Bradyrhizobium japonicum* IRAT FA3 Alters *Arabidopsis thaliana* Root Architecture via Regulation of Auxin Efflux Transporters *PIN2*, *PIN3*, *PIN7*, and *ABCB19

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Beneficial rhizobacteria can stimulate changes in plant root development. Although root system growth is mediated by multiple factors, the regulated distribution of the phytohormone auxin within root tissues plays a principal role. Auxin transport facilitators help to generate the auxin gradients and maxima that determine root structure. Here, we show that the plant-growth-promoting rhizobacterial strain *Bradyrhizobium japonicum* IRAT FA3 influences specific auxin efflux transporters to alter *Arabidopsis thaliana* root morphology. Gene expression profiling of host transcripts in control and *B. japonicum*-inoculated roots of the wild-type *A. thaliana* accession Col-0 confirmed upregulation of *PIN2*, *PIN3*, *PIN7*, and *ABCB19* with *B. japonicum* and identified genes potentially contributing to a diverse array of auxin-related responses. Cocultivation of the bacterium with loss-of-function auxin efflux transport mutants revealed that *B. japonicum* requires *PIN3*, *PIN7*, and *ABCB19* to increase lateral root development and utilizes *PIN2* to reduce primary root length. Accelerated lateral root primordia production due to *B. japonicum* was not observed in single *pin3*, *pin7*, or *abcb19* mutants, suggesting independent roles for *PIN3*, *PIN7*, and *ABCB19* during the plant-microbe interaction. Our work demonstrates *B. japonicum*'s influence over host transcriptional reprogramming during plant interaction with this beneficial microbe and the subsequent alterations to root system architecture.

Keywords: auxin transport, bacteria–plant symbiosis, lateral root primordia, lateral roots, plant-growth-promoting rhizobacteria, plant hormones, primary root

Plant-growth-promoting rhizobacteria (PGPR) are soil microorganisms that stimulate plant growth and can be utilized in crop agriculture to increase yield and provide abiotic and biotic protection (Backer et al. 2018; Gachomo et al. 2014; Ryu et al. 2003).

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Among soil bacteria, the *Bradyrhizobium* genus is the most ubiquitous (Shah and Subramaniam 2018). Because these bacteria are such a major component of the soil microbiota, greater understanding of mutualistic *Bradyrhizobium* microbe–plant interactions has the potential to increase plant production and benefit agriculture.

PGPR are often associated with alterations to root morphology and metabolism of various phytohormones (Loper and Schroth 1986; Ryu et al. 2003; Zamioudis et al. 2013). Specifically, *Bradyrhizobium japonicum* strains are known to produce plant hormones and hormone precursors, including those associated with major auxin, ethylene, or abscisic acid plant signaling pathways (Boiero et al. 2007; Hodge et al. 2009). Stimulation of root growth by PGPR may be the result of volatile or diffusible compounds, produced by the microbes, that serve as phytohormones, precursors, or other cues that affect host cell genetic reprogramming and consequential growth promotion (Shi et al. 2010; Spaepen et al. 2007).

Previous research has established the key roles of auxin synthesis, transport, and signaling in root growth (Du and Scheres 2018; Hodge et al. 2009; Overvoorde et al. 2010). Auxin, predominantly found as indole-3-acetic acid (IAA) in plants, is transported between plant cells and tissues, resulting in a regulated distribution that directs plant development (Petrásek and Friml 2009). In its protonated form, IAA (IAAH) can diffuse through the plasma membrane but, in its anionic form, IAA[−] is actively transported by auxin influx facilitators (Delbarre et al. 1996). Inside the neutral cellular environment (approximately pH 7), anionic IAA[−] predominates, requiring auxin efflux carriers to transport it out of the cell (Ng et al. 2015; Žažimalová et al. 2010). Plasma membrane (PM) location of influx and efflux transporters decides directionality of auxin ion movement but polar localization of the efflux carrier proteins can change as internal or environmental cues direct or redirect auxin flow through plant tissues (Geisler et al. 2017; Kleine-Vehn et al. 2008; Tanaka et al. 2006). Auxin transport determines auxin distribution, localized accumulation, and gradient formation in the plant (Benková et al. 2003; Grieneisen et al. 2007; Overvoorde et al. 2010). The auxin efflux system and auxin feedback mechanisms are essential for maintaining these auxin gradients and constructing the root system architecture (Benková et al. 2003; Tanaka et al. 2006). Root polar auxin transport takes place as IAA moves from cell to cell through root tissues. IAA acropetal flux (toward the root tip) predominantly travels through the stele, endodermis, and cortex, while major basipetal transport (from the root tip toward the base) occurs in the lateral root cap, epidermis, and cortex (Jozef et al. 2017; Laskowski and ten Tusscher 2017; Leyser 2006; Muday and DeLong 2001).

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Two groups of auxin efflux carriers are found in the PIN-FORMED (PIN) protein family and ATP-binding cassette subfamily B (ABCB)-type members of the multidrug resistance/phosphoglycoprotein (ABCB/MDR/PGP) protein family (Noh et al. 2001; Petrásek and Friml 2009; Žažimalová et al. 2010). PIN proteins transport auxin across cellular membranes and have various functional roles in shoots and roots (Mohanta et al. 2018; Zhang et al. 2020). PINs are redundantly involved in stages of primary root (PR) and lateral root (LR) development (Blilou et al. 2005). Distinct root architecture phenotypes may not be apparent or vary in many single *pin* mutants but multiple *pin* gene mutations have shown clear disruptions to PR and LR patterning (Benková et al. 2003; Blilou et al. 2005; Laskowski et al. 2008; Lewis et al. 2011). Among the eight PIN proteins of the model plant *Arabidopsis thaliana*, PIN1 to PIN4 and PIN6 and PIN7 localize predominantly to the PM in a polar orientation that guides directional auxin flow (Křeček et al. 2009; Zhou and Luo 2018). ABCB4 and ABCB19 (and other ABCBs) are also shoot- and root-expressed intercellular auxin transporters; however, they exhibit mostly nonpolar localization in the PM (Blakeslee et al. 2007; Cho and Cho 2013; Jenness et al. 2020). ABCBs, having more stable and often symmetrical PM distribution, may predominantly regulate cellular auxin homeostasis, while PINs, highly responsive to internal or external signals and with polar PM localization, direct auxin flow to form auxin gradients in root tissues (Cho and Cho 2013). PIN-ABCB interactions and PIN protein turnover have been shown to enhance auxin transport levels or specificity (Blakeslee et al. 2007; Cho and Cho 2013; Geisler et al. 2017).

PIN and ABCB regulation of auxin distribution leads to root system architecture development. PR and LR morphogenesis are determined by auxin transport facilitator maintenance and alterations of auxin gradients, complex subcellular molecular interactions, internal developmental programs, and external stimuli (Sauer and Kleine-Vehn 2019; Tanaka et al. 2006). Auxin increases PR length at very low concentrations but high auxin doses inhibit PR elongation (Simon et al. 2013; Stoeckle et al. 2018; Waidmann et al. 2020). Moreover, auxin is the dominant hormonal regulator of LR development (Cavallari et al. 2021; Fukaki and Tasaka 2009; Overvoorde et al. 2010). LR growth encompasses LR primordia (LRP) initiation and progression, as well as LR emergence and outgrowth; these, together with higher-order LR branching, are the major contributing factors to root system size (Waidmann et al. 2020). LR development can be stimulated by both local and overall auxin increases (Boerjan et al. 1995; Dubrovsky et al. 2008). Root development heavily influenced by PGPR often leads to increases in root system size which can enhance nutrient uptake that supports increases in shoot growth and yield (Bailly et al. 2014; Gachomo et al. 2014; Ryu et al. 2003). Auxin can be one of the major factors produced by PGPR, yet it is likely that additional compounds crosstalk with auxin, in concert with the plant, to promote growth (Asari et al. 2017; Semerádova et al. 2020). Many bradyrhizobia are known for symbiotic associations with plants, inducing nodule formation in legumes. *B. japonicum* IRAT FA3 (*B. japonicum*) has been shown to promote growth in *A. thaliana* and maize (Gachomo et al. 2014). We are further characterizing it here to better understand the mechanisms it uses to promote plant development, with emphasis on enhanced lateral root formation, in *A. thaliana*. By profiling host transcripts in *B. japonicum*-inoculated roots of the wild-type *A. thaliana* accession Columbia (Col-0), we identified genes potentially contributing to a diverse array of auxin-related responses. We used transgenic *A. thaliana* lines with mutations in genes known to have roles in root development and auxin transport to further elucidate auxin pathway points of influence by *B. japonicum* and found four specific auxin efflux transporters to have nonredundant functions required for

B. japonicum-stimulated root growth modification. This work proposes mechanisms by which *B. japonicum* strain IRAT FA3 promotes alterations in *A. thaliana* root architecture.

