J Plant Growth Regul (2010) 29:441–454
DOI 10.1007/s00344-010-9156-x

Plant Immunity Induced by Oligogalacturonides Alters Root Growth in a Process Involving Flavonoid Accumulation in *Arabidopsis thaliana*

Georgina Hernández-Mata · María Elena Mellado-Rojas · Alan Richards-Lewis · José López-Bucio · Elda Beltrán-Peña · Eva Luz Soriano-Bello

Received: 29 August 2009 / Accepted: 14 April 2010 / Published online: 30 May 2010

Abstract Plants adapt to challenging environmental factors by modulating morphogenetic processes. Although it has been speculated that activation of defense responses against pathogens leads to plant growth adjustment, little is known about developmental and architectural responses to defense stimulators. In this report we evaluated the activity of oligogalacturonides (OGs), a class of molecules directly involved in plant immunity, to modulate root system architecture in *Arabidopsis thaliana*. We show that OGs induce *PAD3* expression and camalexin synthesis, two well-known markers of defense responses. These effects were related to primary root growth inhibition and increased lateral root and root hair formation, which are reminiscent of altered auxin responses. Cellular analysis showed that the effect of these compounds on primary root growth was due to changes in cell elongation and increased flavonoid accumulation at the root elongation region. Moreover, the observations that similar changes in primary root growth were induced by naphthylphthalamic acid supply and that auxin- or flavonoid-related mutants *tir1*, *doc1*, *pgp1*, *pgp4*, *pgp19*, and *tt4-1* show differential responses to primary root growth inhibition by OGs suggest that auxin homeostasis plays a role in the oligogalacturonide-induced alteration of root cell patterning. Our results suggest that OGs might play a dual function in adaptation of plants to pathogen challenge by inducing defense responses and plant architecture adjustment.

G. Hernández-Mata · M. E. Mellado-Rojas · A. Richards-Lewis · J. López-Bucio · E. Beltrán-Peña · E. L. Soriano-Bello (✉)
Instituto de Investigaciones Químico Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, 58030 Morelia, Mich., Mexico
e-mail: esoriano@umich.mx

Keywords *Arabidopsis thaliana* · Oligogalacturonides · Root architecture · Flavonoids · Auxin transport · Auxin signaling · Defense responses · Camalexin

Introduction

The capacity of plants to survive adverse conditions and reach reproductive maturity critically depends on their ability to continuously adapt to changes in the environment. Therefore, plants have evolved an array of intricate regulatory mechanisms that involve the generation of signaling molecules mediating the activation of adaptive responses. In particular, the activation of pathogen-specific defense mechanisms upon microbial infection and the acquisition of architectural and physiological adjustments to environmental changes permit survival, development, and reproduction of plants.

Physiological and ecological constraints play key roles in plant growth and developmental patterns, especially in relation to defense against pathogens or in interactions with beneficial microorganisms. Plant activity at the cellular level can be classified as growth (cell division and enlargement) and differentiation (chemical and morphological changes leading to cell maturation and specialization). These processes are often affected in response to microorganisms because plants must grow fast enough to compete, yet maintain the defenses necessary to survive in the presence of pathogens or symbiotic organisms (Herms and Mattson 1992).

The plant cell wall is a complex extracellular structure that plays important roles in plant growth and development (Humphrey and others 2007). It is also the first line of defense against pathogens (Ridley and others 2001). At the early stages of infection, phytopathogenic microorganisms

produce enzymes capable of degrading the plant cell wall (Vorwerk and others 2004); among these enzymes, polygalacturonases (PGs) cleave the α-1,4 glycosidic bonds present between the galacturonic acid units of homogalacturonan, the main component of pectin. PGs not only enable pathogens to enter, they also activate defense responses in the plant. These plant defense responses are triggered by fragments of homogalacturonans with a wide degree of polymerization of 3 to 15 residues called oligogalacturonides (α-1,4-D-oligo-GalUA) or OGs, which accumulate in the plant apoplast (Spiro and others 1998; Cabrera and others 2008; Denoux and others 2008). OG actions include the production of reactive oxygen species, glucanases, chitinases, and phytoalexins, and expression of inducible genes involved in the production of antimicrobial compounds (Ridley and others 2001; Nafisi and others 2007; Denoux and others 2008). In *Arabidopsis thaliana*, OGs induce the expression of several defense genes, including *PAD3*, which encodes the cytochrome P450 (CYP71B15) enzyme that catalyzes the last step in camalexin biosynthesis (Ferrari and others 2007). In addition to these defense events, OGs have also been shown to induce changes in plant growth and development (Mauro and others 2002; Spiro and others 2002). Treatment of bean plants with a decagalacturonide (OG_{10}) has shown a marked reduction in primary root growth and alterations in lateral root formation (Hernández and others 2006). Increasing experimental evidence indicates that exogenously added OGs are able to antagonize the action of the phytohormone auxin (indole-3-acetic acid, IAA) (Ridley and others 2001; Hernández and others 2006). Because auxin plays an essential role in growth and developmental processes in plants, the changes effected by OGs in the root system architecture appear to be mediated at least in part by auxin signaling. Auxin controls many different aspects of plant development; recent reports have shown that these distinct auxin responses are defined by complex and interdependent relationships between auxin metabolism, transport, and signaling (Kieffer and others 2010). Specifically, directional (polar) transport of the auxin between cells is a plant-specific form of developmental regulation. Transport-based asymmetric auxin distribution within tissues produces auxin gradients, which play important roles in many developmental processes, including cell patterning and tropisms (Tanaka and others 2006). IAA moves throughout the plant by phloem or by a more controlled polar transport system (PAT). PAT is a process regulated by AUXIN RESISTANT 1/LIKE AUX1 (AUX1/LAX) uptake proteins, PIN-FORMED (PIN) efflux carriers, and P-GLYCOPROTEIN (MDR/PGP/ABCB) efflux/conditional transporters (Swarup and others 2001, 2004; Kleine-Vehn and others 2006; Wu and others 2007; Mravec and others 2008; Kieffer and others 2010). There are several *Arabidopsis* mutants defective in the production of auxin transport proteins or in the correct location of these proteins that show auxin-related phenotypes, including *aux1-7* and *doc1/tir3*. The *aux1-7* mutant is defective at the *AUX1* locus (Swarup and others 2001), whereas *doc1/tir3* is a mutant allele of *BIG*, which encodes a protein important for the correct location of the PIN1 auxin transport protein (Gil and others 2001). Auxin is perceived by the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX PROTEIN 1-3 (TIR1/AFB1-3) receptor family. TIR1 is part of the ubiquitin-ligase complex $SCF^{TIR1/AFB}$ that catalyzes the ubiquitination and destruction of AUXIN/INDOL-3-ACETIC ACID (AUX/IAA) proteins. These proteins, under low auxin concentrations, form dimers with AUXIN RESPONSE FACTORS (ARF) transcription factors, thereby blocking the activity of ARFs. Once freed from the AUX/IAAs, ARFs regulate the expression of auxin-responsive genes (Lau and others 2008; Kieffer and others 2010). *Arabidopsis* mutants affected in components of auxin signaling complexes such as *tir1-1* (Dharmasiri and others 2005) show auxin-related phenotypes. Although much has been learned about auxin signaling in plants, the mechanisms underlying the antagonistic effect of OGs on auxin-induced morphogenetic programs remain uncertain.

