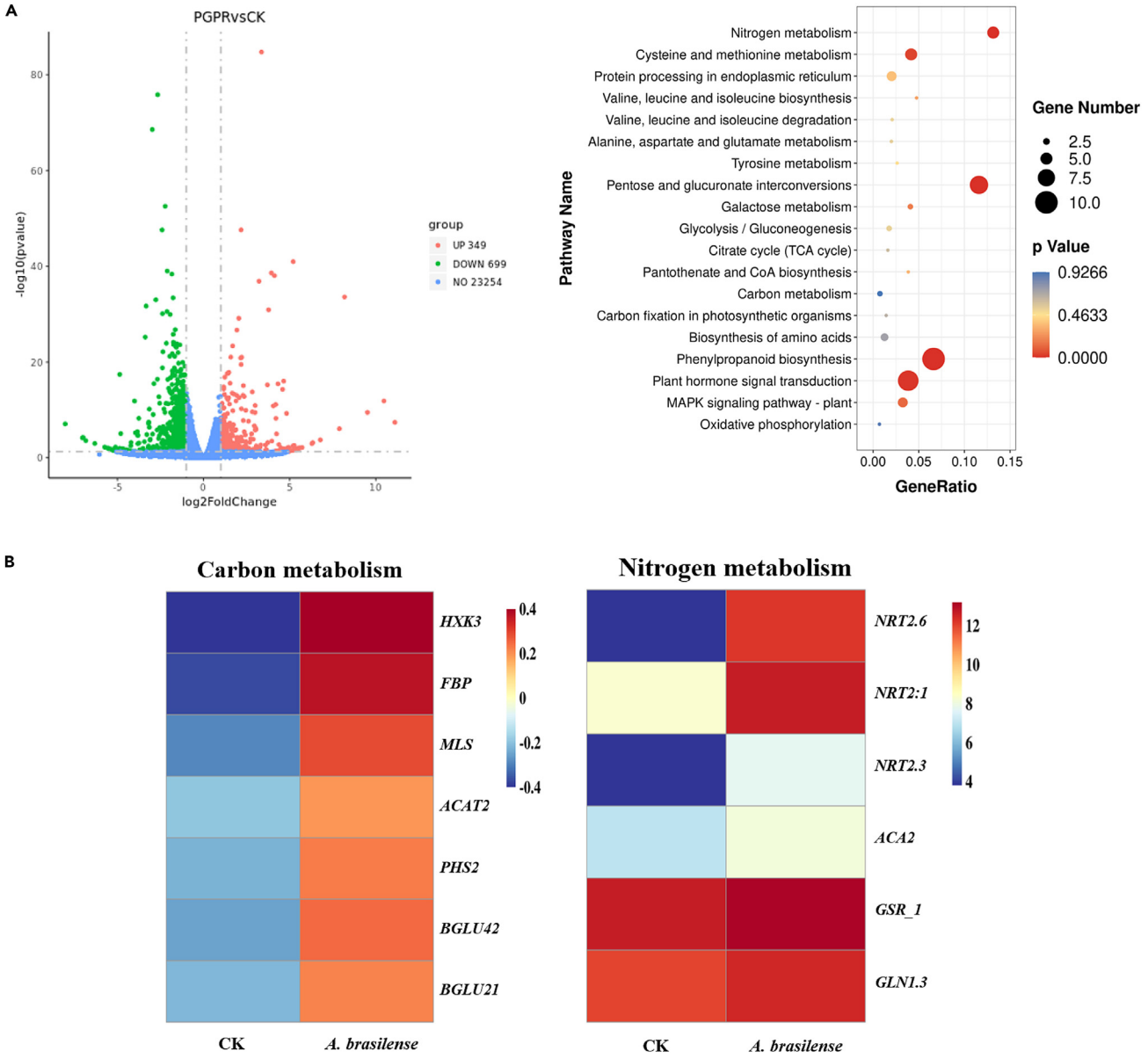

Figure 2. RNA-seq analysis in *Arabidopsis* treated with or without *A. brasilense*

Four-day-old seedlings were treated with *A. brasilense* for 2 days, and the RNA was extracted and analyzed.

(A) Genes differentially expressed in *Arabidopsis* treated with or without *A. brasilense* and KEGG analysis for up-regulated genes.

(B) Heatmap of DEGs involved in carbon metabolism, nitrogen metabolism and sugar metabolism. Each color spot reflected the differential expression level of the corresponding gene: red for up-regulated genes and blue for downregulated genes.

A. brasilense-treated group had 1048 differentially expressed genes (DEGs) based on both *P* and $\log_2$ (treatment/control) values (see the STAR Methods section), including 349 up-regulated genes and 699 down-regulated genes (Figure 2A). To gain insights into the biological processes associated with *A. brasilense*-promoting effects, a KEGG pathways analysis was performed on these up-regulated genes, which can depict the characteristics of *A. brasilense*-mediated biological processes linked to plant growth. We found that *A. brasilense* significantly enhanced the expression of genes involved in nitrogen metabolism, amino acid metabolism, sugar metabolism, phenylpropanoid biosynthesis and plant hormone signal transduction after 4 days inoculation (Figure 2A). In sucrose metabolic pathways, *β*-glucosidase synthesis related genes *BGLU21* and *BGLU42* were significantly upregulated (Figure 2B). In plants, nitrogen is essential for plant growth and *NRT* family play important roles in nitrate uptake and transport. We found

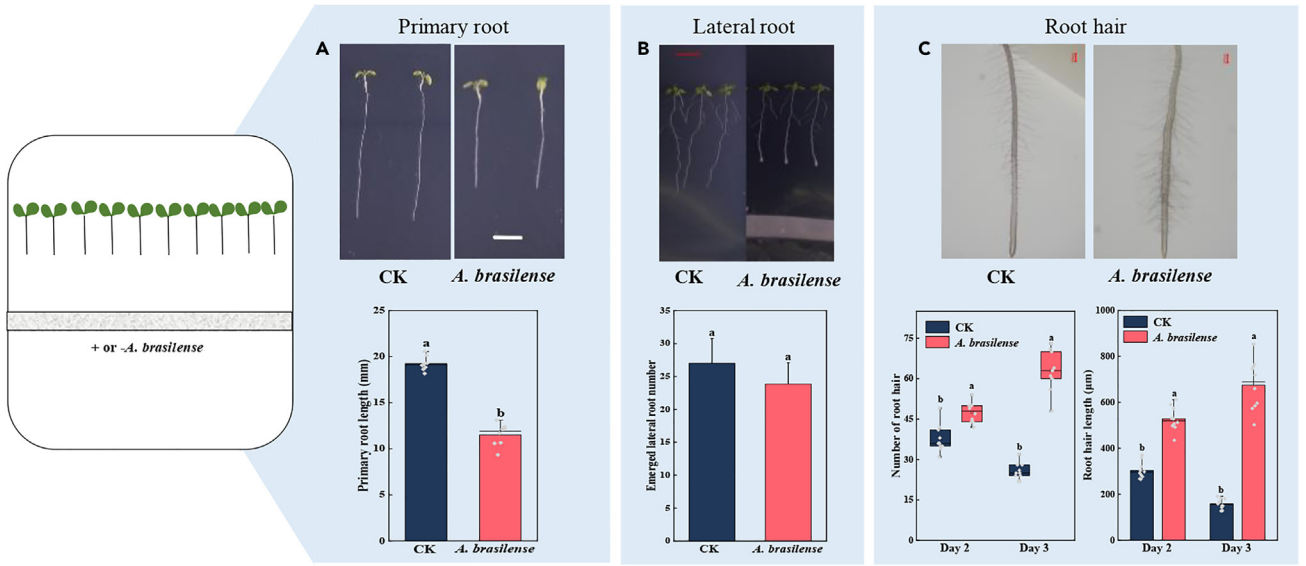

Figure 3. Effects of *A. brasilense* inoculation on root system architecture of *Arabidopsis* seedlings

(A) Effects of *A. brasilense* on primary root elongation. Four-day-old seedlings were treated with *A. brasilense* for 3 days, the primary root length was determined by ImageJ. Bar, 5 mm.