RESULTS

B. japonicum stimulates root architecture alterations in *A. thaliana*.

B. japonicum strain IRAT FA3 has been shown to increase plant biomass (Gachomo et al. 2014). To closely examine specific root growth responses to *B. japonicum*, bacteria were applied directly to *A. thaliana* seedling roots (cocultivation) grown on agar plates. Inoculation of wild-type (Col-0) plant roots decreased the PR length and increased LR development (Fig. 1). Following 6 days of cocultivation, PR length was significantly reduced by 44.8% compared with control-treated roots (Fig. 1B). One of the most prominent *B. japonicum*-mediated morphological alterations in the root system architecture was the stimulation of LR formation (Fig. 1). The LR number was significantly higher as early as 2 days postinoculation (dpi) in *B. japonicum*-inoculated plants. After 8 dpi, the average number of total LR per seedling was 70% higher than that of the noninoculated plants (Fig. 1C).

B. japonicum produces auxin and activates auxin responses in *A. thaliana*.

A relationship between IAA accumulation and rhizobacteria-stimulated plant growth has been established between certain PGPR and plants (Loper and Schroth 1986; Spaepen et al. 2007). The root phenotype induced by *B. japonicum* is a typical response to auxin (Woodward and Bartel 2005). The increased LR phenotype can be mimicked by the exogenous addition of 1 μ M naphthalene-1-acetic acid (NAA) or 10 μ M indole-3-butyric acid (IBA), as seen in our data presented in Supplementary Fig. S1, as well as in previous work (Simon et al. 2013). The auxin compound solvent 0.1% dimethyl sulfoxide (DMSO) did not appear to affect *B. japonicum*'s ability to induce LR production (Supplementary Fig. S1). Serving as a potential negative control for *B. japonicum*, we also tested *B. diazoefficiens* (previously *B. japonicum*) strain USDA 110 LI with *A. thaliana*. USDA 110 LI did not induce increased LR production in Col-0 plants in our LR counting assay, and its IAA production in culture was undetectable (Supplementary Fig. S2). USDA 110 LI, a USDA 110 derivative, is considered an ineffective strain because it lacks symbiotic N₂-fixation ability (Kuykendall and Elkan 1976).

Tryptophan is a precursor of IAA in higher plants that has been found in root exudates (Kamilova et al. 2006; Vacheron et al. 2013). IAA can be synthesized from tryptophan by some rhizobacteria (Kamilova et al. 2006; Spaepen et al. 2007). We quantified the actual (no L-tryptophan addition) and potential (2 mM L-tryptophan added) production of IAA by *B. japonicum* grown in Luria-Bertani (LB) broth using spectrophotometry (Gordon and Weber 1951). The potential IAA production was approximately three to four times the actual at 24 and 48 h, respectively, in culture (Fig. 2A). The auxin-responsive gene *GH3.3*, involved in auxin conjugation, was significantly upregulated by *B. japonicum* in *A. thaliana* roots within the first 24 h of cocultivation (Fig. 2B).

To further determine whether *B. japonicum* could influence internal plant auxin levels, transgenic *A. thaliana* plants expressing the synthetic *DIRECT REPEAT 5* (*DR5*) auxin-responsive promoter fused to the β -glucuronidase (*GUS*) reporter gene (*DR5:GUS*) were employed (Casimiro et al. 2001; Ulmasov et al. 1997; Zamioudis et al. 2013). At 4 dpi, *GUS* staining in control plants was detected in the PR tip, in LRP, and at the tips of emerged LR (Fig. 2C). In *B. japonicum*-inoculated seedlings, *GUS* staining was remarkably enhanced in the root tip and along

the entire root, including LRP and LR (Fig. 2C). Because *DR5* activity correlates with auxin content in roots, accumulation of the *DR5* reporter indicated increased root auxin levels with *B. japonicum* treatment, likely due to enhanced environmental IAA produced by the bacteria surrounding the roots (Casimiro et al. 2001). The increase in auxin responsiveness in the presence of *B. japonicum* suggests microbial interference with plant auxin concentration and subsequent auxin-regulated gene expression.

Inhibition of auxin efflux transport reduces *B. japonicum*-induced LR development.

Auxin movement in and out of cells is controlled by membrane diffusion and carrier-mediated transport (Gutknecht and Walter 1980; Kramer and Bennett 2006). Application of the auxin efflux inhibitor N-1-naphthylphthalamic acid (NPA)

(1 μ M) was used to investigate the role of auxin efflux transport in *B. japonicum*-induced root alteration. NPA has been shown to block basipetal auxin transport and arrest LR growth (Casimiro et al. 2001; Teale and Palme 2018). Our data substantiated previous findings, as demonstrated by a decrease in LR number in control plants grown on agar media containing 1 μ M NPA (Fig. 3A). The presence of NPA in the growth media also significantly reduced the number of LR in plants treated with 1 μ M NAA, *B. japonicum* culture filtrate, or *B. japonicum* cells at 4 and 8 dpi (Fig. 3A). NPA decreased *B. japonicum*-stimulated LR formation to the level of untreated control plants (Fig. 3A), suggesting that auxin efflux transport inhibited by NPA is required for *B. japonicum*-induced LR increases. The number of LR formed by the addition of NAA was higher than those formed after treatment with *B. japonicum* or its culture filtrate.

Fig. 1. *Bradyrhizobium japonicum* cocultivation induces primary root (PR) length reduction and increases lateral root (LR) number in *Arabidopsis thaliana*. **A**, *A. thaliana* Columbia (Col-0) seedlings were grown with either *B. japonicum* bacterial suspension or 10 mM MgSO₄ (control). **A**, Representative photos of Col-0 root architecture 14 days postinoculation (dpi). **B**, Average PR length 6 dpi, gray = control and black = *B. japonicum*, $P < 0.05$. **C**, Col-0 LR number was counted under microscopy every 2 dpi. Solid line = *B. japonicum* and dotted line = control; error bars represent standard error for six biological replicates, $n > 10$. Student's t test, *, **, and *** indicate $P < 0.05$, 0.01, and 0.001, respectively.

Fig. 2. *Bradyrhizobium japonicum* produces auxin in culture and enhances auxin responses in plants. **A**, Indole-3-acetic acid (IAA) concentration increased in the bacterial culture when L-tryptophan (L-Trp) was added, as measured by spectrophotometry. **B**, Reverse-transcription PCR measurement of *GH3.3* expression relative to the *ACTIN* reference gene in roots of control and *B. japonicum*-inoculated plants at 4, 8, and 24 h postinoculation (hpi). Student's t test determined significant differences at each time point, $P < 0.05$. **C**, Images of stained *DR5::β-glucuronidase* roots 4 days postinoculation (dpi). LRP = lateral root primordia, LR = lateral root, and PR = primary root; bar = 50 μ m.

The greatest percent LR reduction by NPA was observed in control and NAA-treated plants, while the lowest was in plants treated with *B. japonicum* or its culture filtrate (Supplementary Fig. S3). Primary root length was reduced by NPA, *B. japonicum*, and culture filtrate and was arrested by the combination of *B. japonicum* plus NPA (Fig. 3B). Because NPA and DMSO did not hinder the growth of *B. japonicum* cells in culture (Supplementary Fig. S4), the compounds do not appear to disrupt bacterial function. In addition, *B. japonicum* displayed a lack of microbe sensitivity to the treatment compounds when grown on

Fig. 3. Inhibition of auxin efflux transport affects *Bradyrhizobium japonicum*-stimulated lateral root (LR) growth and primary root (PR) length. Columbia (Col-0) 5-day-old plants were transferred to agar plates containing the auxin efflux inhibitor N-1-naphthylphthalamic acid (NPA; 1 μM) in 0.1% dimethyl sulfoxide (DMSO), or solvent alone. These plants were treated with 10 μl of either 1 μM naphthalene-1-acetic acid (NAA), *B. japonicum* culture filtrate, *B. japonicum*, or their control, added to root tips. **A**, LR numbers were counted at 0, 4, and 8 days postinoculation (dpi). **B**, PR lengths were measured at 0, 4, and 8 dpi. **C**, *B. japonicum* CFU were recovered from roots treated with no compound (NC), 0.1% DMSO, or 1 μM NAA at 0 and 8 dpi. Graphs are averages from two independent experiments; standard error bars, $n > 10$. Data-appropriate tests (A and B, Welch's analysis of variance [ANOVA] and Games-Howell; C, ANOVA and Tukey), $P < 0.05$, were used to determine significant differences. For A and B, statistical analysis was performed separately for each time point.

plates (Fig. 3C). No significant differences were seen between *B. japonicum* CFU recovered from roots treated with either no compound (NC), 0.1% DMSO, or 1 μM NAA at 8 dpi (Fig. 3C).