Many plants accumulate secondary metabolites in their tissues, possibly to protect the rapidly growing seedling from pathogen attack; these metabolites can also act as mediators of growth and developmental processes. Flavonoids, a class of phenol-like compounds produced by plants in response to developmental and environmental factors, are considered natural regulators of cellular auxin efflux and PAT (Brown and others 2001; Buer and Muday 2004; Peer and Murphy 2007). It was found that flavonoid treatment inhibits ABCB-mediated auxin transport in heterologous expression systems (Bouchard and others 2006; Bailly and others 2008). In *abcb4* and *abcb19* mutants, quercetin was accumulated at the same region where ABCB4 and ABCB19 would be localized (Titapiwatanakun and Murphy 2008). Because flavonoids are important negative regulators of PAT at the plasma membrane by inhibiting the activity of auxin transport proteins and OGs have been reported to affect auxin responses in plants, it seems possible that the relationship between OGs and auxin transport would be an important factor linking plant immunity to growth and development. However, the mechanisms involved in this interaction remain to be investigated.

To test a potential OG-auxin interaction and its role in flavonoid biosynthesis and plant development, in this study we evaluated the effects of OGs in *Arabidopsis* root system architecture. *PAD3* gene expression and camalexin quantification in *Arabidopsis* wild-type (WT) plants treated with

OGs were analyzed to determine whether changes in developmental processes correlate with activation of plant immunity. In addition, *DR5::uidA* auxin-inducible marker expression and fluorescence analyses in WT and mutant plants were carried out to determine whether flavonoid accumulation modulates auxin responses in the primary root. Collectively, our results indicate that OGs might play a dual function in adaptation of plants to pathogen challenge by inducing defense responses and plant architecture adjustment.

Materials and Methods

Plant Material and Growth Conditions

WT *Arabidopsis thaliana* (Col-0 and Ler ecotypes) seedlings, *DR5::uidA* (Ulmasov and others 1997) transgenic line, and *tir1-1* (Dharmasiri and others 2005), *aux1-7* (Pickett and others 1990), *pgp4* (Terasaka and others 2005), *pgp1* and *pgp19* (Geisler and others 2003), *doc1/tir3* (Gil and others 2001), and *tt4-1* (from the ABRC at Ohio State University, At5G13930 CS85) mutant lines were used for the experiments. Seeds were surface disinfected with 1 ml 95% ethanol (v/v) for 5 min and 1 ml sodium hypochlorite 5% and SDS 10% solution. After six washes with 1 ml sterile distilled water and 2 days of stratification at 4°C, seeds were germinated and grown on agar plates containing 0.2× Murashige and Skoog medium (1962), (MS, Sigma), 2% Suc (w/v), pH 5.7, 200 µl/l vitamin stock (thiamin 1 mg/ml, pyridoxine 5 mg/ml, and nicotinic acid 5 mg/ml), and 1% phytagar (Sigma). The medium was supplemented with 1-*N*-naphthylphthalamic acid (NPA, 10^{-6} M), synthetic auxin naphthalene acetic acid (NAA, 10^{-6} M), and different concentrations of OGs ranging from 0.1 to 100 ng/ml. Plates were placed in a vertical position to allow root growth along the agar surface in a chamber with a photoperiod of 16-h light and 8-h darkness at 22–24°C.

Isolation, Chemical Determination, and Inactivation of OGs

Cell wall preparation was done from the French bean (*Phaseolus vulgaris* L.) "Flor de Mayo." Plant material was obtained from seeds germinated in pots with aseptic moist conditions in a growth chamber with a 16-h light, 8-h dark photoperiod at 22–24°C. The seeds had been surface sterilized for 15 min at 5% NaOCl and then washed with abundant sterile distilled H_2O.

Hypocotyls were collected from plants when the first pair of true leaves emerged, frozen in liquid N_2, and stored at −70°C. Cell walls were prepared from 35 g of bean hypocotyls that were ground to a fine powder in a mortar, resuspended in 500 ml 0.5 M Na_2HPO_4, pH 7.0. The suspension was centrifuged at 10,000 rpm for 15 min at 4°C. The pellet was washed twice with the same solution and three times with deionized H_2O. The pellet was then resuspended in 500 ml boiling ethanol, stirred for 1 h, and filtered on Wathmann #3 paper. The collected walls were resuspended in 500 ml ethanol, washed with 500 ml chloroform:methanol (1:1 v/v) and 500 ml acetone, and the solvent was discarded by suction. The wall sample was air-dried and used for OG preparation (Hahn and others 1981).

Preparation of α-D-oligogalacturonides (OGs)

OGs were prepared from 0.30 g of cell wall samples dissolved in 35 ml sodium acetate 50 mM, pH 4.0 buffer, and 24.5 units of pectinase from *Rhizopus* sp. (Sigma), stirred with a glass spindle for 1 min, incubated at 25°C, and stirred at 100 rpm for 1 h. The sample was filtered on Whatmann #3 paper. The solution was boiled for 5 min and centrifuged for 5 min at 5000 rpm. The supernatant volume was reduced to 1 ml under vacuum evaporation at 40°C and then loaded onto a chromatograph Bio-gel P6 medium (Bio-Rad) matrix column (1 × 132 cm) previously equilibrated with deionized water. The sample was eluted with deionized water. Two-milliliter fractions were collected and galacturonate content was spectrophotometrically determined ($\lambda = 525$ nm) in a reaction mix with sodium tetraborate and carbazol, according to Davidson (1966).

The sample was separated into four peaks (fractions 15-27 **I**, 28-31 **II**, 32-35 **III**, 37-41 **IV**). Fractions from each peak were concentrated and adjusted to 2.5 µg/µl and elicitor activity determined. Because peak III had the highest activity to elicit phaseollin production, it was selected and purified by filtration in a Bio-gel P4 medium column (1 × 140 cm) equilibrated and eluted with deionized water. Pool III was resolved into six peaks as determined by galacturonate content. Fractions in each peak were pooled and named IIIa to IIIf and the activity was newly determined. The approximate molecular weight of the most active peak IIId OGs was determined by filtration on a Bio-gel P4 medium column (0.7 × 228 cm) calibrated with mannooligosaccharides of molecular weight ranging from 504.5 (3mer) to 1801 (11mer) Da, in addition to mannose (180.2 Da) and cyanocobalamin (1350 Da) (Dixon and others 1989).

OGs were inactivated by reducing their C-1 side to a primary alcohol with sodium borohydride (OG-R). OG-R was prepared essentially according to the procedure described by Spiro and associates (2002) and used as specificity control for OG bioactivity.

Molecular Analysis of OGs by MALDI TOF-TOF

Molecular analysis of the OGs was performed in a 4800 *plus* MALDI TOF-TOF analyzer (Applied Biosystems) under the following conditions: Positive mode with laser intensity of 4200 and 500 total shots/spectrum. An α-cyano-4-hydroxycinnamic acid matrix (5 mg/ml) was used. OG concentration was 0.01 μg/μl and 1 μl matrix-dry plus 1 μl sample-dry was applied.

Histochemical Analysis

To analyze β-glucuronidase reporter gene activity (GUS), *CycB1::uidA* and *DR5::uidA* transgenic seedlings were incubated overnight at 37°C in the reaction buffer (0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate). Seedlings with the blue characteristic staining reaction were subjected to tissue clarification, which consisted of incubating the seedlings in an acidic solution (HCl 0.24 N in methanol 20%) for 60 min at 60°C, then in 60% ethanol and 7% NaOH solution for 20 min at room temperature, followed by 40, 20, and 10% ethanol treatments, 15 min each. Finally, the tissue was incubated overnight in 50% glycerol (Malamy and Benfey 1997a). At least 20 seedlings were analyzed from each treatment in two independent experiments. Seedling images were captured using a camera adapted to a microscope Optiphot-2 (Nikon, Tokyo).