(B) Effects of *A. brasilense* on lateral root number. Four-day-old seedlings were treated with *A. brasilense* for 6 days, and then the number of lateral roots was calculated. Bar, 10 mm.

(C) Root hair development after *A. brasilense* inoculation. Four-day-old seedlings were treated with *A. brasilense*, and the root hair density and length were characterized at 2 and 3 days. Bar, 200 μm. Values represent means ± SD (n = 10). Different letters indicate statistically significant differences.

that the expression of *NRT2.1*, *NRT2.3* and *NRT2.6* in roots was significantly up-regulated by *A. brasilense* (Figure 2B). These results suggest that *A. brasilense* inoculation induced a marked response of *Arabidopsis* roots at the gene expression levels, and the enhanced and coordinated carbon and nitrogen metabolism might be associated with its growth-promoting effects.

A. brasilense alters root system architecture

To more closely analyze the effect of *A. brasilense* on *Arabidopsis* root development, several root architecture traits were analyzed, including primary root length, lateral root number, root hair length and density. After 3 days post inoculation, the primary root length of *A. brasilense*-treated seedlings was reduced by 50% compared to non-treated plants (Figure 3A). No significant effects of inoculation with *A. brasilense* were observed for lateral root formation over a 6-day period (Figure 3B), while *A. brasilense* may stimulated lateral root elongation as evidenced by the increased lateral root length after a long period. However, a 1.5- and 4.27-fold increase in root hair density and length was observed in *Arabidopsis* roots inoculated with *A. brasilense*, respectively (Figure 3C). As a whole, these results together suggest that *A. brasilense* alters root architecture with obviously increased root hair development and reduced primary root length.

A. brasilense has no effect on the organization and function of root meristem

Cessation of root growth can result from decreases in stem cell niche activity and meristematic cell division potential in root tips. To unravel the molecular mechanism underlying *A. brasilense*-induced primary root growth cessation, we firstly examined stem cell activity using the quiescent center (QC)-specific marker *pQC25::GUS*. We found that the expression patterns of *QC25* were similar between *A. brasilense*- and control-treated seedlings (Figure 4A). We also detected the expression of *WOX5* which is critical for maintaining stem cell niche in the QC using the marker *pWOX5::GFP*. Confocal microscopy of *pWOX5::GFP* showed a similar expression pattern in roots with or without *A. brasilense* inoculation (Figure 4A).

Since PLETHORA (PLT) acts in concert with SHORT ROOT (SHR) to control QC identity, we further examined the influence of *A. brasilense* on *PLT1* and *SHR* expression using *PLT1::GFP* and *SHR::GFP* transgenic lines. Expression patterns of these marker genes were unaltered by *A. brasilense* in *Arabidopsis* roots, which further confirmed the neutral effects of *A. brasilense* on QC and root stem cell niche specification

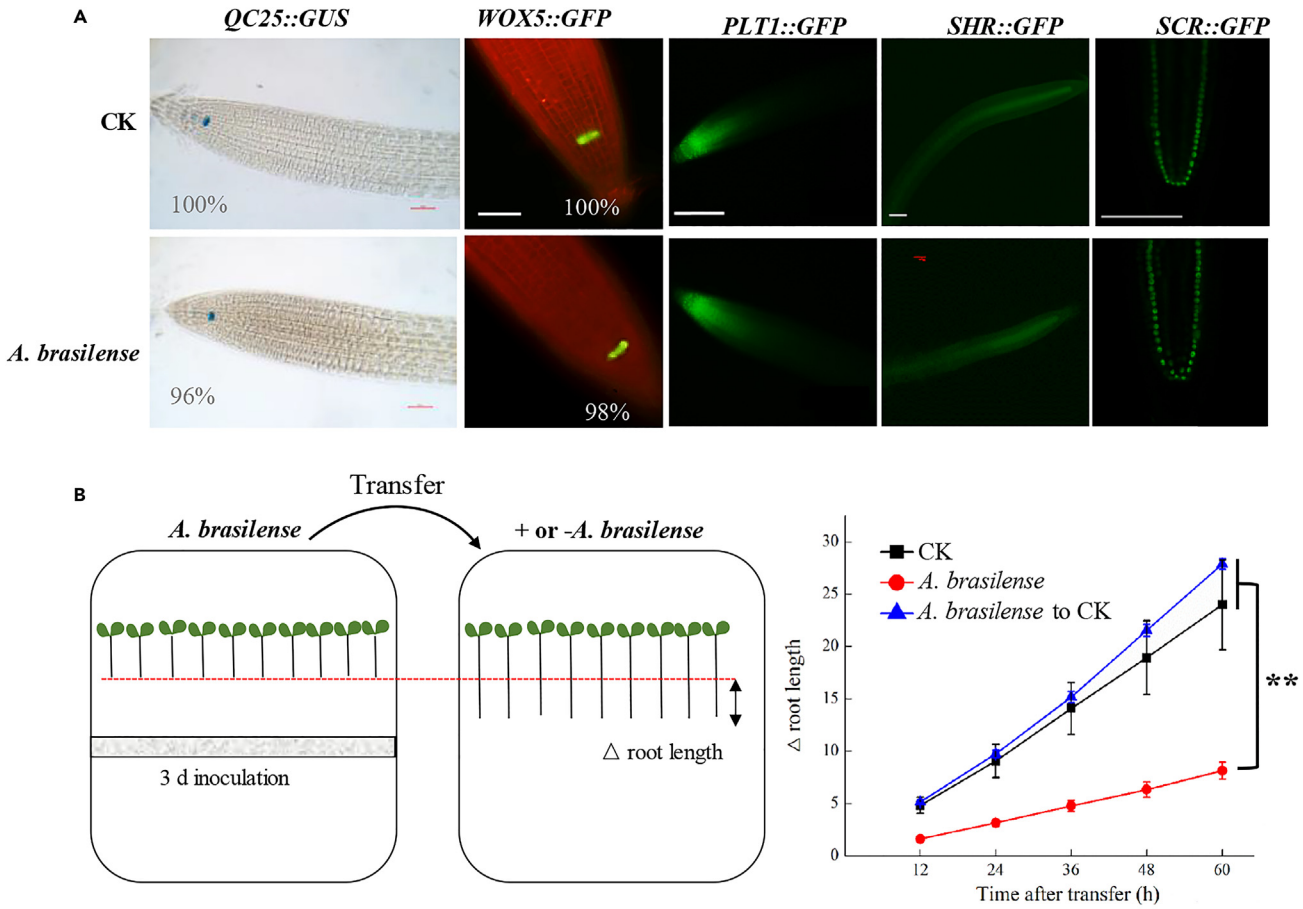

Figure 4. Effects of *A. brasilense* on the organization of the root meristem

Four-day-old seedlings were treated with *A. brasilense* for 3 days, and the *QC25::GUS* staining and *WOX5::GFP*, *PLT1::GFP*, *SHR::GFP* and *SCR::GFP* expression were determined.

(A) GUS staining of *QC25::GUS* and *WOX5::GFP* expression seedlings inoculated with *A. brasilense*. Bar, 50 μm. Representative images showing the expression patterns of *PLT1::GFP*, *SHR::GFP* and *SCR::GFP*. Bar, 100 μm.