B. japonicum increases PIN and ABCB gene expression.

Our results strongly suggested that auxin efflux carriers play a role in *B. japonicum*-induced root architecture alterations. Therefore, we investigated *B. japonicum*-stimulated gene expression modifications by performing RNA-sequencing (RNA-seq) analysis of Col-0 whole-root tissue. A general increase in auxin-related gene transcript levels was evident in the *B. japonicum*-inoculated roots (Supplementary Fig. S5), and more than 70 differentially regulated distinct auxin-related genes were identified at 14 dpi (Table 1). In the RNA-seq data, significant increases in transcripts of auxin efflux transporters *PIN2* (1.44 log₂-fold-change [FC]), *PIN7* (4.15 FC), and *ABCB19* (1.22 FC) were observed under *B. japonicum* cocultivation (Table 1). *PIN3* transcripts increased (mean read counts were nearly 41% higher than control) but the data were not statistically significant. *ABCB4* and *PIN1* transcript levels did not show a clear change between inoculated and control roots. Other auxin-related genes upregulated by *B. japonicum* included *ARF7* (6.60 FC), *IAA14* (4.31 FC), *PILS7* (4.15 FC), *ICR1* (3.68 FC), *GH3.6* (2.41 FC), and *ARF5* (2.27 FC), while among the downregulated genes were *IAA28* (−1.08 FC), *PILS3* (−1.10 FC), and *BIG* (−6.88 FC) (Table 1). Sequential activation of auxin response modules during LR development is a highly coordinated event (Lavenus et al. 2015). Our RNA-seq data are a snapshot of *B. japonicum*'s influence over host transcriptional reprogramming in the root system (Table 1; Supplementary Fig. S5).

Quantitative real-time PCR (qRT-PCR) gene expression studies also illustrated transcriptional changes in auxin transporters after treatment with *B. japonicum*. We measured transcript levels of six major auxin efflux transporters in the roots of Col-0 plants at 12 h postinoculation (hpi). The 12-h time point was chosen to catch early transcriptional changes in response to *B. japonicum* cocultivation. *PIN3*, *PIN7*, and *ABCB19* were upregulated in *B. japonicum*-treated whole-root tissues (Fig. 4). *ABCB4* expression increased, but not significantly. There were no significant differences between inoculated and noninoculated root transcript levels of *PIN1* or *PIN2* in the qRT-PCR data (Fig. 4). qRT-PCR and RNA-seq analyses detected significant increases in *PIN7* and *ABCB19* expression with *B. japonicum*. RNA-seq analysis detected an increase in *PIN2* expression whereas *PIN3* upregulation was found to be significantly higher only in the qRT-PCR experiments ($P < 0.01$) in inoculated roots. Differential observations between the two assays reflect gene expression fluctuations over time with *B. japonicum* cocultivation.

Auxin efflux transport mutants display altered *B. japonicum*-induced LR growth.

To further demonstrate putative roles for auxin efflux carriers in *B. japonicum*-induced root alterations, we investigated the effect of *B. japonicum* on LR formation in *A. thaliana* single-gene mutants with interrupted function in *PIN2* (*pin2* and Salk_122916C), *PIN3* (*pin3-3* and *pin3-4*), *PIN7* (*pin7* and Salk_048791C), *ABCB4* (*abcb4-2* and *pgp4-1*), and *ABCB19* (*abcb19-3* and *atmdr1-101*) (Fig. 5). Disruption of auxin efflux transport genes in *A. thaliana* mutants resulted in differential LR growth when cocultivated with *B. japonicum* (Fig. 5). Although higher in the *pin2* alleles, the LR number per seedling in noninoculated *pin3*, *pin7*, *abcb4*, and *abcb19* mutant plants was similar to Col-0 at 8 dpi (Fig. 5). In *B. japonicum*-inoculated plants, LR numbers increased in wild type, *pin2*, and *abcb4* lines (Fig. 5). However, *B. japonicum* did not increase LR numbers in *pin3*, *pin7*, and *abcb19* as it did in wild-type plants (Fig. 5). Without functional *PIN3*, *PIN7*, or *ABCB19* genes, the inoculated LR

Table 1. RNA-sequencing differentially expressed auxin-related genes

AGI	Log ₂ -fold change	Gene	Gene name and descriptive phrases ^a
AT5G05730.2	6.78	<i>ASA1</i> ^b	<i>Anthranilate synthase α subunit 1</i> ; tryptophan synthesis, lateral root formation, response to bacterium
AT5G20730.1	6.60	<i>ARF7</i>	<i>Auxin response factor 7</i> ; auxin-regulated transcriptional activator
AT1G28560.3	4.70	<i>SRD2</i> ^b	<i>Shoot redifferentiation defective 2</i> ; organ morphogenesis, polar transport, cellular response to auxin stimulus, lateral root formation, meristem initiation
AT4G02570.1	4.70	<i>AXR6</i>	<i>Auxin-resistant 6</i> ; mediating responses to auxin
AT1G14350.4	4.70	<i>FLP</i>	<i>Four lips</i> ; auxin polar transport, regulation of lateral root development
AT3G44310.2	4.59	<i>NIT1</i>	<i>Nitrilase 1</i> ; auxin biosynthetic process, indole-3-acetonitrile (IAN) to indole-3-acetamide (IAM) conversion
AT4G13260.1	4.34	<i>YUC2</i>	<i>Yucca 2</i> ; indole-3-pyruvic acid (IPA) to indole-3-acetic acid (IAA) conversion, auxin biosynthetic process
AT4G14550.2	4.31	<i>SLR</i>	<i>Solitary root</i> , (aka <i>IAA14</i>); lateral root development, expressed in stele and root tip epidermis
AT5G65980.1	4.15	<i>PILS7</i>	<i>Pin-like 7</i> ; auxin efflux carrier
AT3G23050.2	4.12	<i>AXR2</i> ^b	<i>Auxin-resistant 2</i> , (aka <i>IAA7</i>); repressor of auxin-inducible gene expression
AT5G62380.1	3.98	<i>NAC101</i>	<i>NAC-domain protein 101</i> ; response to auxin
AT2G46370.1	3.95	<i>JAR1</i> ^b	<i>Jasmonate-resistant 1</i> ; member of the GH3 family of proteins, cellular response to auxin stimulus
AT5G48070.1	3.72	<i>XTH20</i>	<i>Xyloglucan endotransglycosylase/hydrolase 20</i> ; expressed in lateral root primordia, cell growth, cellular response to auxin stimulus
AT1G17140.2	3.68	<i>ICR1</i>	<i>Interactor of constitutively active ROP1</i> ; regulation of auxin polar transport
AT1G25220.2	3.55	<i>ASB1</i> ^b	<i>Anthranilate synthase β subunit 1</i> , (aka <i>WEI7</i>); auxin biosynthetic process, lateral root formation, response to bacterium
AT5G63160.3	3.52	<i>BT1</i>	<i>BTB and TAZ domain protein 1</i> ; response to auxin
AT2G23260.1	3.38	<i>UGT84B1</i>	<i>UDP-glucosyltransferase 84 B 1</i> ; regulation of auxin metabolic process
AT2G01940.3	3.25	<i>IDD15</i>	<i>Indeterminate(ID)-domain 15</i> ; regulation of auxin biosynthesis and transport
AT1G23080.3	2.89	<i>PIN7</i>	<i>Pin-formed 7</i> ; auxin efflux, pattern specification during root development
AT1G25250.3	2.85	<i>IDD16</i>	<i>Indeterminate(ID)-domain 16</i> ; regulation of auxin biosynthesis and transport
AT2G22330.1	2.69	<i>CYP79B3</i>	<i>Cytochrome P450 family 79 subfamily B polypeptide 3</i> ; tryptophan metabolism
AT5G54490.1	2.46	<i>PBP1</i>	<i>Pinoid-binding protein 1</i> ; upregulated by auxin
AT5G54510.1	2.41	<i>GH3.6</i>	<i>Gretchen Hagen 3.6</i> ; auxin homeostasis, auxin-activated signaling pathway, response to auxin, unidimensional cell growth
AT1G28130.1	2.39	<i>GH3.17</i>	<i>Gretchen Hagen 3.17</i> ; auxin homeostasis, response to auxin
AT1G19850.1	2.27	<i>ARF5</i>	<i>Auxin response factor 5</i> , (aka <i>IAA24</i>)
AT1G75580.1	2.15	<i>SAUR51</i>	<i>Small auxin upregulated RNA 51</i> ; response to auxin
AT3G62100.1	2.15	<i>IAA30</i>	<i>IAA inducible 30</i> ; response to auxin, root quiescent center
AT4G39950.1	1.84	<i>CYP79B2</i>	<i>Cytochrome P450 family 79 subfamily B polypeptide 2</i> ; tryptophan metabolism, indoleacetic acid biosynthetic process, response to bacterium
AT1G68130.1	1.70	<i>IDD14</i>	<i>Indeterminate (ID)-domain 14</i> ; regulation of auxin biosynthesis and transport
AT4G31500.1	1.69	<i>SUR2</i>	<i>Superroot 2</i> ; adventitious root development, defense response to bacterium, indoleacetic acid biosynthetic process, tryptophan biosynthetic process
AT4G37390.1	1.67	<i>AUR3</i>	<i>Auxin-regulated 3</i> ; regulation of auxin activity
AT1G02335.1	1.67	<i>PDGLP2</i>	<i>Plasmodesmal germin-like protein 2</i> ; primary root growth regulation by controlling phloem-mediated allocation of resources between the primary and lateral root meristems
AT5G05730.1	1.55	<i>ASA1</i> ^b	<i>Anthranilate synthase α subunit 1</i> ; tryptophan synthesis, lateral root formation, response to bacterium
AT1G12140.1	1.46	<i>DAO2</i>	<i>Dioxygenase for auxin oxidation 1</i> ; auxin homeostasis, expressed in root caps
AT2G22240.1	1.45	<i>MIPS2</i> ^b	<i>Myo-inositol-1-phosphate synthase isoform 2</i> ; defense response to bacterium, response to auxin
AT5G57090.1	1.44	<i>PIN2</i>	<i>Pin-formed 2</i> , (aka <i>EIR1</i>); auxin efflux, auxin polar transport, response to auxin
AT1G59750.2	1.39	<i>ARF1</i>	<i>Auxin response factor 1</i> ; mediator of auxin response gene expression, negative regulation of transcription, regulation of transcription
AT3G23050.1	1.36	<i>AXR2</i> ^b	<i>Auxin-resistant 2</i> , (aka <i>IAA7</i>); repressor of auxin-inducible gene expression
AT2G04160.1	1.35	<i>AIR3</i>	<i>Auxin induced root cultures 3</i> ; lateral root morphogenesis, response to auxin
AT2G21050.1	1.24	<i>LAX2</i>	<i>Like auxin-resistant 2</i> ; auxin influx carrier
AT4G14740.1	1.24	<i>FL3</i>	<i>Forked-like 3</i> ; auxin-activated signaling pathway
AT1G48690.1	1.23	No name	Auxin-responsive GH3 family protein
AT3G28860.1	1.22	<i>ABC19</i>	<i>ATP-binding cassette B19</i> ; auxin transport
AT2G37080.3	1.18	<i>RIP2</i>	<i>ROP interactive partner 2</i> ; response to auxin
AT5G66700.1	1.16	<i>HB53</i>	<i>Homeobox 53</i> ; auxin-inducible, root development
AT2G06850.1	1.14	<i>XTH4</i>	<i>Xyloglucan endotransglucosylase/hydrolase 4</i> ; secondary cell wall biogenesis, response to auxin