Root Analysis

Analysis of root system architecture (RSA) of *Arabidopsis* seedlings was performed using a stereomicroscope (Iroscope ES-24, Leica). Lateral roots emerging from primary roots in WT and mutant seedlings were observed in a 4× objective and the total lateral root number was recorded. The primary root length was measured using a ruler in at least 20 seedlings. The primary root meristem length was determined as the length of cortex meristematic cell files from the stem cell niche to the transition zone where cells leave the meristem and enter the elongation zone of primary roots in WT (Col-0) plants. Cortical images were captured from the primary root differentiation zone of cleared seedlings using a camera connected to a microscope to determine its length (Ramírez-Chávez and others 2004). From the same zone of Col-0 plants, the length and number of root hairs were measured from photographs recorded with a digital camera connected to a microscope. Tissue clearing and mounting were performed as previously described (Malamy and Benfey 1997a). Independent cell measurements were done for at least 20 seedlings from two independent experiments. The root gravitropic response was determined in 3-day-old seedlings grown in vertical plates under the above conditions and then subjected to gravity stimulation by rotating the plates 90° for 24 h and measuring the rotation angle of each root to determine the response to that stimulus.

Flavonoid Fluorescence Staining and Quantification

Flavonoids were visualized in vivo by the diphenyl boric acid 2-amine ethyl ether (DPBA) fluorescence method. Five-day-old *A. thaliana* Col-0 and *tt4-1* mutant seedlings grown in 10 ng/ml OGs were stained with DPBA. Fluorescent staining of whole seedlings and quantification of fluorescent intensities were performed according to the procedure described by Buer and others (2007). Fluorescent intensities were analyzed from 20 independent seedlings in two independent experiments and representative photographs were used to construct panel figures.

Camalexin Determination

Camalexin was extracted from 3-day-old seedlings grown in MS 0.2× medium with 10 ng/ml OGs or from rosette leaves from 4-week-old *A. thaliana* plants growing in soil and sprayed with 1 ml 10 ng/ml OGs; OG-R or distilled water were used in control plants. Fresh-weight rosette leaves (18 mg) were used for phytoalexin extraction. The extraction was done essentially according to the procedure described in Glazebrook and Ausubel (1994). Camalexin concentration was calculated by comparison with a standard curve obtained by using purified camalexin kindly provided by Professor J. Glazebrook (University of Maryland). This analysis was performed using three samples from each condition in two independent experiments.

Real-time qRT-PCR Analysis of *PAD3* Gene Expression

Total RNA was isolated using TRIzol (Invitrogen) following the manufacturer's protocol. qRT-PCR was performed using the Superscript III Platinum SYBR® Green One-Step qRT-PCR kit (Invitrogen) and the Applied Biosystems 7500 Real-Time PCR machine. The thermal cycling program was as follows: 50°C for 30 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and a one-cycle dissociation stage at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The primers used were *Actin2* (At3g18780), 5′-CGTACAACCGGTATTG TGCTGGAT-3′ and 5′-CCCCAGCTTTTTAAGCCTTT-3′; *PAD3* (At3g26830), 5′-TGCTCCCAAGACAGACAA TG-3′ and 5′-GTTTTGGATCACGACCCATC-3′. *Actin2* was used as the internal reference gene, and expression values were normalized to those of *Actin2*. After the establishment of a dynamic range and validation of

endogenous control, an analysis of the relative quantification of the genes in the different conditions was performed. Data were then subjected to analysis of variance in a completely randomized design and the treatment means separated by Duncan's multiple-range tests.

Statistical Analysis

For all experiments, the overall data were analyzed statistically using the SPSS 10 program (SPSS Inc., Chicago, IL). Univariate and multivariate analyses of variance (ANOVA) and post-hoc Tukey tests were used for testing differences in cellular and developmental traits under OG treatments in WT and mutant plants. Different letters are used to indicate means that differ significantly ($p < 0.05$).

Results

OGs Induce Camalexin Accumulation and *PAD3* Expression

Phytoalexins are low-molecular-weight antimicrobial compounds produced by plants in response to pathogen attack. In *Arabidopsis*, the phytoalexin camalexin is produced in response to attack by bacterial or fungal pathogens (Glazebrook and Ausubel 1994; Nafisi and others 2007). To test the effect of OGs on camalexin production, we evaluated camalexin accumulation in both 3-day-old *Arabidopsis* seedlings grown in vitro and in rosette leaves of adult WT (Col-0) plants grown in soil, which were treated with 10 ng/ml OGs at different time points. Camalexin fluorospectrometry quantification analyses showed a significant accumulation of camalexin at 24 h after treatment in plants from the two experimental conditions (Fig. 1a, b). To determine the specificity of OG bioactivity, the accumulation of camalexin was also tested in response to inactive OGs, in which the reducing end had been chemically reduced by sodium borohydride (OG-R). Camalexin accumulation was absent in *Arabidopsis* seedlings treated with OG-R for 24 h (Fig. 1c). To determine whether camalexin production elicited by OGs was associated with the induction of the camalexin biosynthetic gene *PAD3*, which encodes a cytochrome P450 monooxygenase and catalyzes the last step in camalexin biosynthesis (Ferrari and others 2007; Nafisi and others 2007), we used qRT-PCR to monitor expression of this gene over the course of OG induction in *Arabidopsis* WT plants grown in soil. The levels of *PAD3* expression were determined immediately after OG treatment and 6, 24, and 48 h later. As shown in Fig. 1d, the expression of *PAD3* was clearly induced in plants 24 and 48 h after treatment and correlates with camalexin accumulation (Fig. 1a–c). These data show that OGs are able to elicit defense responses in *Arabidopsis*.

OGs Alter *Arabidopsis* Root System Architecture

The root system is an essential part of the plant, providing anchorage and playing an important role in water and nutrient uptake. The primary root is formed during embryogenesis; in contrast, lateral roots and root hairs are formed post-embryonically and increase the exploratory capacity of the root system (Malamy and Benfey 1997a; Parker and others 2000; López-Bucio and others 2005; Osmont and others 2007).

To determine whether activation of defense responses by OGs could alter plant growth and developmental processes, the changes in the root system architecture in *Arabidopsis* seedlings grown in agar plates containing MS 0.2× medium with active OGs were evaluated. Seedlings were germinated and grown in vertically oriented Petri dishes containing 0.2× MS agar media with OG concentrations ranging from 0.1 to 100 ng/ml. A remarkable effect on the *Arabidopsis* root architecture—inhibition of primary root growth and promotion of lateral root formation—was observed (Fig. 2a–e). To analyze more closely the effects of OGs on root development, primary root length, number of emerged lateral roots, and lateral root density were quantified in *Arabidopsis* seedlings grown under the same conditions. After 10 days of growth, it was observed that treatments of 1, 10, and 100 ng/ml OGs had a dose-dependent effect on primary root growth, in which a 100-ng/ml concentration showed a 28% inhibition of growth (Fig. 3a). Interestingly, OG treatments induced the formation of more lateral roots, suggesting a compensatory effect of the elicitor on lateral organs (Fig. 3b). The density of lateral roots was also calculated by dividing the number of lateral roots by the length of the primary root to normalize for the effects of OGs on primary root length. These results also showed a dose-dependent increase in lateral root density in seedlings treated with OGs when compared with the control (Fig. 3c).