(B) Root length changes in *Arabidopsis* seedings after transferring *A. brasilense*-inculcated seedlings to either + *A. brasilense* or - *A. brasilense* agar. Values represent means ± SD (n = 10). Asterisk indicates statistically significant differences (**p < 0.01).

(Figure 4A). These data suggest that *A. brasilense* inoculation has no profound influence on the overall organization of root meristem. These results were further confirmed by transferring *A. brasilense*-inculcated seedlings to *A. brasilense* free agar. Compared to *A. brasilense* treatment, primary root growth recovered when *A. brasilense* was removed (Figure 4B).

Alterations of meristematic cell division potential after *A. brasilense* inoculation were analyzed with a cell cycle marker *CYCB1;1:GUS* transgenic line. The GUS activity of *CYCB1;1:GUS* was not altered by *A. brasilense* (Figure 5A). Likewise, the number of meristematic cells in *A. brasilense*-treated roots was similar to that in the control roots (Figure 5B). These data indicated that *A. brasilense* unaffected cell division rates in the root meristem.

A. brasilense inhibits cell elongation in *Arabidopsis* primary root

After initiation, the continuous grow and development of primary root rely on meristem cell division combined with cell elongation and differentiation. To test the possibility that reduction of cell elongation is responsible for the significant cessation of primary root, we measured the length of the first 6 cells of the elongation zone after 4 days of *A. brasilense* inoculation. The total length of the first six cells in the elongation zone in presence of *A. brasilense* was only 48% of the control values, and each of the six measured cells was shorter in bacteria-treated roots than its corresponding cell in the roots under control treatment

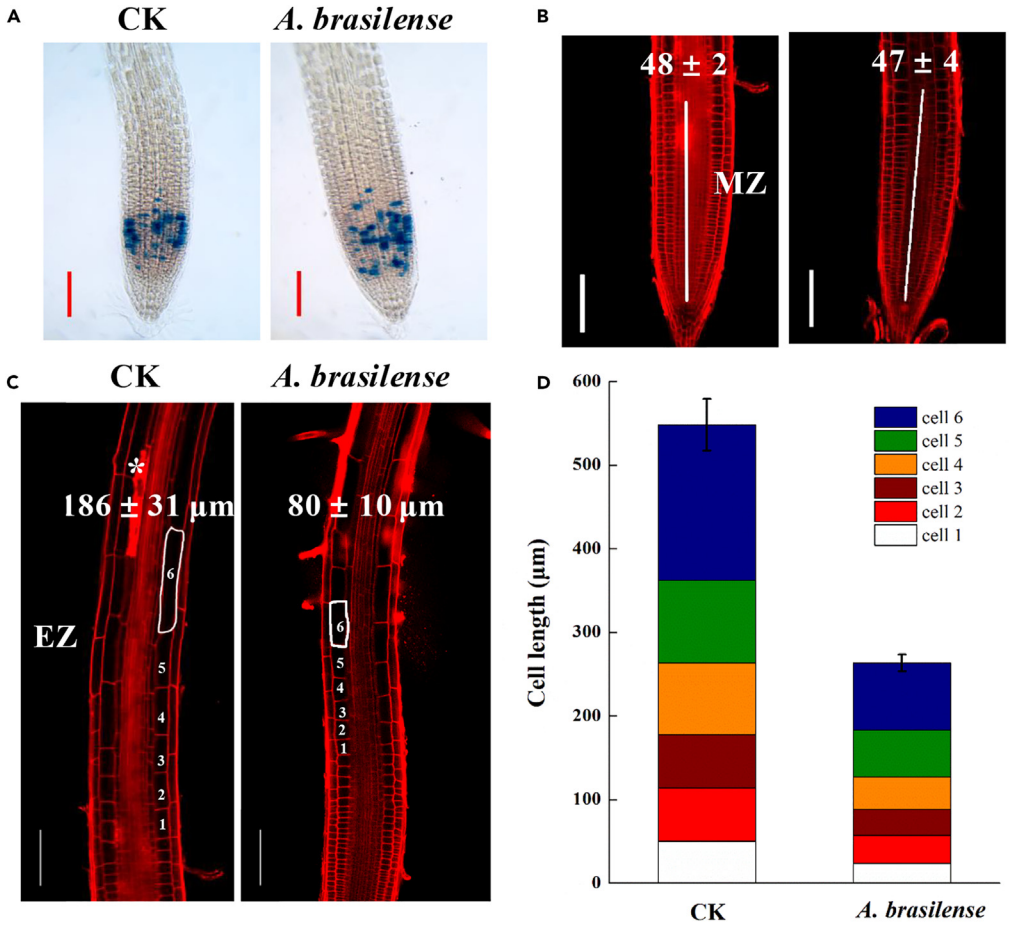

Figure 5. Effects of *A. brasilense* on the potential of cell division and cell elongation

Four-day-old seedlings were treated with *A. brasilense* for 3 days, and the size of different zone as well as their corresponding activity were monitored.

(A) GUS staining of *CYCB1::GUS* seedlings inoculated with *A. brasilense*. Bar, 100 μm.

(B) The alterations of the size of meristematic zone by *A. brasilense*. Values represent average numbers ±SD of cortical cells in meristematic zone (n = 6). Bar, 100 μm.

(C and D) The size of cortical cell in the elongation zone in the presence or absence of *A. brasilense*. Values represent average length ±SD of the cortical cells in elongation zone (n = 6). Bar, 100 μm.

(Figure 5D). The length of the sixth cell in elongation zone treated by *A. brasilense* was only 80 ± 10 μm, which was 57% lower than that of the control-treated cells (Figure 5C). Findings from the influence of *A. brasilense* on the organization and function of root meristem and current results together suggest that primary root growth cessation induced by *A. brasilense* results from inhibitory effects on cell elongation of the elongation zone rather than on the organization and function of the root meristem.

A. brasilense enhances peroxidase activity and stiffens cell walls

In *Arabidopsis* roots, the rapidly elongating cells have considerably thinner cell walls compared to slow-growing cells.[57] To investigate how cell walls respond to *A. brasilense* inoculation, we examined the epidermal root cell walls using transmission electron microscopy. *A. brasilense* treatment led to comparably increased cell wall thickness, and the cell wall thickness measurements of the control and *A. brasilense*-treated seedlings were 0.07 ± 0.02 and 0.13 ± 0.02 μm, respectively (Figure 6A). Furthermore, expansins (EXPAs) and xyloglucan endotransglucosylases/endohydrolases (XTHs) are the best characterized cell wall proteins that drive cell expansion. To get more insight into the *A. brasilense*-inhibited cell elongation, we analyzed the expression of several genes related to cell wall metabolism. Of interest, a remarkable decrease in the expression of *EXPA4*, *EXPA10*, *XTH24*, *XTH7* and *XTH32* was observed in