(Continued on next page)

^a Minimal phrases representative of genes from The Arabidopsis Information Resource; aka = also known as.^b Splice variants are differentially regulated.

Table 1. (Continued from previous page)

AGI	Log ₂ -fold change	Gene	Gene name and descriptive phrases ^a
AT1G56220.4	1.11	No name	Dormancy/auxin associated family protein
AT1G04240.1	1.08	<i>SHY2</i>	<i>Short hypocotyl 2</i> , (aka <i>IAA3</i>); regulates multiple auxin responses in roots
AT1G04250.1	1.05	<i>AXR3</i>	<i>Auxin-resistant 3</i> , (aka <i>IAA17</i>); repressor of auxin-inducible gene expression, auxin signaling
AT1G09560.1	1.04	<i>PDGLP1</i>	<i>Plasmodesmal germin-like protein 1</i> ; primary root growth regulation by controlling phloem-mediated allocation of resources between the primary and lateral root meristems
AT2G20610.2	1.02	<i>SUR1</i>	<i>Superroot 1</i> , (aka <i>ALF1</i>); auxin production, lateral roots, adventitious root development, indoleacetic acid biosynthetic process, regulation of cell growth by extracellular stimulus
AT1G25220.1	1.01	<i>ASB1^b</i>	<i>Anthranilate synthase β subunit 1</i> , (aka <i>WEI7</i>); auxin biosynthetic process, lateral root formation, response to bacterium
AT5G25620.1	-1.01	<i>YUC6</i>	<i>Yucca 6</i> ; auxin biosynthesis, response to oxidative stress
AT1G51950.1	-1.03	<i>IAA18</i>	<i>IAA inducible 18</i> ; response to auxin
AT1G22530.2	-1.06	<i>PATL2</i>	<i>Patellin 2</i> ; cellular response to auxin stimulus, auxin polar transport
AT5G25890.1	-1.08	<i>IAA28</i>	<i>IAA inducible 28</i> ; negative regulation of auxin responsive lateral root formation, resistance to inhibition of root elongation by auxin
AT3G44300.1	-1.10	<i>NIT2</i>	<i>Nitrilase 2</i> ; indoleacetic acid biosynthetic process, response to bacterium
AT1G76520.1	-1.10	<i>PILS3</i>	<i>Pin-like 3</i> ; auxin efflux carrier family protein
AT4G28720.1	-1.13	<i>CKRC2</i>	<i>Cytokinin induced root curling 2</i> , (aka <i>YUC8</i>); auxin biosynthetic process regulation
AT1G77850.2	-1.13	<i>ARF17</i>	<i>Auxin response factor 17</i> ; adventitious root development, auxin-activated signaling pathway
AT5G35735.1	-1.14	No name	Auxin-responsive family protein
AT5G56750.2	-1.22	<i>NDL1</i>	<i>N-MYC downregulated-like 1</i> ; response to auxin, regulation of auxin polar transport
AT2G47460.1	-1.23	<i>MYB12</i>	<i>MYB domain protein 12</i> ; positive regulation of transcription, response to auxin
AT5G17300.1	-1.24	<i>RVE1</i>	<i>Reveille 1</i> ; auxin-activated signaling pathway, auxin biosynthetic process regulation
AT5G13370.1	-1.27	<i>GH3.15</i>	<i>Gretchen Hagen 3.15</i> ; conjugates glutamine to IBA, auxin homeostasis
AT5G08640.1	-1.63	<i>FLS1</i>	<i>Flavonol synthase 1</i> ; response to auxin
AT3G55120.1	-1.94	<i>CHI</i>	<i>Chalcone flavanone isomerase</i> ; response to auxin
AT1G04180.1	-1.97	<i>YUC9</i>	<i>Yucca 9</i> ; auxin biosynthetic process, response to ethylene
AT2G42330.1	-2.10	<i>TMK4</i>	<i>Transmembrane kinase 4</i> ; auxin signaling, auxin biosynthetic process regulation
AT4G17980.1	-2.97	<i>NAC071</i>	<i>Nac domain containing protein 71</i> ; cellular response to auxin stimulus
AT1G74650.3	-3.84	<i>MYB31</i>	<i>MYB domain protein 31</i> ; response to auxin
AT2G46370.4	-4.07	<i>JAR1^b</i>	<i>Jasmonate-resistant 1</i> ; member of the GH3 family of proteins, cellular response to auxin stimulus
AT2G22240.3	-4.08	<i>MIPS2^b</i>	<i>Myo-inositol-1-phosphate synthase isoform 2</i> ; defense response to bacterium, response to auxin
AT3G02875.3	-4.57	<i>ILR1</i>	<i>IAA-leucine-resistant 1</i> ; IAA metabolic processes
AT1G28560.1	-4.99	<i>SRD2^b</i>	<i>Shoot redifferentiation defective 2</i> ; organ morphogenesis, polar transport, cellular response to auxin stimulus, lateral root formation, meristem initiation
AT3G02260.1	-6.88	<i>TIR3</i>	<i>Transport inhibitor response 3</i> , (aka <i>BIG</i>); auxin polar transport, lateral root formation, unidimensional cell growth
AT5G14420.4	-8.25	<i>RGLG2</i>	<i>Ring domain ligase 2</i> ; auxin metabolic process

numbers were similar to those of noninoculated *pin3*, *pin7*, *abcb4*, and *abcb19* mutants and wild-type plants (Fig. 5). This indicated that PIN3, PIN7, and ABCB19 may have nonredundant roles in the *A. thaliana*–*B. japonicum* interaction and that functional auxin efflux transport through PIN3, PIN7, and ABCB19 was required for *B. japonicum*-induced LR production. The 8-dpi LR numbers of noninoculated *pin2* plants were not significantly different from control or inoculated wild-type, *pin2*, or *abcb4* plants (Fig. 5). Significant differences were found between Col-0 inoculated and noninoculated plants using Welch's analysis of variance (ANOVA) (Fig. 5) and the Student's *t* test ($P < 0.0001$). No significant differences were observed between the two independent alleles for each gene under control or inoculation treatments (Fig. 5). Interestingly, application of 1 μ M NAA did not show the same LR growth trends seen with *B. japonicum* cocultivation in *pin3*, *pin7*, or *abcb19* (Supplementary Fig. S6).

***B. japonicum* enhances LR initiation and emergence efficiency.**

To find out whether the *B. japonicum*-increased number of emerged LR was due to enhanced LRP (stages I to VII) formation

or promotion of emergence of already formed LRP, we counted the number of LRP at different developmental stages and emerged LR in auxin efflux mutants at 4 dpi. LRP stages were determined according to Torres-Martínez et al. (2019). When these data were graphed as a percentage of the total (total = all LRP + emerged LR), both the proportions of each stage and the general distribution trend remained largely the same between control and inoculated roots (Supplementary Fig. S7).

Summation of two independent experiments revealed that the number of LRP (stages I to VII) at 4 dpi was slightly lower in inoculated relative to control wild-type Col-0 roots whereas the number of emerged LR was increased by *B. japonicum* (Table 2). In inoculated Col-0 plants, the increase (72.6%) of emerged LR surpassed the decrease (5.7%) in LRP stages I to VII. If this represented solely an acceleration of LRP progression toward emergence, the decrease in LRP would have likely closely matched the increase in emerged LR. Because the number of LRP was nearly maintained while the number of emerged LR increased, *B. japonicum* appears to have stimulated LR initiation as well as LR emergence, suggesting an overall acceleration in the rate of LR and LRP growth throughout LRP morphogenesis.