Effect of OGs on Root Hair Development

Root hairs are tubular outgrowths of root epidermal cells and play a role in water and nutrient acquisition. It has been reported that root hair formation is affected by auxin (Parker and others 2000). Therefore, it was possible that root hair development could also be altered by OGs. To analyze whether OGs could alter root hair development, *Arabidopsis* seedlings were grown on the surface of solidified MS 0.2× medium supplied with different

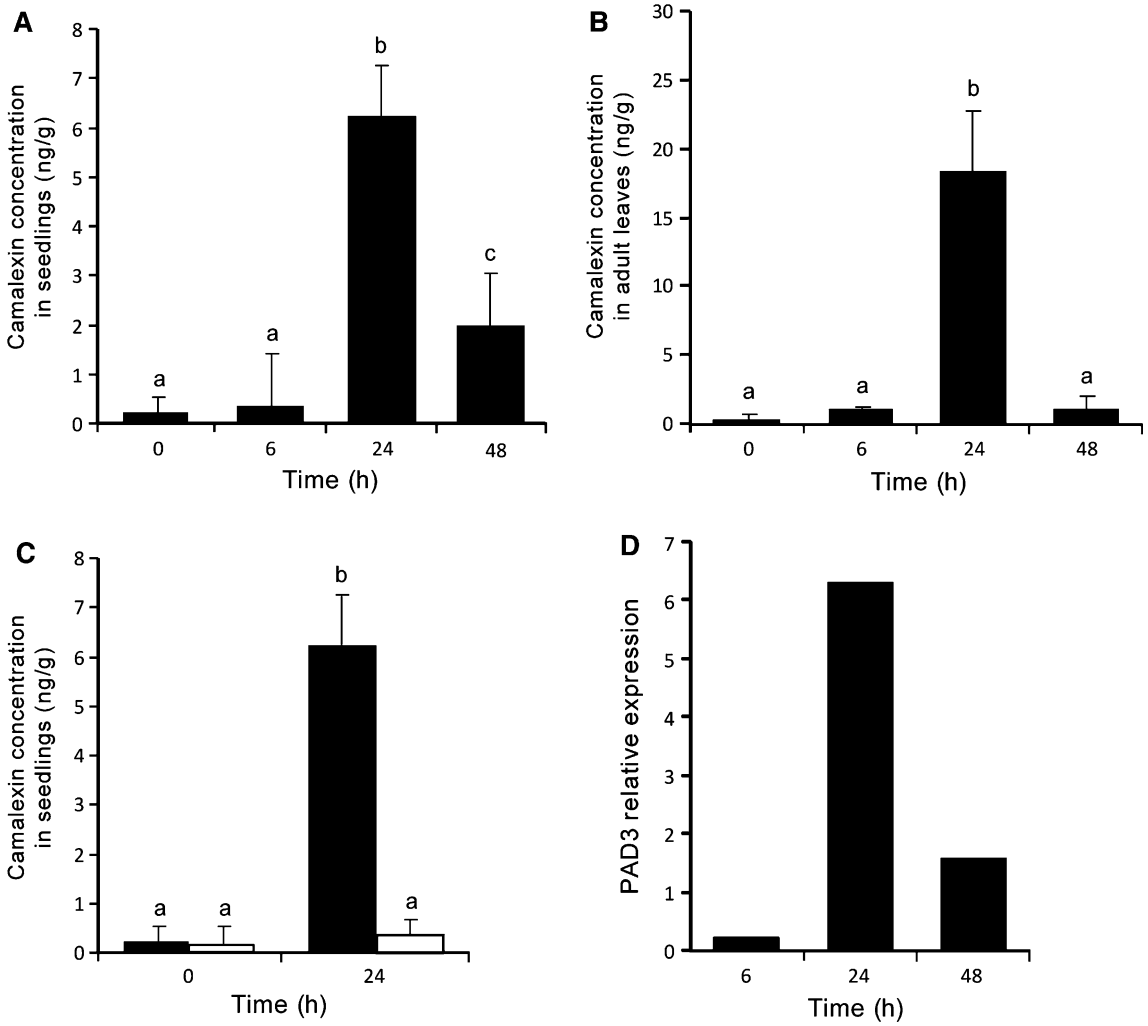

Fig. 1 Effects of OGs and reduced OG-R on camalexin accumulation and *PAD3* expression. **a** Camalexin accumulation in 3-day-old *Arabidopsis* Col-0 seedlings that were transferred for the indicated times to MS medium supplied with 10 ng/ml OGs. **b** Camalexin accumulation in rosette leaves of adult WT (Col-0) plants sprayed with 10 ng/ml OGs or water and harvested at the indicated times after treatment. **c** Camalexin accumulation in seedlings treated with OG-R (*white bars*) and OGs (*black bars*) 10 ng/ml. **d** Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of *PAD3* expression level at various times in response to 10 ng/ml OG treatment. Plant material was processed as indicated and qRT-PCR analysis performed using three independent samples from each treatment. Values shown represent the mean of 20 seedlings ± SD. *Different letters* represent means statistically different at the 0.05 level. The experiment was repeated twice with similar results

concentrations of OGs. Root hair parameters, including trichoblast length, root hair number, and root hair length, were analyzed after 6 days of growth using primary roots of control and OG-treated plants. Our data show that treatment with OGs decreased trichoblast length (Fig. 4a), which was related to a substantial increase in both the number and length of root hairs, except at 100 ng/ml, which was inhibitory (Fig. 4b, c). In these experiments, the effect was most evident in roots grown in 10 ng/ml OGs in which a threefold increase in root hair length was observed compared to the control (Fig. 4c). These results show that trichoblast length and root hair development are also modulated by OGs, perhaps as part of the compensatory growth mechanisms that are activated after primary root growth inhibition.

OGs Alter Cell Elongation

To determine whether OG treatment could alter root development by altering cell division, the length of the primary root meristem was determined in WT (Col-0) seedlings. The results show that OG treatment does not alter cell proliferating activity in the primary root meristem of *Arabidopsis*. Cell elongation was also analyzed by measuring cortical cell size in the differentiation and maturation regions of primary roots in WT (Col-0) plants.

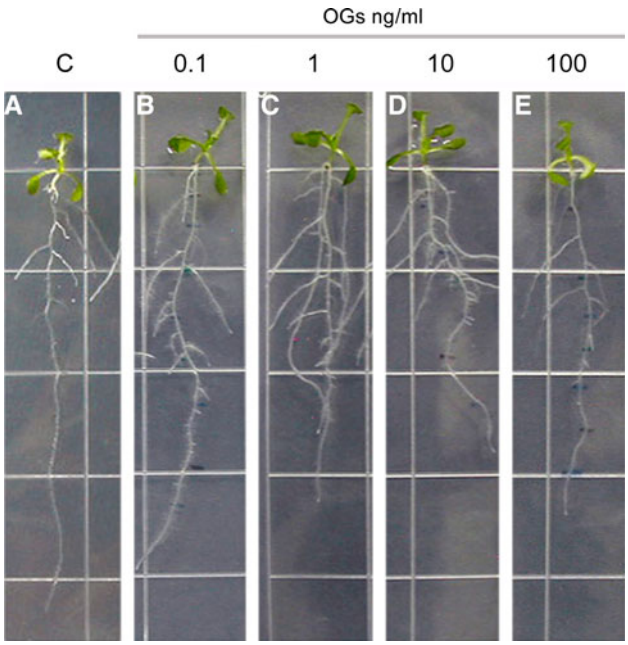

Fig. 2 Effects of OGs on *Arabidopsis* growth and development. **a–e** Wild-type (Col-0) seedlings were grown for 10 days under increasing OG concentrations or water treatment on vertically oriented agar plates. Representative photographs were taken of at least 20 plants analyzed. The experiment was repeated three times with similar results

Interestingly, the elongation zone length showed a significant decrease in plants treated with 10 and 100 ng/ml OGs (Fig. 5b). Therefore, the observed inhibitory effect of OGs on primary root growth seems to be caused by an inhibition of cell elongation.