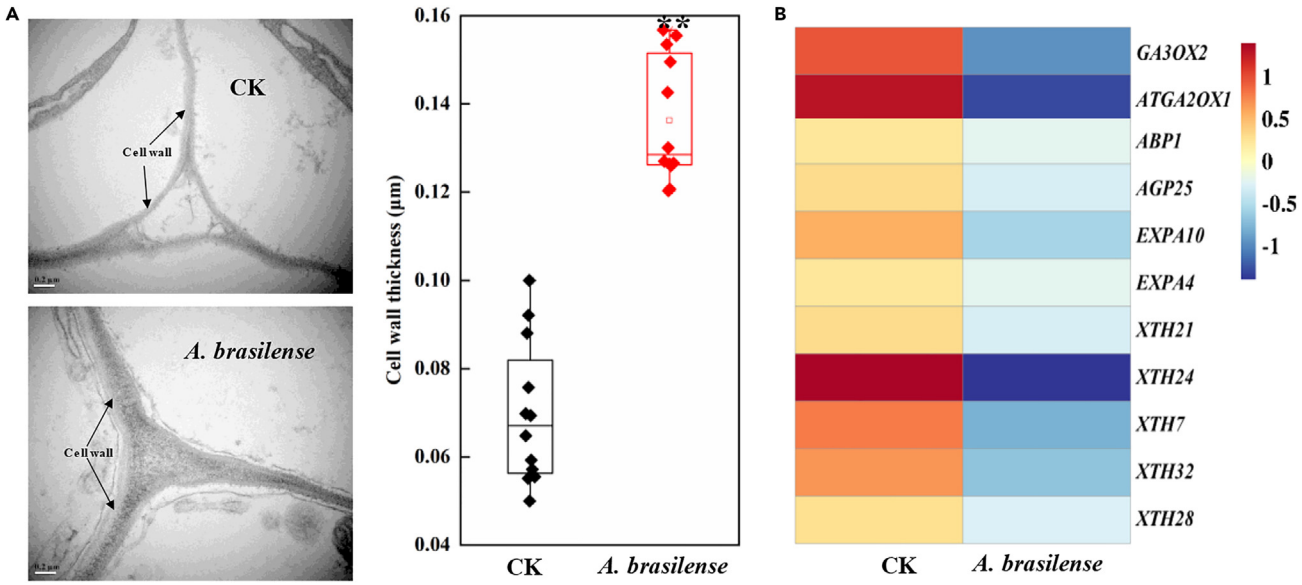

Figure 6. Effects of *A. brasilense* on cell wall thickness, and the expression levels of several EXPAs and XTHs

(A) Transmission electron microscopy of epidermal root cell walls thickness in the presence or absence of *A. brasilense*. Images and average cell wall thickness were produced using ten or more root tips in each treatment (n = 20). Bar, 0.2 μm. Asterisk indicates statistically significant differences (**$p < 0.01$). (B) Heatmap of DEGs involved in root cell elongation of *Arabidopsis* under *A. brasilense* treatment. Each color spot reflects the differential expression level of the corresponding gene: red for up-regulated genes and blue for downregulated genes.

roots of seedlings inoculated with *A. brasilense* (Figure 6B). These results indirectly suggest a simple causal scenario where *A. brasilense* triggers cell wall thickness of elongated cells and inhibited cell wall-loosening enzymes to restrict their elongation.

Class III peroxidases are considered to impede cell enlargement by catalyzing crosslinks between cell wall components, thereby stiffening the cell wall.[41] Next, we detected peroxidase activity in root apex after *A. brasilense* inoculation by histological 4-chloro-1-naphtol staining. It was observed that peroxidase staining was more intense in *A. brasilense*-treated root tips compared to control treatment, and the activity increasing with extended incubation times (Figures 7A and 7C). Simultaneously, we found that a large number of peroxidase genes were up-regulated by *A. brasilense* (Figure 7D). To further confirm the idea that *A. brasilense*-induced peroxidase activity correlates with the cessation of primary root elongation, we inhibited peroxidase activity by salicylhydroxamic acid (SHAM).[58] It was nothing that the SHAM restored primary root growth under *A. brasilense* inoculation (Figure 7E). Peroxidases inhibit cell elongation via cell wall cross-linking that consume H_2O_2. We also observed accumulation of H_2O_2 in the transition and elongation zone of primary roots by 3,3′-diaminobenzidine staining after *A. brasilense* inoculation (Figures 7A and 7B). These observations support the view that *A. brasilense*-caused enhancement of peroxidase activity prevent cell wall loosening, thereby reducing cell expansion.

Cadaverine produced by *A. brasilense* are probably related to primary root cessation

We next investigated how *A. brasilense* inhibited primary root elongation. As the beneficial bacterium did not directly attached to *Arabidopsis* roots in the present study, it is reasonable to suppose that microbial originated volatile organic compounds modulate primary root growth. Surprisingly, when wild type seedlings co-cultivated with *A. brasilense* on bipartite Petri dishes (Figure 8A), where seedlings and bacteria were separated and had contact only through a shared headspace, primary root elongation is similar to control treatment (Figures 8A and 8B). These results indicate that non-volatile compounds produced and released by *A. brasilense* induced the arrest of root cell elongation.

Production of polyamines such as spermine and spermidine by *Azospirillum* species has considered to be responsible for the plant growth-promoting effects.[47,59] Unlike spermine and spermidine, the secretion and physiological functions of bacterial cadaverine in plant-rhizobacteria is still unclear. To further challenge the idea that cadaverine sourced from *A. brasilense* is linked to modification of primary root growth,