Emerged LR significantly increased in Col-0, *pin2*, and *abcb4* with *B. japonicum* cocultivation but decreased in *pin7* (Table 2), similar to our observations at 8 dpi (Fig. 5). Following *B. japonicum* inoculation, wild-type plants had a higher increase in the number of emerged LR than the mutants (Col-0 = 72.6% compared with *pin2* = 17.8%, *pin3* = 2.6%, *pin7* = -9.3%, *abcb4* = 24.7%, and *abcb19* = 2.9%) (Table 2). Apart from *abcb4*, there was a greater reduction in LRP in the tested mutants than in Col-0 following *B. japonicum* inoculation (Col-0 = 5.7% compared with *pin2* = 12.1%, *pin3* = 12.6%, *pin7* = 26.5%, and *abcb19* = 28.5%) (Table 2). The percentage differences observed between Col-0 and the loss-of-function mutants were most robustly demonstrated in *pin3*, *pin7*, and *abcb19*. Because *B. japonicum* had little effect on the overall proportions and distribution of LRP stages I to VII (Supplementary Fig. S7) but the number of total LRP decreased more dramatically in the mutants than in Col-0 (Table 2), these data suggest that *PIN3*, *PIN7*, and *ABCB19* were integral to the acceleration of LR growth throughout the LRP developmental stages and emergence.

PR length was significantly lower in *B. japonicum*-treated than control plants in Col-0, *pin3*, *pin7*, and *abcb19* (Table 2). However, PR length remained unchanged in *pin2* and insignificantly lowered in *abcb4* with *B. japonicum* inoculation (Table 2), suggesting involvement of *PIN2* in *B. japonicum*-induced PR reduction. The tested auxin efflux facilitators, as single-gene mutants, demonstrated differential roles in *B. japonicum*-activated PR and LR alterations.

Fig. 4. *Bradyrhizobium japonicum* induced *PIN3*, *PIN7*, and *ABCB19* gene expression in *Arabidopsis thaliana* root tissue at 12 h postinoculation. *A. thaliana* roots were treated with either *B. japonicum* or 10 mM MgSO₄ (control) for 12 h. Quantitative reverse-transcription PCR expression data relative to the *IPP2* reference gene showed significantly increased RNA transcripts in *PIN7*, *PIN3*, and *ABCB19*. Student's *t* test, *, **, and *** indicate *P* < 0.05, 0.01, and 0.001, respectively.

Fig. 5. Mutations in auxin efflux transport genes alter *Bradyrhizobium japonicum*-induced lateral root (LR) growth. Two independent mutant alleles were used for each gene (*PIN2* [*pin2* and Salk_122916C], *PIN3* [*pin3-3* and *pin3-4*], *PIN7* [*pin7* and Salk_048791C], *ABCB4* [*abcb4-2* and *pgp4-1*], and *ABCB19* [*abcb19-3* and *atmdr1-101*]). The LR number per seedling was averaged at 8 days postinoculation (dpi) for at least two independent experiments. Different letters indicate statistical differences between treatments or lines analyzed by Welch's analysis of variance, Games-Howell (*P* < 0.05); standard error bars; *n* > 10.

***B. japonicum* alters *PIN3:GFP*, *PIN7:GFP*, and *ABCB19:GFP* expression localization.**

Confocal microscopy of green fluorescent protein (GFP) reporter line fluorescence was investigated after *B. japonicum* was found to increase *PIN3*, *PIN7*, and *ABCB19* transcript abundance in whole-root Col-0 tissue, and LR and LRP data with *pin3*, *pin7*, and *abcb19* mutants indicated roles for the genes in *B. japonicum*-stimulated root alterations. Imaging of three reporter lines demonstrated altered expression localization with *B. japonicum* cocultivation at 12 hpi in root tips (Fig. 6). Differential expression localization between control and inoculated plants was seen in the elongation zone of *PIN3:GFP* and *PIN7:GFP* and the meristematic zone of *ABCB19:GFP* (Fig. 6). Unlike control plants, fluorescent signals of inoculated *PIN3:GFP* and *PIN7:GFP* were minimal in the elongation zone (Fig. 6; Supplementary Fig. S8). Under control conditions, *ABCB19:GFP* is predominantly expressed in the central core of the differentiation zone and broadly expressed in the root apex and meristematic zone (Supplementary Fig. S9A and B) (Wu et al. 2007). When cocultivated with *B. japonicum* for 12 h, *ABCB19:GFP* fluorescence nearly disappeared in the meristematic zone where it was confined to the columella and lateral root cap cells (Fig. 6). *ABCB19:GFP* fluorescence in the root central cylinder was visible and the *B. japonicum*-induced *ABCB19:GFP* fluorescence pattern was maintained at 24 hpi (Supplementary Fig. S9) (Sukumar et al. 2013b; Wu et al. 2007). A schematic diagram of *B. japonicum*-stimulated *ABCB19:GFP*, *PIN3:GFP*, and *PIN7:GFP* expression distribution, based on our confocal microscopy imaging and gene expression data, is illustrated in Figure 7. The diagram also shows previously reported *PIN2*, *PIN3*, *PIN7*, *ABCB4*, and *ABCB19* root tissue location and associated auxin flux directionality under control conditions (Fig. 7) (Geisler et al. 2017; Laskowski and ten Tusscher 2017).

Note that *B. japonicum*-stimulated PR reduction results in a significantly shorter elongation zone, as measured between the transition zone and the first apical root hair, compared with control roots (Supplementary Fig. S8) (Barrada et al. 2015; Di Mambro et al. 2017). No significant difference was observed in lengths between the quiescent center and the transition zone between inoculated and noninoculated plants (Supplementary Fig. S8).

DISCUSSION

Traits observed in roots inoculated with the *B. japonicum* strain IRAT FA3 phenocopy the exogenous auxin application increase and decrease to plant LR number and PR length, respectively. Not all strains of *B. japonicum* produce IAA and,

arguably, one of the distinguishing factors between *B. japonicum* and the later-named species *B. elkanii* (previously *B. japonicum*) has been IAA production in culture (Minamisawa and Fukai 1991; Minamisawa et al. 1996). In *B. japonicum* E109, a widely used soybean inoculation strain, genome analysis revealed tryptamine pathway genes, IAA did not accumulate in culture, and auxin was degraded by this strain in the presence of added IAA, IBA, and NAA (Torres et al. 2018). Unlike E109, the *B. japonicum* IRAT FA3 strain used in this study produces IAA in culture and, with the IAA precursor L-tryptophan provided, IAA production increased (Dobbelaere et al. 1999). Our *B. japonicum* strain enhanced auxin responses in the plant root, as shown by intensified *DR5*-reporter staining and increased expression of *GH3.3*, a gene that helps plants cope with excess auxin by synthesizing IAA-amino acid conjugates (Casimiro et al. 2001; Ulmasov et al. 1997; Zamioudis et al. 2013).

RNA-seq analysis revealed transcriptional changes in response to *B. japonicum* cocultivation in more than 70 differentially regulated auxin-related genes, several of which have previously demonstrated roles in either LR formation, PR length regulation, or auxin transport. For example, upregulation of *ARF7*, *ARF5*, and *IAA14* suggests activation of LRP initiation, LRP cell fate specification, and LRP development (Dubrovsky et al. 2008; Lavenus et al. 2015; Torres-Martínez et al. 2019). Increased *GH3.6* expression may be related to feedback mechanisms that regulate auxin homeostasis during LRP initiation (Vanneste et al. 2005). An increase in *ICR1*, involved in the recruitment of PINs to polar plasma membrane domains, implies further regulation of auxin polar transport (Hazak et al. 2010). Differential regulation of *PIN-LIKE* genes *PILS7* and *PILS3* is indicative of intracellular auxin homeostasis management (Barbez et al. 2012). Downregulation of both *IAA28*, a repressor of LR initiation, and *BIG*, with roles in auxin-mediated PIN cycling to increase PIN proteins at the plasma membrane and PR elongation, may promote progression of *B. japonicum*-mediated LR number increases and PR length reduction, respectively (Guo et al. 2013; Paciorek et al. 2005; Rogg et al. 2001; Zhang et al. 2019). *ABCB4* and *PIN1* transcript levels did not change with inoculation, suggesting a lack of involvement of these genes at this point in the *B. japonicum*-plant interaction. Chen and coworkers (2015) proposed a mechanism of regulation of transcriptional auxin sensitivity in which a *PIN3* pathway, together with *ARF7* and *FLP*, may maintain locally enhanced auxin flux during LR growth. Given that the *PIN3*, *ARF7*, and *FLP* genes were upregulated by *B. japonicum*, the proposed circuitry in Chen and coworkers (2015), if amplified by *B. japonicum* in wild-type plants, would be severed in the *pin3* mutant, preventing a *B. japonicum*-stimulated LR number increase.