Involvement of Auxin in *Arabidopsis* Responses to OGs

The effects of OGs on root development observed in this study are similar to that described for auxin in plants (Casimiro and others 2001). Because auxin controls plant morphogenesis via its activity gradients, we next tested whether OGs could alter auxin responses in *Arabidopsis* roots. The auxin response was monitored by using the auxin-inducible marker gene *DR5::uidA* (Ulmasov and others 1997). Figure 6 shows histochemical staining for transgenic *DR5::uidA* plants that were grown 8 days under naphthalene acetic acid (NAA), 1-*N*-naphthylphthalamic acid (NPA), or OG treatments. As previously reported (Ulmasov and others 1997), in untreated control plants *DR5::uidA* was expressed in the primary root meristem and the elongation zone corresponding to the provasculature in root tips (Fig. 6a). When *DR5::uidA* seedlings were exposed to auxin treatment, we found that a concentration of 10^{-6} M NAA induced an extended GUS

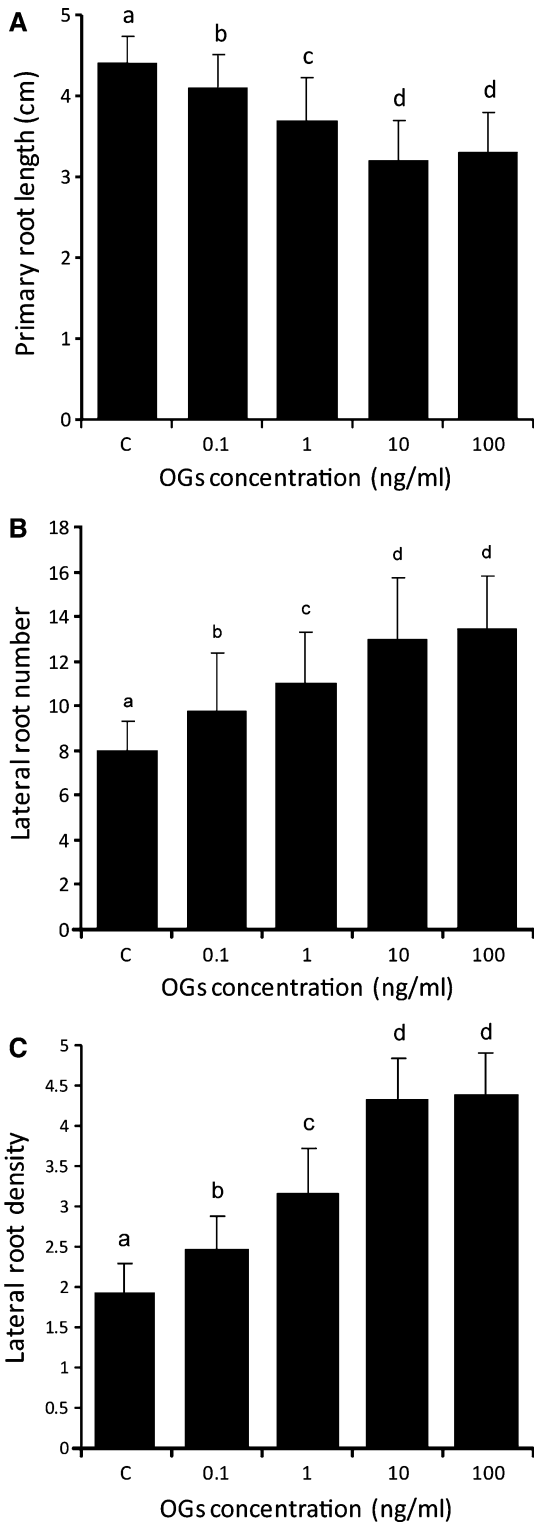

Fig. 3 Effects of OGs on *Arabidopsis* root system architecture. Wild-type (Col-0) seedlings were grown for 10 days under increasing OG concentrations or water treatment on vertically oriented agar plates. Data are given for **a** the length of the primary root, **b** lateral root number per seedling, and **c** emerged lateral root density. Values shown represent the mean of 20 seedlings ± SD. *Different letters* represent means statistically different at the 0.05 level. The experiment was repeated twice with similar results

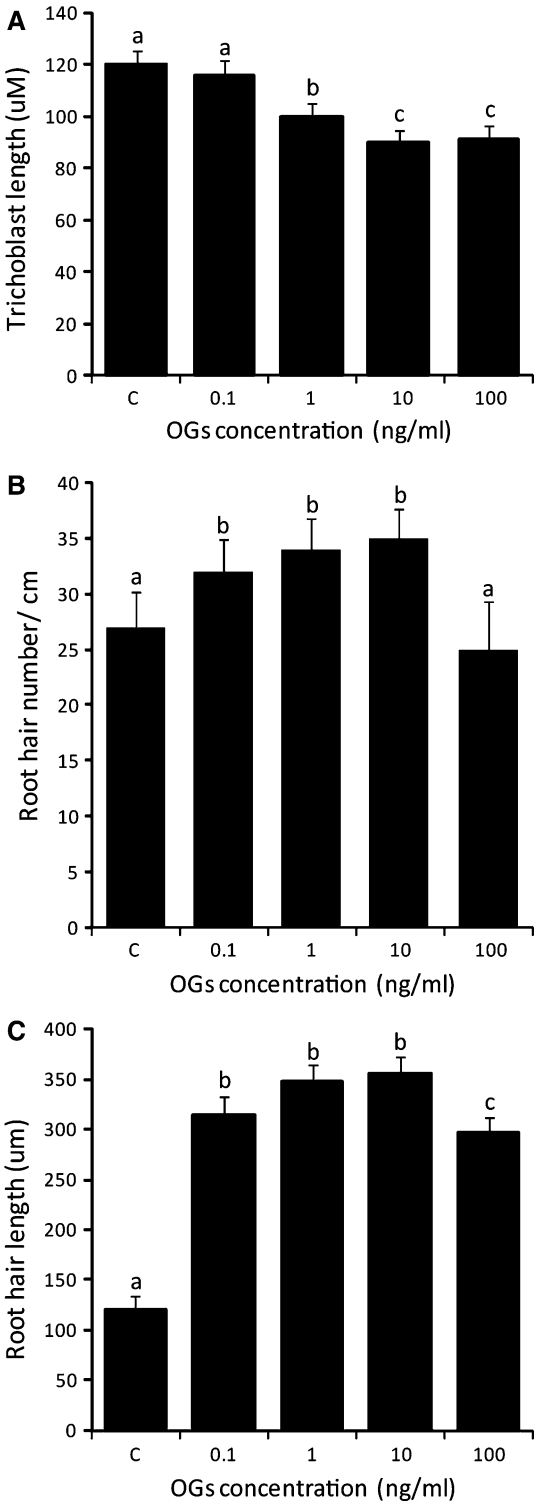

Fig. 4 Effects of OGs on root hair development. **a** Trichoblast length. **b** Root hair number. **c** Root hair length. *A. thaliana* (Col-0) seedlings were grown for 6 days on MS 0.2× medium supplemented with the indicated concentrations of OGs. Data points indicate mean of 20 seedlings ± SD. The results show means of ten epidermal cells located in the root hair forming zone of the primary root. *Different letters* indicate statistical differences at $p < 0.05$. The experiment was repeated twice with similar results

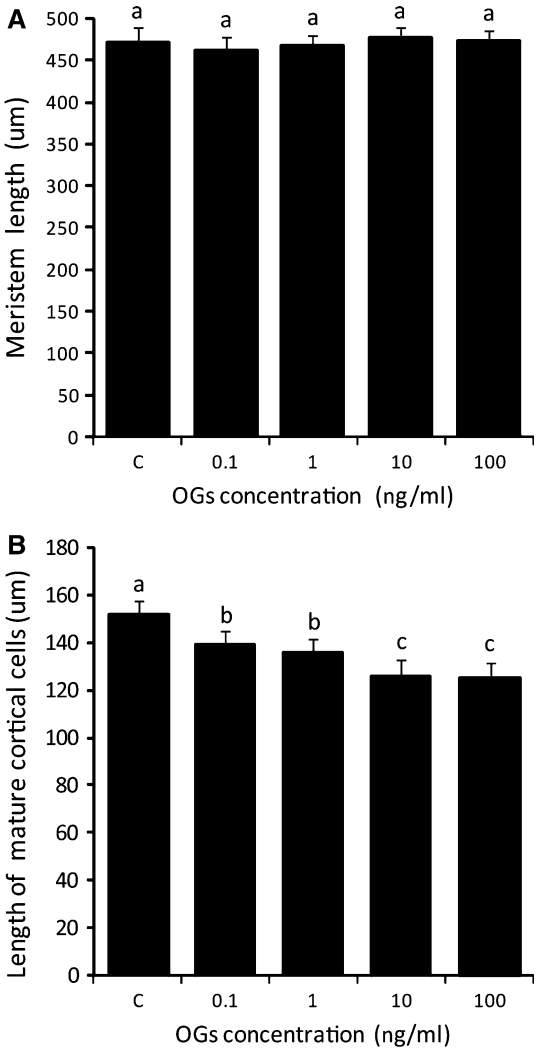

Fig. 5 Effects of OGs on cell division and elongation in the primary root. WT (Col-0) seedlings were grown for 8 days under increasing OG concentrations or water treatment on vertically oriented agar plates. The seedlings were cleared and determinations were done for **a** the meristem length and **b** the length of mature cortical cells. Values shown represent the mean of 20 seedlings ± SD. *Different letters* represent means statistically different at the 0.05 level. The experiment was repeated twice with similar results