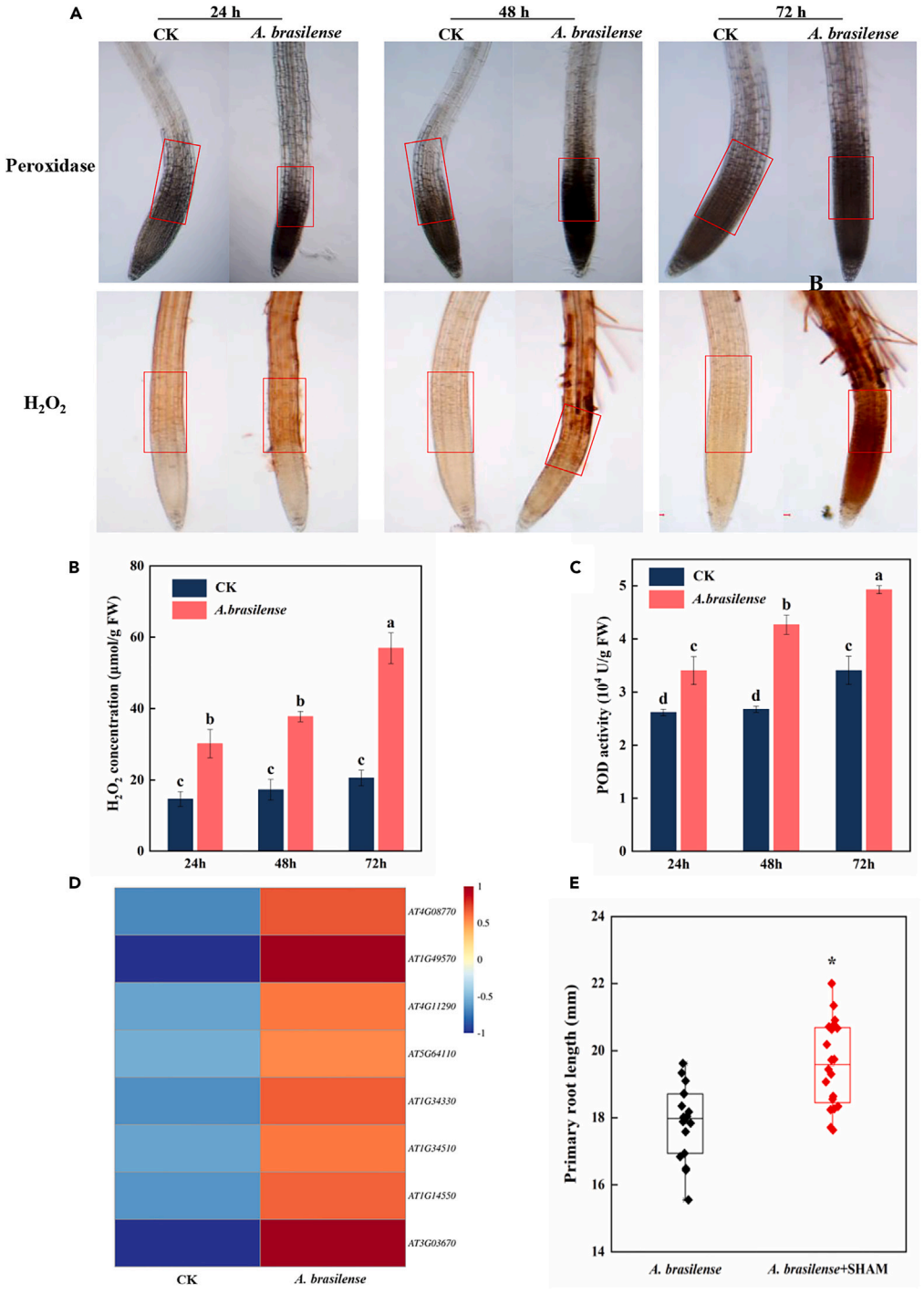

Figure 7. Effects of *A. brasilense* on peroxidase activity, H_2O_2 accumulation

Four-day-old seedlings were treated with *A. brasilense*, and then the root samples were collected and analyzed. Four-day-old seedlings were treated with or without *A. brasilense* for indicated time, and then the root was collected. (A) Visualization of peroxidase activity and H_2O_2 content. Plant roots treated with *A. brasilense* at 24, 36 and 72 h were stained with 4-chloro-1-naphtol and DAB solution to detect peroxidase activity and H_2O_2, respectively. Red square indicates the position of the elongation zone.

(B) Detection of H_2O_2 content in plant roots.

(C) Peroxidase activity was monitored by oxidation of guaiacol.

Figure 7. *Continued*

(D) Heatmap of several genes encoding peroxidase in *Arabidopsis* roots under *A. brasilense* treatment. Each color spot reflects the differential expression level of the corresponding gene: red for up-regulated genes and blue for downregulated genes.

(E) Effects of peroxidase inhibitor salicylhydroxamic acid (SHAM, 100 μM) on *A. brasilense*-mediated primary root elongation. Four-day-old seedlings were transferred to plates containing *A. brasilense* application with or without SHAM for 3 days, and the primary root length was determined. Values represent means ± SD (n = 15). Asterisk indicates statistically significant differences (*$p < 0.05$).

we evaluated cadaverine production in the culture medium. Cadaverine production was clearly observed by HPLC-DAD after pre-column derivatization with dansyl chloride (Figure 8C). Exogenous cadaverine exhibited strong inhibitory effect on primary root elongation (Figures 8D and 8E), and caused enhancement of peroxidase activity (Figure 8F). An RNA-sequencing approach was also utilized to examine overall gene expression in 500 μM cadaverine-treated *Arabidopsis* seedlings. Genes related to cell wall modification and extension like *XTH21*, *XTH24*, *EXPA10* and *GA3OX2* induced by *A. brasilense* were also up-regulated after cadaverine treatment (Figure 8G). Moreover, several genes encoding peroxidase were also enhanced by cadaverine (Figure 8G). Based on Venn diagrams analysis, we analyzed differences and cross talk of cell wall-related gene expression between *A. brasilense* and cadaverine treatment, we identified that *A. brasilense* and cadaverine shared more than 27.3% of these genes (Figure 8G). Taken together, these results suggest that cadaverine secreted by *A. brasilense* may activate peroxidase to inhibit root cell elongation in *Arabidopsis*.

DISCUSSION

Modification of root architecture could improve the ability of plants to explore the soil, which is a fundamentally important strategy to enhance nutrient acquisition and consequently crop productivity. In the present study, the beneficial rhizobacteria *A. brasilense* leads to significant changes in root architecture traits and shows a remarkably growth-promoting effect in *Arabidopsis* plants (Figure 1). It was noticeable that inoculation with *A. brasilense* leads to a dense shallow root system with an inhibited primary root and elongated lateral roots and root hairs (Figure 3). These observations were consistent with Spaepen et al.,[20] who found *A. brasilense* Sp245 significantly reduced the length of primary root but increased the fresh weight of roots and shoots. In addition, other PGPR strains such as *P. brassicacearum*, *P. fluorescens* and *V. paradoxus* have been well-known to inhibit primary root growth while promote the formation of secondary roots. Despite strong evidence that PGPR influence overall root development, the local primary response and molecular mechanisms underpinning cessation of primary root is still less explored.

Our transcriptomics data showed that *A. brasilense* enhances major transcriptional changes in carbon and nitrogen metabolism in *Arabidopsis* roots, which might lead to increases of root biomass. Beneficial bacteria of the genus *Azospirillum* are mainly isolated from grasses and cereals, but they exert growth promoting effects on a wide range of plant species.[22] For example, the stimulatory effect of *Azospirillum* strains on plant growth have been observed in wheat, maize, rice and *Arabidopsis*.[7,20,25] For a long time, the enlargement of root surface by altering root morphology has been considered to be an important mechanism for growth-promoting effects, because of enhanced uptake capacity of nutrient and water by roots.[26,27] In a previous study, Defez et al.[9] suggested that the increased contents of nitrogen, carbon, amino acids, soluble sugars and organic acids in *Medicago sativa* plants after *Ensifer meliloti* strain RD64 inoculation may be associated with increased plant biomass. Here, transcriptomic approaches were applied to unravel the primary molecular responses induced by *A. brasilense* in *Arabidopsis* roots. Compared to control seedlings, 349 and 699 genes were at least two times up- and down-regulated after 2-day inoculation, respectively (Figure 2A). Among the most highly upregulated transcripts were genes involved in biological processes linked to nitrogen metabolism, amino acid metabolism, sugar metabolism, phenylpropanoid biosynthesis and plant hormone signal transduction. As carbon and nitrogen accumulation are the most critical processes for plant growth and development, the transcriptome data indicate that the stimulated and balanced carbon and nitrogen metabolism after *A. brasilense* inoculation might be associated with its growth-promoting effects. Similarly, Schwachtje et al.[28] found that the global transcriptional profile of *Arabidopsis* after beneficial bacteria *Pseudomonas* sp. G62 inoculation strikingly resembled with that on carbohydrate starvation. In a detailed analysis of phenotypic and gene expression, Desrut et al.[29] suggested that PGPR strain *Pseudomonas simiae* WCS417-stimulated growth and development of *Arabidopsis* are associated with sugar transport and carbon allocation. Of interest, a number of genes related to carbon metabolism such as *BGLU21* and *BGLU42*, which encode *β*-glucosidase in plants, were significantly