Treatment of inoculated plants with the auxin efflux inhibitor NPA significantly reduced the number of LR, pointing to the major role of polar auxin transport in *B. japonicum*-induced LR formation. NPA did not prevent *B. japonicum* cells or culture filtrate from inducing nearly the same number of LR as control plants without NPA, indicating that *B. japonicum* may rely, at least partially, on mechanisms other than those disrupted by NPA to increase LR development. The lack of significant differences in the number of LR formed by plants treated with the culture filtrate or *B. japonicum* cells, with or without NPA, demonstrated the importance of compounds produced by *B. japonicum* rather than the presence of the bacterial cells in stimulating LR growth. Auxin, produced by *B. japonicum*, may contribute to rescuing the NPA LR phenotype (Casimiro et al. 2001). NPA has been shown to have a greater effect on NAA than on IAA, which correlates with NPA's relatively lower effect on *B. japonicum*- and culture filtrate-stimulated LR production in our assay (Delbarre et al. 1996). Although the exact mechanism is still debated, there is evidence for NPA sensitivity, direct or indirect activity, or binding affinity among members of both the PIN and ABCB families (Abas et al. 2021; Blakeslee et al. 2007; Noh et al. 2001; Rojas-Pierce et al. 2007; Teale and Palme 2018). Auxin efflux inhibition causes LRP defects similar to those observed in *pin1pin3pin4* mutants, indicating that PIN-dependent auxin efflux is necessary for LRP development (Benková et al. 2003). Benková et al. (2003) also observed that mutations of *PIN* genes interfered with auxin gradients in LRP and with LRP development, indicating that LRP growth was dependent on PIN-regulated auxin gradients. *B. japonicum*-induced LR increases may largely be due to its auxin production and NPA may have reduced *B. japonicum*-induced LR growth by inhibiting auxin efflux transporters. Therefore, we investigated auxin transport gene cooperation in controlling the root growth responses to *B. japonicum*.

B. japonicum-treated auxin efflux transport mutants showed differential LR phenotypes compared with control plants. Three of the single mutant lines tested (*pin3*, *pin7*, and *abcb19*) had LR numbers similar to those of the wild-type Col-0 seedlings when noninoculated but significantly fewer LR than Col-0 when cocultivated with *B. japonicum*. Single *pin* mutants have been shown to have altered expression of the remaining functional PIN proteins that masks the defect of the mutation, demonstrating a somewhat compensatory expression in *pin* mutants under control conditions (Benková et al. 2003; Blilou et al. 2005). Such auxin transport system redundancy may account for the lack of differentiation in LR numbers between noninoculated individual mutants and wild-type plants, as well as the LR number increases seen in *pin3* and *pin7* with NAA application in our study (Benková et al. 2003). However, that functional redundancy was unable to prevent an absence

Table 2. Lateral root (LR) and LR primordia (LRP) count and average primary root (PR) percent reduction summary^a

Line	Treatment	Sums	Emerged	Total	PR reduction ± SE (%)
Columbia (Col-0)	Control	351	73	424	—
	<i>Bradyrhizobium japonicum</i>	331	126	457	10.3 ± 1.6
<i>pin2</i>	Control	306	101	407	—
	<i>B. japonicum</i>	269	119	388	0.0 ± 2.4
<i>pin3</i>	Control	231	76	309	—
	<i>B. japonicum</i>	202	78	280	13.3 ± 3.7
<i>pin7</i>	Control	268	75	343	—
	<i>B. japonicum</i>	197	68	265	16.7 ± 3.0
<i>abcb4</i>	Control	238	77	315	—
	<i>B. japonicum</i>	239	96	335	3.7 ± 3.8
<i>abcb19</i>	Control	375	70	445	—
	<i>B. japonicum</i>	268	72	340	13.3 ± 3.8

^a Data from two independent experiments of seedlings treated with either 10 mM MgSO₄ (control) or *B. japonicum* for 4 days were combined. LR count data are presented as the sums of LRP stages I to VII (Sums), emerged LR (Emerged), or both (Total). PR length percent reductions are averages; SE = standard error. Student's *t* tests comparing each line's inoculated and noninoculated PR lengths gave the following *P* values: Col-0 (*P* = 7.62E-04), *pin2* (*P* = 1.00), *pin3* (*P* = 8.52E-04), *pin7* (*P* = 4.79E-07), *abcb4* (*P* = 0.45), and *abcb19* (*P* = 2.69E-02).

of *B. japonicum*-stimulated LR growth in single *pin3*, *pin7*, or *abcB19* mutants, suggesting independent roles for PIN3, PIN7, and ABCB19 during the plant–*B. japonicum* interaction.

Although the synthetic auxin NAA serves as a positive control in activating Col-0 LR growth, it is not likely to fully capture the nuances, feedback, or reactivity between living organisms over time (Spaepen et al. 2007). NAA did not reduce Col-0 PR length, nor did it lower LR numbers in *pin3* or *pin7* as *B. japonicum* did. These differences could reflect (i) the mode of treatment (i.e., a single, topical 1-μM NAA application differs from inoculum containing thousands of microbes that grow over time), (ii) specific auxin compound differences between those produced by bacteria and synthetic NAA, (iii) NAA and IAA diffusive influx capacity and efflux transporter affinity differences, or (iv) unidentified compounds produced by *B. japonicum* (Asari et al. 2017; Blake-slee et al. 2007; Boiero et al. 2007; Delbarre et al. 1996). Although this study identifies IAA as a major factor produced by *B. japonicum*, it is likely that additional compounds also have roles in *A. thaliana* growth promotion, the analyses of which deserve future study (Asari et al. 2017; Boiero et al. 2007; Delbarre et al. 1996). A variety of priming signals are capable of specifying cells to become founder cells and prebranch sites that will form LR, and such cues may be produced or stimulated by *B. japonicum* (Laskowski and ten Tusscher 2017). Likewise, an increase in exogenous IAA produced by the bacteria may promote LRP initiation, speed LR development or emergence, and shorten PR.

Previous studies have shown that treatment of plants with auxins increased expression of *PIN3* and *PIN7*, which corroborates our gene expression analysis of *A. thaliana* cocultivated with the IAA-producing strain of *B. japonicum* (Lewis et al. 2011; Vieten et al. 2005). Despite the close evolutionary relationship and pathway-level coexpression between *PIN3* and *PIN7*, plus their known roles in root development, redundant cooperation between these two genes in *B. japonicum*-inoculated plants was not obvious (Chen et al. 2015; Friml et al. 2002; Paponov et al. 2005; Ruiz Rosquete et al. 2018; Zhou and Luo

2018). *PIN3*, as a mediator of auxin flux between the pericycle and endodermis, is a major player in LR initiation following founder cell specification and in LR emergence facilitation (Marhavý et al. 2013; Péret et al. 2013). *PIN3:GFP* and *PIN7:GFP* reporter lines displayed a change in root tip expression localization of *PIN3* and *PIN7*, respectively, with *B. japonicum* cocultivation. Fluorescent signals in both lines were minimal in the elongation zone (i.e., basipetal of the auxin minimum of the transition zone and acropetal to the first apical root hair) in inoculated compared with control roots (Barrada et al. 2015; Di Mambro et al. 2017). The elongation zone is significantly shorter in *B. japonicum* cocultivated roots at 12 hpi. To promote cell expansion, auxin levels are lower in the elongation zone; however, increases in auxin can rapidly inhibit cell elongation (Motte et al. 2019). Blilou et al. (2005) demonstrated that PIN-mediated auxin distribution controls cellular elongation and found the elongation zone to be reduced in the *pin3pin4pin7* triple mutant. Although inoculated *pin3* and *pin7* did not demonstrate a PR length phenotype that was different from that of wild-type plants, altered GFP expression localization suggests a putative role for *PIN3* and *PIN7* related to auxin accumulation in the elongation zone with *B. japonicum* cocultivation.

B. japonicum-stimulated PR reduction may be due to microbially produced exogenous auxin levels, because IAA has been shown to reduce elongation zone size (Rahman et al. 2007). Nevertheless, differential regulation of PIN proteins by root architecture-altering soil bacteria has been recognized; for example, *Achromobacter* sp. 5B1 repressed expression of *PIN7* in the root tip while increasing *PIN3* expression in columella cells (Jiménez-Vázquez et al. 2020; Sukumar et al. 2013a). However, several PGPR studies have identified non-PIN plant interaction points. In plants treated with another PGPR (*Bacillus megaterium*), reduction in *A. thaliana* PR length was a result of decreased cell proliferation and cellular elongation in the root meristem (López-Bucio et al. 2007). In a study with a *Pseudomonas* sp. named WCS417, the PGPR promoted root cell division yet decreased the PR length by reducing cell

Fig. 6. Green fluorescent protein (GFP) expression localization changes in response to *Bradyrhizobium japonicum*. **A to D**, *PIN3:GFP*; **E to H**, *PIN7:GFP*; and **I to L**, *ABCB19:GFP* seedlings were grown for 12 h with either *B. japonicum* bacterial suspension or 10 mM MgSO_4 (control) and imaged with confocal microscopy. GFP is shown in green and propidium iodide was used as a counterstain (red). Images are representative of three independent experiments. Scale bars = 50 μm.

elongation in the elongation and differentiation zones (Zamioudis et al. 2013).