activity, which correlated with prolific root hair development close to the root tip (Fig. 6b). In contrast, PAT inhibition by treatment with 10^{-6} M NPA led to enhanced *DR5::uidA* expression in the QC and columella cells but decreased expression in provasculature (Fig. 6c). Interestingly, *DR5::uidA* plants grown in a concentration of 10 ng/ml OGs showed a reduced GUS activity in the QC, columella, and provasculature cells compared to the control (Fig. 6d). These data suggest that OGs did not increase auxin responsiveness in primary root tips but actually decreased auxin responses as revealed by marker-regulated gene expression.

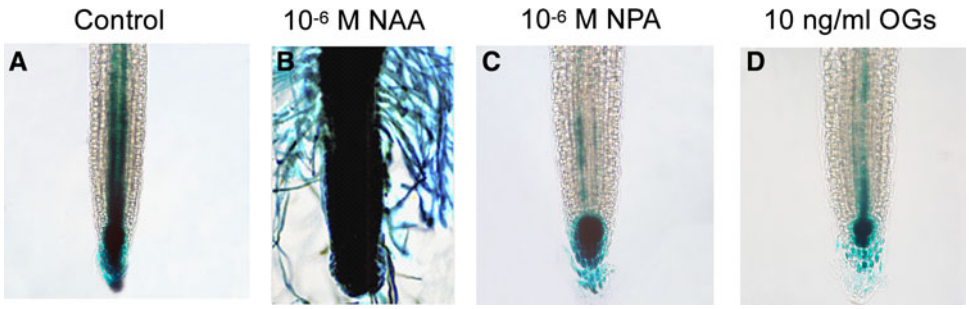

Fig. 6 Effect of OGs on auxin response in *Arabidopsis* primary root tips. Twelve hours of GUS staining of *DR5::uidA* primary roots grown for 8 days on MS 0.2× medium supplied with **a** water, **b** 10^{-6} M NAA, **c** 10^{-6} M NPA, or **d** 10 ng/ml OGs. *Photographs* are of representative individuals of at least 15 plants stained. The experiment was repeated twice with similar results

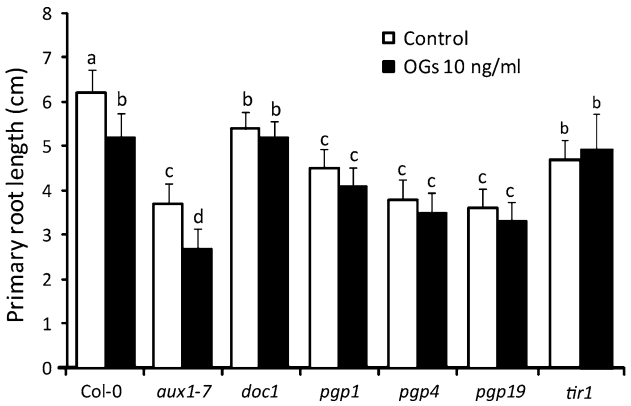

Fig. 7 Primary root growth response of WT (Col-0) and auxin-related mutants to OGs. Col-0 and *aux1-7*, *doc1*, *pgp1*, *pgp4*, *pgp19*, and *tir1* mutant seedlings were grown for 8 days in 10 ng/ml OGs or water-containing media on vertically oriented agar plates and the length of the primary root determined for 20 seedlings. Values shown represent the mean ± SD. *Different letters* represent means statistically different at the 0.05 level. The experiment was repeated twice with similar results

PAT Mutants Show Differential Responses to Root Architecture Changes Induced by OGs

Comparing the effects of OGs and NPA on auxin responses in the root tip suggests that these compounds act in different ways to alter auxin signaling in *Arabidopsis*. To explore the role of auxin transport and signaling in mediating the effects of OGs on root architecture adjustment, we analyzed the primary root growth response of *Arabidopsis* WT (Col-0) and mutant seedlings defective in auxin transport, that is, *aux1-7*, *doc1*, *pgp1*, *pgp4*, and *pgp19,* or auxin signaling, that is, *tir1-1*. As shown in Fig. 7, the mutant defective in the auxin influx carrier, *aux1-7*, showed similar primary root growth inhibition when treated with 10 ng/ml OGs compared to WT seedlings, indicating that this mutant is as sensitive to OGs as the WT. In contrast, the mutant lines *doc1*, *pgp1*, *pgp4*, *pgp19*, and *tir1-1* showed decreased primary root growth in both the presence and the absence of OGs, indicating that these genes are important for root growth independent of the supply of OGs and that both auxin transport and signaling are important for root developmental responses to OGs (Fig. 7).

Root Architecture Responses in *tt4 Arabidopsis* Mutants

We also tested the growth response of the *transparent testa* mutant *tt4-1*, which is defective in flavonoid biosynthesis, to investigate if OGs could affect auxin distribution and signaling via synthesis of flavonoids. Flavonoids have been implicated as natural regulators of auxin transport in vivo and the absence of flavonoids in this mutant line results in elevated auxin transport (Murphy and others 2000; Peer and others 2004; Buer and Muday 2004). Our results revealed that *tt4-1* mutant seedlings supplied or not with 10 ng/ml OGs show reduced primary root growth when compared to WT (Ler) seedlings (Fig. 8a). Interestingly, OG application did not result in increased lateral root formation in the mutant (Fig. 8b, c), indicating that flavonoid biosynthesis is important in normal and OG-induced lateral root formation.

OGs Induce Flavonoid Accumulation in Primary Root Tips

The result that *tt4-1* is resistant to OG-mediated root architectural changes prompted us to determine whether OGs affect flavonoid accumulation. We performed experiments to determine if OGs could induce flavonoid accumulation in WT and *tt4-1* plants. Flavonoids can be visualized using diphenyl boric acid 2-amino ethyl ester (DPBA), a flavonoid-specific dye (Buer and others 2007). In *Arabidopsis*, DPBA forms a fluorescent complex with quercetin and kaempferol generating golden and green fluorescence, respectively (Buer and others 2007). WT (Col-0) seedlings grown without 10 ng/ml OGs showed flavonoid fluorescence in all three assayed zones from the