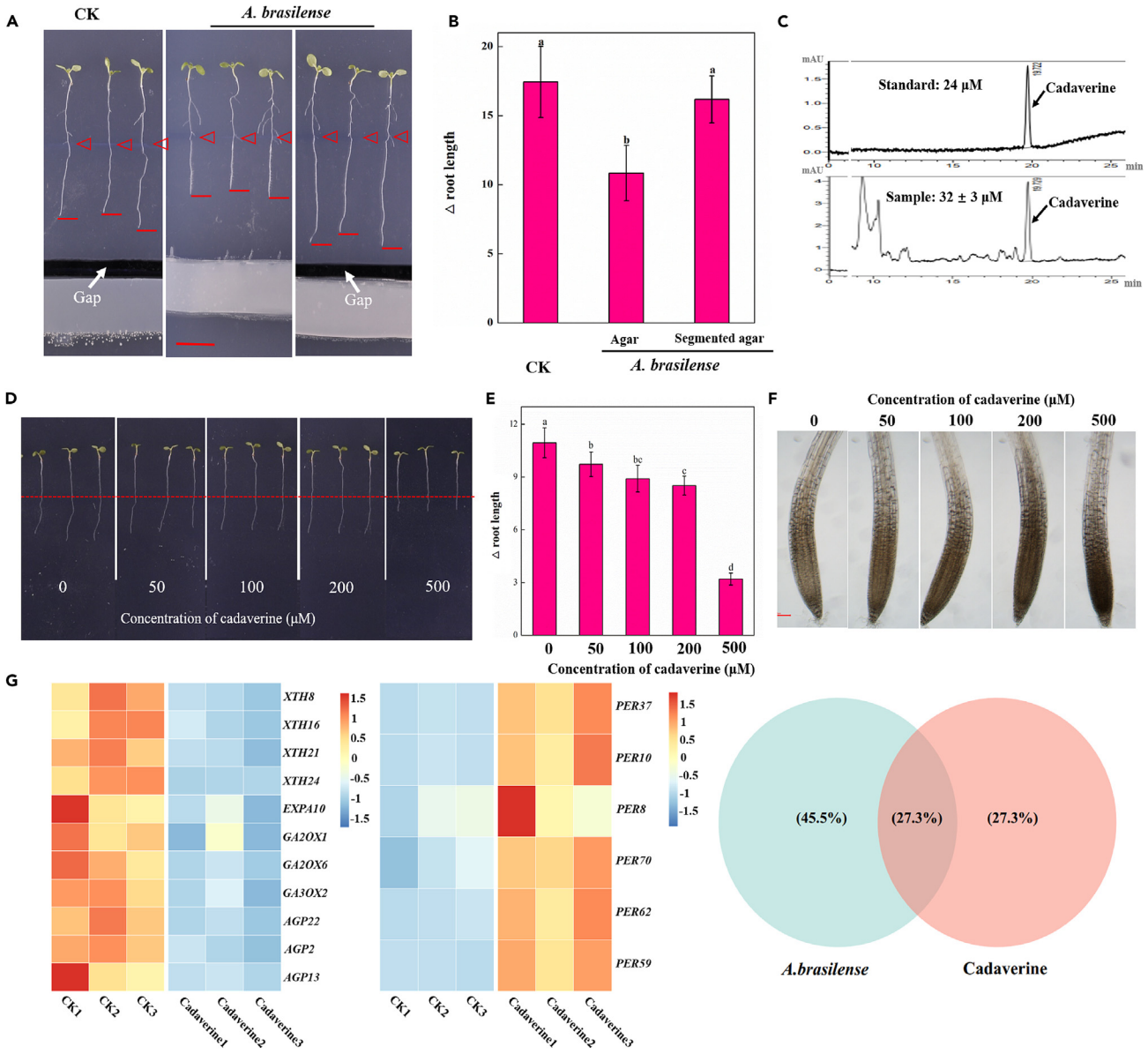

Figure 8. Effects of cadaverine on the development of primary root in *Arabidopsis* seedings

(A and B) Effects of different inoculation methods of *A. brasilense* on the primary root. Four-day-old seedlings were treated with or without *A. brasilense*, sterile scalpel was used to separate the seedlings from the *A. brasilense* by excising the agar strip above the location of the *A.brasilense* inoculation.

(C) Determination of cadaverine produced by *A. brasilense* through PLC-DAD after pre-column derivatisation with dansyl chloride. The bacterial culture of *A. brasilense* was centrifuged and derivatized, and the cadaverine produced by the *A. brasilense* was determined.

(D and E) Effects of different concentrations of cadaverine on the growth of primary root in *Arabidopsis* seedings. Four-day-old seedlings were treated with different concentrations of cadaverine for 2 days, and the length of primary root was measured. Values represent means ± SD (n = 10). Different letters indicate statistically significant differences.

(F) Effects of cadaverine on peroxidase activity in *Arabidopsis* primary roots.

(G) Effects of cadaverine on several genes related to root cell elongation and peroxidase activity.

up-regulated by *A. brasilense*. In addition to carbon metabolism, *A. brasilense* also promotes nitrogen uptake in *Arabidopsis* as the NO_3^- transporter genes of the *NRT2* family, *NRT2.1*, *NRT2.3* and *NRT2.6*, are significantly up-regulated (Figure 2B). Incidentally, this result is consistent with the fact that *P. brassicacearum* STM196-inoculated *Arabidopsis* exhibited increases in plant nitrogen content.[30] Moreover, the putative NO_3^- transporter genes, *NRT2.5* and *NRT2.6*, are significantly enhanced by STM196 in *Arabidopsis* plants.[30,31] However, contrary to *NRT2.1*, *NRT2.6* appeared to promote plant growth

independent on nitrate uptake but dependent on an unknown mechanism.[31] Collectively, findings from the previous studies and the present study suggest that the enhanced and coordinated carbon and nitrogen metabolism might be linked to PGPR growth-promoting effects.

A. brasilense inoculation significantly alters *Arabidopsis* root architecture by reducing primary root length and increasing root hair density and lateral root elongation, presumably enlarging root surface. Proliferation of lateral roots and root hairs by PGPR has been widely observed and comprehensively studied in various plants.[10–13] However, reports on primary root development in response to PGPR are contradictory. It was found that PGPR strain *Burkholderia phytofirmans* PsJN significantly promoted primary root length in *Arabidopsis* plants.[19] In other studies, however, it has been reported that some PGPR strains shows little effect on primary root development, for example, *Phyllobacterium brassicacearum* STM196.[18] In contrast, our results showed that *A. brasilense* inoculation significantly inhibited primary root elongation (Figure 3). For *Arabidopsis*, cessation of primary root growth has also been observed after inoculation of *Pseudomonas* spp. WCS417 and *A. brasilense* Sp245.[10,20] Of particular note, PGPR-induced deep rooting because of primary root promotion might facilitate nitrogen and water uptake for plants grown under certain conditions, whereas dense shallow roots as a result of primary root cessation would increase topsoil foraging and improve the uptake of nutrient of low mobility in soils such as phosphorus. Thus, improved understanding of the mode of action and molecular mechanisms controlling primary root development would facilitate developing bacterial inoculants and microbial consortia for different abiotic stress arising from future climatic conditions.

Postembryonic root growth is maintained by coordinating cell division in the meristem and cell elongation in the elongation zone.[32] Root meristem size and activity are determined by meristematic cell division potential combined with the activity of root stem cell niche, which comprises a quiescent center (QC) and the surrounding stem cells.[33,34] Our results clearly showed that primary root cessation caused by PGPR differed from inhibition-induced by abiotic stresses such as salt, drought and metal stress. Loss of the normal organizing center QC and stem cells, as evidenced by the decreased expression levels of *QC25* which is a QC-specific marker and *WOX5* which is critical for maintaining the stem cell niche, mostly accounts for abiotic stress-repressed root growth.[35–37] Surprisingly, even though the overall organization of the root meristem were targeted for analysis, there was no changes of *pQC25::GUS* and *pWOX5::GFP* after *A. brasilense* inoculation (Figure 4A). Furthermore, neither SHR nor PLT1 protein, coordinately regulating QC identity and stem cell niche activity, was altered by *A. brasilense* (Figure 4A). This observation was consistent with Zamioudis et al.,[10] who found that *P. fluorescens* WCS417 did not impact the organization of the root meristem despite inhibition of primary root growth. Next, we examined whether *A. brasilense* influences cell division potential. The GUS staining of the *CYCB1;1*, an excellent marker for cells undergoing mitosis, and meristem size revealed that *A. brasilense* has no effects on cell division activity (Figures 5A and 5B), and the primary root growth would recover once *A. brasilense* was removed (Figure 4B). These data clearly indicate that neither the overall organization nor cell division potential of the root meristem is affected by *A. brasilense*.