In our study, *abcb19* had a similar number of LR as Col-0 under control conditions (Wu et al. 2007) but, unlike Col-0, the LR number did not increase in *abcb19* with *B. japonicum* cocultivation. Thus, *ABCB19* appears to be required for *B. japonicum*-induced LR formation. To our knowledge, this is one of the first reports of *ABCB19* involvement in LR formation in a plant–PGPR interaction. It has been shown that *ABCB19* is important for acropetal auxin transport and LR elongation and that *ABCB19* stabilizes PIN PM localization, an interaction that enhances the rate of auxin transport, all of which could possibly be involved in *B. japonicum*-stimulated LR growth (Titapiwatanakun et al. 2009; Wu et al. 2007). A putative *B. japonicum*-activated *ABCB19* pathway in the *abcb19* mutant would lack

the PIN PM stabilization; hence, the levels and rates of auxin transport needed to enhance the LR number would be absent.

Confocal microscopy of *ABCB19:GFP* roots with and without *B. japonicum* cocultivation revealed a change in the location of *ABCB19* promoter activity at 12 and 24 hpi. In the meristematic zone, fluorescence changed from diffuse distribution to more defined expression limited to the columella and lateral root cap in inoculated plants. Using a translational fusion reporter line, Wu et al. (2007) found an increase in *ABCB19* protein when treated with IAA for 5 h in the location of the PR tip where our *ABCB19* promoter expression dissipated with *B. japonicum* at 12 hpi. Differential gene and protein expression localizations are not necessarily contradictory (Wu et al. 2007). Plus, the timing (7-h difference), growth conditions, and other factors at work during the plant–microbe interaction could contribute to the observed differences between our work and that of Wu et al. (2007). Both polar and nonpolar *ABCB19* root cell localizations have been described (Blakeslee et al. 2007; Teale and Palme 2018; Titapiwatanakun et al. 2009; Wu et al. 2007). Both types of transport could be involved in the interaction with *B. japonicum*, such that differential *ABCB19* expression in the meristematic zone may be an indication of redirected auxin accumulation in regions where it is needed to increase LR formation. In addition, because *ABCB* proteins can affect mechanisms involved in PIN protein subcellular localization, *B. japonicum*-stimulated redirection of *ABCB19* expression may have downstream effects on other auxin transporters (Noh et al. 2003). Just as *mdr4/abcb4*'s amplified auxin asymmetry was proposed as an explanation for that mutant's hypertropism (Lewis et al. 2007), redistribution of *ABCB19* expression by *B. japonicum* may account for some of the microbially induced root phenotypes. The *B. japonicum*-stimulated change in *ABCB19:GFP* root tip localization should not be confused with *ABCB19* expression levels throughout the entire root. The *ABCB19* transcript abundance increase with *B. japonicum* was observed in RNA extracted from whole-root tissues.

Although changes were observed in root tip localization, *ABCB19*, *PIN3*, and *PIN7* expression increased in whole-root tissue according to our RNA-seq and qRT-PCR data. Genes expressed in distinct root tissues, such as LRP, may have been diluted by the use of entire roots for qRT-PCR and RNA-seq analyses. Nevertheless, *PIN2*, *PIN3*, *PIN7*, and *ABCB19* were upregulated by *B. japonicum*, an indication of roles for these genes in the plant–microbe interaction. The inability of *B. japonicum* to stimulate increased LR numbers in *pin3*, *pin7*, and *abcb19* or PR reduction in *pin2* is evidence of their respective involvement in *B. japonicum*-stimulated root alterations.

PIN2 does not appear to be required for *B. japonicum*-stimulated LR growth. According to Bilou et al. (2005), although *PIN2* is crucial for basipetal auxin transport as well as regulation of cell division, this is only evident in *pin1pin2* double mutants and in other multimutants containing *pin2*, because ectopic expression of other PIN proteins masks the defects. Such functional redundancy between PIN proteins may be the reason why we did not see significant changes in the LR numbers between *pin2* and Col-0 after inoculation with *B. japonicum*. On the other hand, *pin2* significantly differed from Col-0 with regard to the *B. japonicum*-stimulated PR reduction phenotype. A role for *PIN2* in PR length determination was also seen in Vieten et al. (2005), in which *pin2* PR length was the same as *pin1* and similar to WT but lower in *pin1pin2*. *Pin2* and wild-type PR length decreased while LR number increased in response to the PGPR *Bacillus megaterium* in a study in which the *pin2* mutant had fewer LR than wild-type plants under control conditions (López-Bucio et al. 2007). *B. megaterium* inhibited PR growth by limiting cell proliferation and elongation (López-Bucio et al. 2007). In a separate study also using a *Bacillus* sp. (LZR216), inoculation reduced meristem length and cell division but

Fig. 7. Root tip auxin efflux system schematic diagram. Arrows indicate previously identified efflux transporter locations and auxin flux directionality of *PIN2* (purple), *PIN3* (green), *PIN7* (blue), *ABCB4* (black), and *ABCB19* (tan) under control conditions (Geisler et al. 2017; Jozef et al. 2017; Laskowski and ten Tusscher 2017). Shading represents *Bradyrhizobium japonicum*-influenced localization of *PIN3* (green), *PIN7* (blue), and *ABCB19* (tan) gene expression based on reporter line confocal imaging at 12 hpi with *B. japonicum*. The bacteria are shown surrounding the root in gray; the star (*) indicates the location of the most apical root hair when cocultivated with *B. japonicum* at 12 hpi. DZ = differentiation zone, EZ = elongation zone, TZ = transition zone, and MZ = meristematic zone.

promoted cell elongation in wild-type plants, yet *pin2* PR length was unaffected by LZR216 (J. Wang et al. 2015). In our assay, the *pin2* LR number was consistently higher than (but not significantly different from) Col-0 in noninoculated plants and increased to a similar level as Col-0 with *Bradyrhizobium japonicum*. Although mutant *pin2* PR length and LR number relative to the wild type can vary (depending on the allele, research conditions, and so on), multiple studies have associated loss of *PIN2* function with differentially modulated root system architecture (Blilou et al. 2005; Laskowski et al. 2008; Li et al. 2015; López-Bucio et al. 2007; Qu et al. 2017).

Both *PIN2* and *ABCB4* are involved in basipetal auxin flow, with the *abcb4* mutant, (also known as *pgp4* or *mdr4*), having 50% of normal basipetal transport (Lewis et al. 2007; Müller et al. 1998). The *pgp4-1* (SALK_063720) mutant was described as having shorter PR and fewer LR than wild-type plants, and *PGP4* and *ABCB4* expression increased in response to IAA at 12 hpi in previous work (Terasaka et al. 2005). *Pgp4-1* did not show fewer LR under our assay's control or cocultivation conditions. Our qRT-PCR data show an insignificant *ABCB4* expression increase at 12 hpi with *B. japonicum* cocultivation that is far lower than that of the findings of Terasaka and coworkers (2005) in response to IAA. The auxin concentration applied in the Terasaka and coworkers (2005) qRT-PCR study was likely much higher than that produced by *B. japonicum*, especially at only 12 hpi. The *abcb4-2* allele did not have a short PR under control conditions but it clearly demonstrated a lack of PR reduction in response to *B. japonicum*, a major difference from wild-type Col-0 plants, insinuating a role for *ABCB4* in PR growth related to *B. japonicum* influence, despite no increase in *ABCB4* gene expression in our study (Wu et al. 2007). *ABCB4*'s functional plasticity as an efflux carrier under high IAA and an influx transporter at low IAA concentration could be used by *B. japonicum* at time points other than those recorded in this study (Kubeš et al. 2012).

Various species of PGPR have been shown to stimulate root growth and influence auxin signaling pathways (Grunewald et al. 2009; Jiménez-Vázquez et al. 2020; López-Bucio et al. 2007; Spaepen et al. 2014). Here, we showed that *B. japonicum*, an IAA-producing strain, activates auxin response, signaling, and transport genes; utilizes *PIN2* and possibly *ABCB4* to reduce PR length; and requires *PIN3*, *PIN7*, and *ABCB19* to accelerate LRP development and LR emergence in *A. thaliana* roots. Our results provide insight into the auxin pathway points involved in *B. japonicum*-stimulated alteration of root morphology and a greater understanding of a mutualistic *Bradyrhizobium* microbe-plant interaction.

MATERIALS AND METHODS

Plant material and growth conditions.

All plants used in this study were in the *A. thaliana* Columbia (Col-0) background. Seeds were kindly provided by Gloria Muday (*DR5::GUS*, *eir1-1* [aka *pin2*], *pin3-3*, *pin7*, *abcb19-3*, and *ABCB19::GFP*), Marta Laskowski (*PIN3::GFP* and *PIN7::GFP*), and Edgar Spaulding (*abcb4-2*) (Laskowski et al. 2008; Sukumar et al. 2013b; Wu et al. 2007). Col-0, *pin2*-Salk_122916C, *pin3-4* (CS9363), *pin7*-Salk_048791C, *pgp4-1* (Salk_063720C), and *atmdr1-101* (Salk_033455C) seeds were obtained from the Arabidopsis Biological Resource Center (The Ohio State University). Plants were grown vertically under light at 120 to 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 22°C with a day and night cycle of 12 and 12 h. Seeds were surface sterilized (3 min in 10% [vol/vol] NaClO and 0.05% Tween solution, 1 min in 70% EtOH, and four sterile water rinses) and cold treated at 4°C for 2 days before being sown onto a germination medium (1% [wt/vol] agar [Fisher Scientific], half-strength Murashige and Skoog [1/2 MS] supplemented with 0.01% myo-inositol, 0.5% sucrose, and 0.05% morpholineethanesulfonic acid, pH 5.7). After 4 to 6

days, uniform seedlings were transferred to a treatment medium (1% agar and 1/2 MS, pH 5.7), with eight seedlings per square Petri dish, for each treatment period.