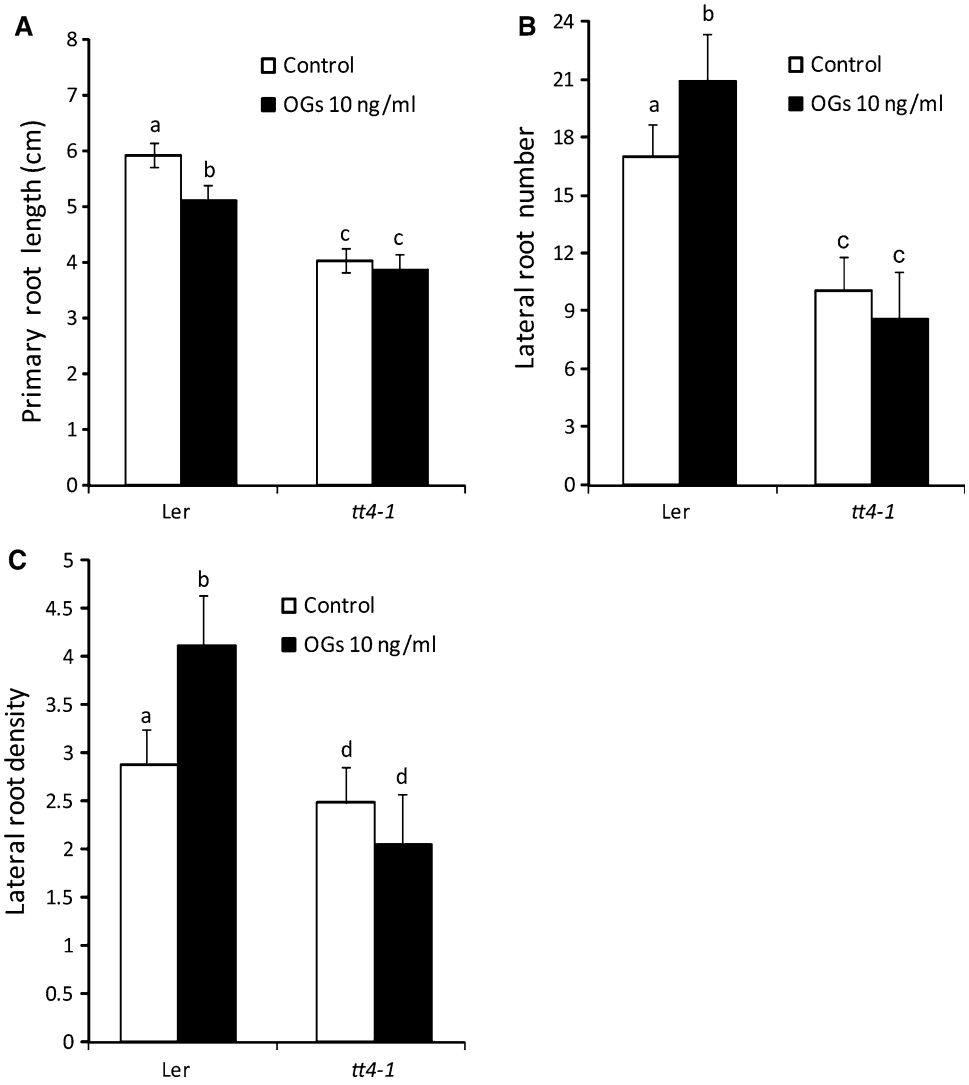

Fig. 8 Effect of OGs on *tt4-1* mutant root system architecture. *A. thaliana* WT (Ler) and *tt4-1* seedlings were grown for 8 days in MS 0.2× medium supplied with 10 ng/ml OGs or water on vertically oriented agar plates. **a** The length of the primary root. **b** Lateral root number per seedling. **c** Emerged lateral root density. Values shown represent the mean of 20 seedlings ± SD. *Different letters* represent means statistically different at the 0.05 level

seedling, namely, the cotyledons, the hypocotyl-root junction zone, and the cell elongation zone of the primary root. Interestingly, OG treatment increased the fluorescence signal in all three evaluated tissues of the plant, whereas in *tt4-1* mutant seedlings, no fluorescence signal was found (Fig. 9a). In *tt4-1* seedlings, only a red autofluorescence distinctive of chlorophyll in cotyledons was observed.

We next evaluated whether flavonoid accumulation in *Arabidopsis* roots treated with OGs correlates with sites of low or high auxin accumulation. We tested flavonoid accumulation in *DR5::uidA* seedlings treated with OGs and compared flavonoid fluorescence with maximum auxin response regions revealed by this marker in primary roots. Our data show that enhanced accumulation of flavonoids in response to OG treatment coincided with decreased GUS expression in the root elongation region (Fig. 9b). We further investigated the role of altered auxin response in the root elongation region in gravitropic responses in WT (Col-0). Gravitropism is a growth response mediated by lateral auxin transport in the elongation region of the primary root stimulated by gravity (Muday 2001). The data obtained from this analysis revealed that seedlings treated with OGs exhibit a delayed gravitropic response in the primary root (Fig. 9c). This indicates that OG treatment can modulate the rate of root gravity response, suggesting that the synthesis of flavonoids by OGs is an important factor for the action of the elicitor, possibly acting as an intermediary that regulates auxin distribution and/or action in the root system.

Discussion

Defense-related responses in plants are stimulated by molecules known as elicitors. A number of reports have shown that OGs, a class of compounds derived from cell wall hydrolysis during plant-microbe interactions, play a

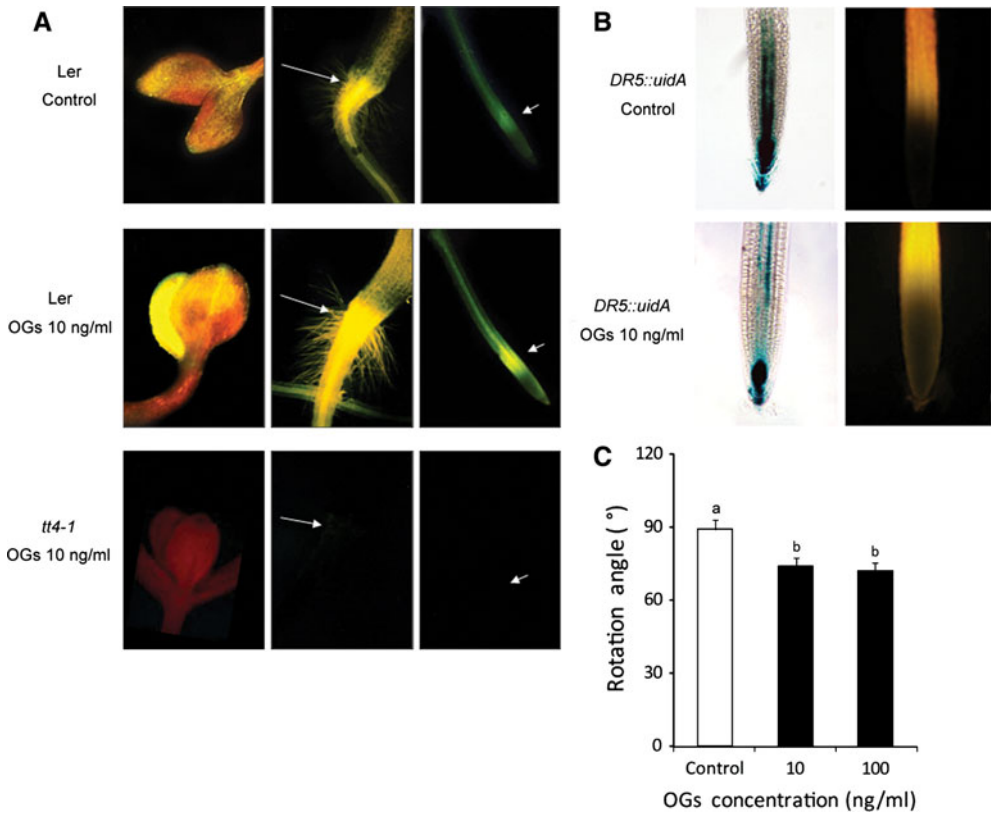

Fig. 9 Effect of OGs on flavonoid accumulation in *Arabidopsis* seedlings. **a** *Arabidopsis* WT (Ler), *tt4-1*, and *DR5::uidA* seedlings were grown for 5 days in MS 0.2× medium supplied with 10 ng/ml OGs or water on vertically oriented agar plates. The seedlings were loaded with DPBA and fluorescence determined by an epifluorescence microscope. *DR5::uidA* seedlings were stained for GUS activity and cleared to show gene expression. Representative photographs were taken from at least 20 plants analyzed. **a** Flavonoid fluorescence in WT and *tt4-1* seedling cotyledons, the hypocotyl-root junction zone, and the cell elongation zone of the primary root. **b** *DR5::uidA* GUS expression and flavonoid fluorescence in the primary root. **c** Effects of OGs on the root gravitropic response. Three-day-old *Arabidopsis* seedlings were grown in MS 0.2× medium supplied with 10 and 100 ng/ml OGs or water on vertically oriented agar plates. The plates were turned 90° for 24 h and the rotation angle of each root measured. The experiments were repeated twice with similar results

role as elicitors and may also act as signals for plant growth and development (Ridley and others 2001; Ferrari and others 2008). However, the cellular and physiological processes that are responsible for developmental responses to these elicitors remain to be clarified. In this study we used *Arabidopsis thaliana* as a model to investigate the effects of OGs on auxin responses and root system architecture.