A. brasilense acts to inhibit primary root growth mainly through reducing cell expansion as a result of peroxidase-mediated cell wall disruption. During cell elongation, apoplast acidification activates cell wall loosening enzymes, which, in concert with turgor pressure, enables cell expansion.[38] Among cell wall loosening enzymes, expansins, and xyloglucan endotransglycosylase (XET) have been characterized to play critical roles in regulating cell wall extension, and some specific genes encoding expansins or XET have been reported in regulating primary root growth.[39,40] Indeed, we found that *A. brasilense* significantly down-regulated *EXPA4*, *EXPA10* and five *XTH* genes in *Arabidopsis* roots (Figure 6B). The suppressed expression of EXPAs and XTHs suggestions that the extension of cellulose microfibrils and hemicellulose might be arrested. Another species of cell wall enzymes associated with cell elongation processes is class III peroxidases, which can induce cell wall cleavage as well as cross-linking, and such bifunctional role can be shifted just by changing the H_2O_2 concentration or pH in the apoplast of plant cells.[41] In the present study, *A. brasilense* inoculation up-regulated the expression levels of a large number of peroxidase genes and enhanced their activity (Figures 7A, 7C, and 7D). Increasing H_2O_2 concentration in the elongation zone (Figures 7A and 7B) and restoring root growth under *A. brasilense* treatment with peroxidase inhibitor SHAM (Figure 7E) together support the view that peroxidase inhibited root growth via inducing cell wall cross-linking, which consume H_2O_2, consequently leads to cell wall thickness and stiffness. Likewise, it was recently reported that low phosphorus activates peroxidase activity to rapidly induce cell wall stiffness, which consequently inhibits root cell elongation.[38] Altogether, these results suggest that *A. brasilense* inhibits primary root growth through the reduction of the cell elongation processes, possibly resulting from disruption of cell wall organization.

PGPR influence host plant root traits via complex mechanisms, and three interaction systems have been established between PGPR and host plants, including direct contact, chemical communication via diffusible compounds, signaling via volatiles.[42] Our investigation suggests that cadaverine produced by *A. brasilense* is probably associated with inhibition of primary root growth through regulating peroxidase activity. As all *Azospirillum* are diazotrophic, this species has been commercially used in agriculture production,[22–24] and the associative biological nitrogen fixation has long been recognized as the major contribution to plant growth promotion.[20,26,43] However, nitrogen transfer from bacteria to plants is small, accumulating evidence suggests that plant-growth-regulating compounds produced by *Azospirillum* rather than nitrogen fixation is mostly responsible for their promoting effects.[20,44,45] Some studies have suggested that the volatile organic compounds (VOCs) are bacterial determinants involved in growth promotion.[11] Our results demonstrated that bacterial non-volatile compounds arrested root cell elongation (Figures 8A and 8B). Polyamines are another important biomolecules produced by rhizobacteria with diverse functions in plants.[25,46,47] For instance, Thuler et al.[48] demonstrated that putrescine and spermine were the most abundant polyamines produced by *Azospirillum* strains isolated from manioc roots. The spermidine produced by *Bacillus subtilis* OKB105 enhanced tobacco root growth by inducing expansin expression.[46] In the present study, we found that *A. brasilense* produced ample amounts of cadaverine (Figure 8C), which inhibited primary root cell elongation in *Arabidopsis* as evidence by exogenous cadaverine treatments (Figures 8D and 8E). A recent study conducted by Gibbs et al.,[49] who demonstrated that cadaverine functions to inhibit *Arabidopsis* primary root growth by regulating biotin synthesis, provides additional evidence that cadaverine produced by *A. brasilense* modulates primary root growth in *Arabidopsis*.

In summary, our study reveals that *A. brasilense* shows a remarkably growth-promoting effect in *Arabidopsis* seedlings, and enhances carbon and nitrogen metabolism in plant roots. It was noticeable that *A. brasilense* leads to a dense shallow root system with an inhibited primary root and elongated lateral roots and root hairs. Our results clearly indicate that *A. brasilense*-induced cessation of primary root elongation results from inhibitory effects on cell expansion rather than on the organization and function of the root meristem. Our results also demonstrate that non-volatile compounds like cadaverine released by *A. brasilense* are responsible for inhibition of cell elongation through activating peroxidase-mediated cell wall metabolism.

Limitations of the study

Despite we detected the cell wall thickness as well as the activity and expression of peroxidase and other cell wall loosening enzymes, measurement of the stiffness of the root surface of the transition zone using atomic force microscopy will provide direct evidence. On the hand, bacteria can secrete a large number of compounds that might be responsible for this response in plants, and cadaverine might not be the only compound contributing to the cessation of primary root.

STAR★METHODS

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AUTHOR CONTRIBUTIONS

C.S., H.Z., and L.H. designed the study; H.Z., L.H., and N.S. performed bioinformatics analyses and sample collection; C.S. led the writing with substantial contributions from H.Z., N.S., L.H., R.Q., X.L., and Y.Z.

DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

INCLUSION AND DIVERSITY

While citing references scientifically relevant fort his work, we also actively worked to promote gender balance in our reference list. We avoided "helicopter science" practices by including the participating local contributors from the region where we conducted the research as authors on the paper.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
A. brasilense	BNCC	BNCC139156
Chemicals, peptides, and recombinant proteins		
Cadaherine	Aladdin	Cat#D121817
Murashige and Skoog Basal Medium	Sigma-Aldrich	Cat#M5519
Agar	Sangon Biotech	Cat#A505255-0250
Sucrose	Aladdin	Cat#S112231-500g
NaClO	Sinoreagent	Cat#80010428
Triton X-100	BioFroxx	Cat#1139ML500
LB Broth	Aladdin	Cat#L113084-250g
4-chloro-1-naphthol	Sigma-Aldrich	Cat#C8890-5g
3,3-diaminobenzid	Sigma-Aldrich	Cat#D12384-5g
Acetonitrile	Sinoreagent	Cat#40064160
Critical commercial assays		
Zorbax SB-C18 column	Agilent	Cat#830990-902
Organic filtration	Agilent	Cat#5191-4294
Deposited data		
RNA-seq data	This study, NCBI Sequence Read Archive (SRA) database	SubmissionID: SUB13490117 BioProject ID: PRJNA978962
Experimental models: Organisms/strains		
Arabidopsis thaliana: Columbia-0	In house stock	N/A
Arabidopsis thaliana: *QC25::GUS*	Sun et al.[50]	N/A
Arabidopsis thaliana: *PLT1::GFP*	Sun et al.[50]	N/A
Arabidopsis thaliana: *WOX5::GFP*	Kong et al.[51]	N/A
Arabidopsis thaliana: *SHR::GFP*	Feng et al.[52]	N/A
Arabidopsis thaliana: *CYCB1;1:GUS*	Zhou et al.[53]	N/A
Software and algorithms		
Fiji	On GitHub	https://imagej.net/software/fiji/
Origin	OriginLab	https://www.originlab.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Chengliang Sun (clsun@zju.edu.cn).

Materials availability

This study did not generate new unique reagents and materials.

Data and code availability

- RNA-seq data have been deposited at NCBI SRA, and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. All the data reported in this paper will be shared by the lead contact upon request.

- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Plant and microbe cultivation

Arabidopsis thaliana cessations Columbia (Col-0) ecotype were used. The transgenic lines *QC25::GUS*, *SHR::GFP*, *PLT1::GFP*, *WOX5::GFP*, *SCR::GFP* and *CYCB1;1:GUS* were used in the study.[50–53] *Arabidopsis* seeds were surface-sterilized with 10% (v/v) NaClO containing 1% Triton X-100 solution for 5 min and 75% ethanol for 1 min, washed three times with sterile deionized water, and stored at 4°C for 2 days before sowing. After cold treatment, the seeds were sown on the half-strength Murashige and Skoog (1/2 MS) medium containing 0.5% sucrose and 1% agar (pH = 5.7). Plants were vertically placed in a plant growth chamber under12 h photoperiod with light intensity of 100 μmol m^{-2} s^{-1} at 23°C. After four days, uniform seedlings were selected and used for further experiments. Cadaherine, which is water soluble, is purchased from Aladdin Company (Shanghai, China) and is used for exogenous cadaherine treatment, and the solvent blank is same to other controls.