Bacterial cultivation and inoculation treatments.

B. japonicum strain IRAT FA3 was originally isolated from Cameroon, Africa, and was characterized at the Institut de Recherches Agronomiques Tropicales (IRAT) in Montpellier, France, as strain number 3 (FA3) (Gachomo et al. 2014). *B. japonicum* was grown in half-LB (1/2 LB) liquid medium at 28°C with shaking at 100 rpm for 12 to 18 h from a previously grown stock. *B. diazoefficiens* (previously *B. japonicum*) USDA 110 LI (kindly provided by Joel Sachs) was grown in yeast-mannitol (YM) liquid medium at 29°C with shaking at 100 rpm for 5 days. Bacteria cells were then collected by room-temperature centrifugation at $1,500 \times g$ for 15 min, the supernatant was removed, and the bacterial pellet was resuspended in 10 mM MgSO_4 . The bacterial titer was adjusted to an optical density at 600 nm (OD_{600}) of 1 ($1.8 \times 10^9 \text{ CFU ml}^{-1}$), and 10 μl of either bacterial inoculation suspension or 10 mM MgSO_4 alone (control treatment) were applied onto each seedling root on treatment plates. For growth-curve analysis, *B. japonicum* with an initial titer at OD_{600} of 0.05 was grown in 1/2 LB liquid medium at 28°C with shaking at 100 rpm, and with or without 0.1% DMSO or 1 μM NPA. Spectrophotometric measurements were read at 4, 8, 12, 24, 48, and 72 h.

Root colonization quantification was performed according to Allard-Massicotte et al. (2016), with modifications. At 5 days old, Col-0 seedlings were treated with 10 μl of either 0.1% DMSO, 1 μM NAA, or NC, followed by adding 10 μl of bacterial suspension. At 0 dpi (1 h following inoculation) and at 8 dpi, PR lengths were measured and roots were separated from their shoots. Roots were placed in tubes with 1 ml of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 2 mM KH_2PO_4). Tubes were placed in an ultrasonic bath (Fisherbrand CPX3800) and sonicated to remove the bacteria with 10 10-s pulses on a low setting. Sonicated solutions were diluted and plated on 1/2 LB agar for CFU recovery counting.

Root phenotypic analysis.

PR length and emerged LR (greater than 0.5 mm) number measurements were taken every 2 dpi for a total period of 8 days of cocultivation. A minimum of 10 seedlings was counted per treatment per replicate for each line. For LRP stages I to VII analysis, 4-day-old seedlings were treated with or without *B. japonicum*. At 4 dpi, the LRP stages were identified under microscopy, as defined by Torres-Martínez et al. (2019), and counted.

Auxin analog (Supplementary Fig. S1) LR number evaluation was performed by placing 5-day-old seedlings on 1/2 MS treatment plates with agar containing either 0.1% DMSO, 1 μM NAA, 10 μM IBA, or NC, followed by adding 10 μl of either bacterial suspension or 10 mM MgSO_4 (control treatment) to each root. Plants were grown for 8 days and LR were quantified under a dissecting microscope.

For inhibition of polar auxin transport, either NPA or DMSO (solvent-only control) were added to the treatment plate medium at a final concentration of 1 μM or 0.1%, respectively. Five-day-old seedlings were placed on the DMSO- or NPA-containing plates and 10 μl of either 10 mM MgSO_4 (control), bacterial suspension, culture filtrate, or 1 μM NAA were topically added to each root. LR were counted and PR were measured at 0, 4, and 8 dpi.

Mean, standard error and Student's *t* test significance were calculated in Microsoft Excel 2007. LR data were analyzed by Welch's ANOVA and Games-Howell post hoc analysis (with bacterial inoculation and noninoculation control as independent variables) using SPSS software (version 11.0; SPSS Inc., Chicago, IL, U.S.A.), $P < 0.05$.

IAA quantification.

Production of IAA by *B. japonicum* or USDA 110 LI was determined by cultivating the bacteria (starting concentration OD₆₀₀ = 0.05) in 1/2 LB or YM, respectively, with or without the addition of 2 mM L-tryptophan. *B. japonicum* or USDA 110 LI were grown for 24 to 48 h at 28°C or for 5 days at 29°C, respectively, with shaking at 100 rpm. Auxin content measurement was carried out following the protocol of Gordon and Weber (1951), with some modifications. Cultures were centrifuged at 14,000 rpm for 5 min; then, 0.5 ml of the supernatant was combined with 0.75 ml of Salkowski's reagent (1:30:50 [vol/vol/vol] of 0.5 M FeCl₃-H₂O-H₂SO₄) and briefly vortexed. The mixture was incubated in the dark for 75 min at room temperature. Absorbance at 535 nm was measured on a spectrophotometer and the IAA content was calculated using an external standard curve generated with known concentrations of IAA.

GUS staining and GFP analysis.

Histochemical detection of GUS in the *DR5::GUS* reporter line was performed according to Zamioudis et al. (2013). Briefly, posttreatment seedlings were submerged in staining solution (50 mM sodium phosphate buffer [pH 7.0], 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid sodium salt, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, and 10 mM EDTA) at 37°C for 6 h, then incubated in 95% EtOH overnight. Imaging was performed using a Leica DM2500 LED optical microscope, a Leica DMC4500 digital camera, and Leica Application Suite X software (Leica Microsystems, Wetzlar, Germany).

PIN3::GFP, *PIN7::GFP*, and *ABC19::GFP* seedlings were treated at 6 days old with either 10 mM MgSO₄ or *B. japonicum* for 12 h (24 h for Supplementary Fig. S9) before incubation in propidium iodide at 10 mg/ml for 10 s, followed by a brief water rinse. Seedlings were mounted in water on slides with coverslips and imaged using a Leica SP5 confocal microscope and Leica Application Suite X software.

RNA-seq and qRT-PCR.

Total RNA was extracted from 21-day-old *A. thaliana* seedling roots at 14 dpi, according to the manufacturer's protocol (GeneJET Plant RNA Purification Kit; Thermo Scientific, Madison, WI, U.S.A.).

For RNA-seq, the RNA was processed (74204 QIAGEN and AM1907 Invitrogen) and libraries were prepared with the NEB-Next Ultra Directional RNA Library Prep Kit for Illumina by following the manufacturer's instruction (E7490S, E7335S, and E7420S; New England Biolabs). Libraries were pooled and sequenced on an Illumina NextSeq 500 platform (Illumina, San Diego, CA, U.S.A.) at the University of California–Riverside Genomics Core Facility.

The program Salmon was used to process raw RNA-seq data to quantify the transcript data from all samples (Patro et al. 2017). The quantified data were analyzed using the R program ThreeDRNA (Calixto et al. 2018; Guo et al. 2021; R Core Team 2020). Briefly, quantified transcript data were uploaded into ThreeDRNA using the integrated tximport program (v. 1.18.0) and the default setting lengthScaledTPM method (Soneson et al. 2016). Low-quality expression data were filtered using the default count per million reads cutoff of 1 and sample number cutoff of 1. The processed data were used to generate a PCA graph using the RUVr method of removing batch effects from the data. Transcriptome data were normalized using the weighted trimmed mean of M values in ThreeDRNA. The data were statistically analyzed using the limma-voom pipeline recommended by ThreeDRNA, which balances outliers in the data that may occur from sample quality differences (Law et al. 2014; Ritchie et al. 2015). For downstream plots, the data were filtered to only include samples with *P* values ≤ 0.05 and log₂ FC ≥ 1 between

the noninoculated controls and samples inoculated with *B. japonicum*. The filtered data were used to generate a heatmap using the program shinyheatmap (Khomtchouk et al. 2017). To identify auxin-related genes within our (adjusted *P* value < 0.05) RNA-seq data for the heatmap, gene ontology (GO) terminology was compiled at the Panther Geneontology website with the annotation version GO database (released 2018-10-09), analysis type PANTHER Overrepresentation Test (released 2020-07-28), and test type GO biological process complete with Fisher's Exact false discovery rate (FDR) multiple test correction displaying only results with FDR < 0.05.

For each qRT-PCR sample, genomic DNA was removed from 1 µg of total RNA prior to RT into first-strand cDNA (iScript Kit; Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's protocol. The first-strand cDNA was used for SYBR green-monitored qRT-PCR (Bio-Rad CFX). Primer sequences for *PIN1*, *PIN2*, *PIN3*, *PIN7*, *ABC4*, and *ABC19* have been described (Terasaka et al. 2005; H. Z. Wang et al. 2015; Xi et al. 2016; Yang et al. 2013). qRT-PCR analysis was performed using the Bio-Rad CFX Maestro Real-Time PCR System.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Panther Geneontology: <http://go.pantherdb.org>

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