A. brasilense (BNCC139156) was cultured in Luria-Bertani (LB) liquid medium at 37°C with 140 rpm overnight. The bacteria cells were collected after centrifugation at 1500 rpm for 10 min and finally re-suspended in double-distilled water to a concentration of OD_{600} = 2 (1.8 ×10^9 colony-forming units mL^{-1}). One milliliter of bacterial solution was evenly smeared in LB solid medium, and cultured in the incubator at 28°C under light for another 2 days. Before inoculation of *A. brasilense*, four-day-old seedlings of *Arabidopsis*, were transplanted to a new 1/2 MS solid medium plate. Then, the LB plate of cultured bacteria was cut into 1 cm wide strips and laid in the 2.5 cm distance from the root tip. The plates were sealed with athletic tape and incubated for 2–12 days under the same conditions as described earlier. As the blank LB strip has no effects on plant growth, we removed the blank strip from the control treatment after 4 days to avoid obstacl sensed by roots after contact with the strip barrier.

To test whether the volatile organic compounds released by *A. brasilense* modulate primary root growth, four-day-old seedlings were treated with or without *A. brasilense*, sterile scalpel was used to separate the seedlings from the *A. brasilense* by excising the agar strip above the location of the *A.brasilense* inoculation. And then primary root were analyzed.

METHOD DETAILS

Phenotypic and data analysis

After cocultivation with *A. brasilense* for an indicated time, the root and shoot fresh weight, the number of root hairs and lateral roots, and the length of root hairs were measured and analyzed. For the fresh biomass detection, root and shoot were cut by a scalpel, and the weight of three separate samples was immediately measured on an analytical balance. After two and three days of co-cultivation, the stereomicroscope (Nikon SMZ800N) was used to photograph the root hairs and then using ImageJ to quantify the number and length of the root hairs. The number of emerged LR (greater than 0.5 mm) of at least 20 seedlings was counted using the stereomicroscope (Nikon SMZ800N). Cell length measurements were performed on single cortical cells located in the elongation zone and differentiation zone. Digital confocal images of propidium iodide-stained roots were analyzed with ImageJ software.

GUS histochemical staining

The GUS Histochemical Stain Kit (MM1001-1KIT) was used to detect the β-glucuronidase activity of transgenic lines. Roots of *QC25::GUS* and *CYCB1;1:GUS* marker lines were incubated overnight at 37°C in the GUS reaction buffer solution. The stained roots were washed three times using 70% ethanol and pictured with a Nikon Eclipse E600 microscopy.[11]

Fluorescence microscopy

The transgenic lines *SHR::GFP*, *PLT1::GFP*, *WOX5::GFP*, *SCR::GFP* after control or *A. brasilense* treatment were observed with a fluorescence microscope (Nikon Eclipse E600). The excitation and emission wavelengths were 488 nm and 505–550 nm, respectively.

Transcriptome analysis

The 4-day-old seedlings of *Arabidopsis* were transplanted to new 1/2 MS solid medium plates inoculated with or without *A. brasilense* for another 4 days, and then roots were harvested and stored at −80°C. Three biological replicates were performed. The samples were entrusted to Beijing Novogene for subsequent RNA-seq. The sequencing platform was Illumina NovaSeq 6000, and the sequencing was completed by double-end 150 bp. After sequencing, the software fastp (version 0.19.7) was used for data filtering and quality control. DESeq2 was used to analyze the differentially expressed genes between control group and treatment group, we used a $\log_2$(fold change) ≥ 1 and an adjusted p value ≤ 0.05 as thresholds to determine the DEGs. The software ClusterProfile was used for GO enrichment and KEGG pathway enrichment analysis of differential genes. Heat maps of gene expression patterns were created using R software.

Transmission electron microscopy

Arabidopsis roots were fixed and dehydrated, the transmission electron microscopy analysis was performed according to the procedure described by Zhang et al.[54] Briefly, fresh roots were immediately fixed in 2.5% (v/v) glutaraldehyde in sodium phosphate buffer (0.2 M), and postfixed in 1% (w/v) OsO_4 for 2 h. After washing with PBS buffer, roots were dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90% and 95%; v/v; 15 min each time). Then, the roots were sequentially treated with ethanol and acetone for 20 min for each treatment. Finally, roots were infiltrated and embedded in Spurr's resin, and polymerized at 70°C for 16 h. Transverse sections were prepared in an ultramicrotome (Leica EM UC7), and cross-sections were examined using a transmission electron microscope (H-7650, Hitachi). The cell wall thickness was calculated with ImageJ software.

Detection of peroxidase activity and H_2O_2 content

Peroxidase activity was visualization by staining *Arabidopsis* roots with 4-chloro-1-naphthol according to Balzergue et al.[38] Briefly, a 1 mL aliquot of a 3% 4-chloro-1-naphthol (30 mg mL^{-1} dissolved in absolute ethanol) and 1 mL of 3% H_2O_2 were added to 10 mL Tris buffer (250 mM pH = 7.4) to prepare the working solution. *Arabidopsis* roots were stained in working solution for 1 min and then washed three times in Tris buffer and pictured with microscopy (Nikon Eclipse E600). The distribution of H_2O_2 in the root tips was characterized with 3,3-diaminobenzid (DAB) staining.[55] Root were incubated in 1 mg mL^{-1} DAB (dissolved in 50 mM Tris-HCl buffer) at 25°C in darkness for 2 h. After washing, the roots were photographed microscopy (Nikon Eclipse E600).

Measurement of cadaverine contents

The LB liquid medium with *A. brasilense* culture for 2 days was centrifuged at 8000 rpm at 4°C for 10 min, and 10 mL of the supernatant was added to 10 mL of 5%TCA to make the sample treatment solution. A 10 mL aliquot of the sample solution or standard solution were taken and adjusted pH to 9.5 with sodium hydroxide solution, then reacted with 10 mL of 10 mg mL^{-1} dansyl chloride (dissloved in acetone) for derivatisation at 60°C for 30 min. Then, the reaction liquid was cooled to room temperature and 1 mL of 25% ammonium hydroxide was added. After standing for 30 min, the reaction liquid was adjusted to 25 mL with acetonitrile, then passed through 0.22 μm organic filtration membrane and stored at −20°C until tested. The quantification of cadaverine was carried out using an Agilent Technologies Agilent 1290 Infinity (Agilent Technologies. SantaClara, California, USA) consisting of a quaternary pump and a diode array detector (DAD). Cadaverine was separated using the Zorbax SB-C18 column (2.1 × 150 mm, 3.5 μm, Agilent Technologies. Santa Clara, California, USA) at a column temperature of 30°C. The mobile phases were ultrapure water (A) and acetonitrile (B). The linear gradient programs of phase A as follows: 0–1 min 40%; 1–9 min 25%; 9–10 min 20%; 10–12 min 5%; 12–15 min 0; 15–20 min 40%. The flow rate was 1.0 mL min^{-1} and the sample injection volume was 20 μL, and the detecting wavelength was 254 nm.[56]

QUANTIFICATION AND STATISTICAL ANALYSIS

The values in the tables and figures were calculated as the mean ± SD. The statistical significance among treatments was performed through one-way ANOVA, followed by Duncan posthoc, and significant difference was defined when P was <0.05 (*$p < 0.05$, **$p < 0.01$).