Denoux and others (2008) showed that OGs play an important role in mediating resistance to the necrothropic fungus *B. cinerea.* This resistance was found to be dependent on *PAD3* gene expression and camalexin accumulation. Phytoalexins are low-molecular-weight antimicrobial compounds that are synthesized in response to pathogen attack. The phytoalexin camalexin, an indole derivative, is also produced by *Arabidopsis* in response to infection by the bacterial pathogen *Pseudomonas syringae* (Zhou and others 1999). The PAD3 protein encodes a cytochrome P450 monooxygenase, which catalyzes the last step in camalexin biosynthesis. In this work we found that OG treatment induced camalexin accumulation in *Arabidopsis* seedlings, which correlated with increased expression of *PAD3* (Fig. 1), showing that OGs were active as elicitors and that plants treated with these compounds activated their defense response. Interestingly, we found that these molecules act as active defense elicitors even in low concentrations, in contrast to what has been reported in other plant systems where OGs have been used at higher concentrations (Ferrari and others 2008).

We presented evidence that OGs disturb the *Arabidopsis* root architecture by inhibiting primary root growth and inducing the formation of new lateral roots (Figs. 2, 3). This appears to be a global effect on cell growth and differentiation processes involving the entire root system, because root hair formation and elongation were also induced after treatment with the defense elicitor in a dose-dependent way (Fig. 4). In a previous study by Hernández-Mata and coworkers (2006), it was shown that OGs inhibited root elongation and the formation of secondary roots in bean seedlings grown in vitro. In the present work

we confirmed the primary root growth inhibitory effect in *Arabidopsis*. However, the effect of OGs on lateral root development was stimulatory rather than inhibitory. This apparent discrepancy can be explained because different media, liquid versus solidified, were used in the previous research with beans, and greater concentrations of OGs, which may repress lateral root development, were used in these previous experiments. The root system displays considerable plasticity in its morphology and physiology in response to variability within its environment (Malamy and Benfey 1997b; Parker and others 2000; López-Bucio and others 2005; Osmont and others 2007). Lateral roots are formed from pericycle cells, which proliferate to give rise to a new lateral organ, which extends the length of the root system. Root hairs are extensions of single epidermal cells, which are formed on the surface of both primary and lateral roots and comprise as much as 77% of the total root surface area of cultivated crops, forming the major point of contact between the plant and the rhizosphere (Parker and others 2000). Both lateral roots and root hairs play a pivotal role in rhizosphere processes, including anchorage to the soil and uptake of water and nutrients.

Interestingly, the decrease in primary root growth observed in OG-treated seedlings was not related to alterations in cell division but rather was caused by significant reduction in cell elongation (Fig. 5), with a further induction of differentiation processes, namely, increased lateral root and root hair formation. Although these root architectural effects are similar to those observed in plants when they are treated with auxins, OG treatment did not increase *DR5::uidA* expression in primary root tips, indicating that the primary root growth inhibition effect was not caused by increased auxin response at this region (Fig. 6). In contrast, reduced *DR5::uidA* expression in OG-treated primary roots correlated with increased expression of this marker in developing lateral roots (data not shown), suggesting that increased growth of lateral roots may be mediated by altered auxin distribution or altered response to auxin, which is an intriguing possibility (Fig. 6).

The similar reported effects of phytotropins such as NPA, a widely used auxin transport inhibitor with those of OGs on root cell patterning, namely, decreased primary root growth and alteration of auxin distribution in root tips, support the hypothesis that inhibition of auxin flow plays a role in RSA adjustment by OGs. Although OG effects suggest an alteration of auxin transport, one important difference in the mode of action of the elicitor when compared to NPA is that NPA leads to inhibition in lateral root development in addition to having an inhibitory effect on primary root growth, whereas OGs promote lateral root formation. This difference can be the result of the fact that the "global" NPA treatment would block auxin efflux in all plant tissues sensitive to NPA (Swarup and others 2004), which guides the auxin efflux, whereas OGs could affect specifically a certain subgroup of the NPA-binding proteins or ABC glycoproteins. Alternatively, because camalexin biosynthesis proceeds from the intermediate indole acetaldoxima (IAOx), a compound that sits at a branch point to IAA synthesis, we cannot exclude the possibility that alterations in the level of IAOx might impact auxin biosynthesis. This could explain why the pattern of response to OGs is more complex than that to NPA.

Certain flavonoids, especially quercetin and kaempferol, have an antagonist effect on auxin transport by direct interaction with the NPA-binding protein of auxin transporters and multidrug resistance (MDR) transporters, also termed ABC transporters (Brown and others 2001). The effects of OGs on primary root growth in WT and auxin transport mutants *aux1-7*, *doc1*, *pgp1*, *pgp4*, *pgp19*, and *tir1* revealed complementary interactions in the genetic responses to the elicitor. The *aux1-7* mutant, which showed altered gravitropism and decreased primary root growth in medium without OGs, showed a significant further reduction in growth in response to the elicitor (Fig. 7). In contrast, the *pgp1*, *pgp4*, and *pgp19* mutants, all of which are considered targets of flavonoid inhibition of root basipetal transport, showed decreased primary root growth when grown in MS medium and in medium supplied with OGs. This indicates that these genes are required for normal root growth in the same way as the auxin signaling gene *tir1-1* in that the primary root growth was reduced in seedlings whether they were treated with OGs or not.

Flavonoids are secondary metabolites found throughout the plant kingdom, where they have diverse roles. Two particular functions of flavonoids are relevant to the present work: the role played in defense (Denoux and others 2008) and the role played in auxin transport (Peer and Murphy 2007). Flavonoid accumulation in different plant regions increased with OG treatment, including the hypocotyl/root junction and the primary root elongation region (Fig. 9). In *Arabidopsis*, the flavonoid pathway is well characterized at the genetic, enzymatic, and product levels (Glazebrook and Ausubel 1994; Buer and others 2007). The *Arabidopsis thaliana transparent testa* (*tt*) mutants are compromised in the enzymatic steps or transcriptional regulators affecting flavonoid biosynthesis. Interestingly, the stimulatory effect of OGs on flavonoid accumulation was absent in the *transparent testa* mutant *tt4-1*, which is defective in the chalcone synthase enzyme that mediates the conversion of 4-coumaril-CoA to naringenin chalcone (Fig. 8). This mutant was also found to be resistant to the growth inhibitory effect induced by OG treatment in primary root growth and lateral root formation, providing genetic evidence of the role played by flavonoid biosynthesis in mediating the OGs root architectural responses. These results show the importance of flavonoids in root

development and are in agreement with previous developmental analysis in *tt4* mutants (Buer and Muday 2004). Recently, Buer and others (2009) revealed a wide variety of architectural phenotypes in root and aerial tissues in *Arabidopsis tt* mutants, including increased inflorescences, siliques, and lateral roots. Further analysis of *tt4* demonstrated that flavonols can act in specific cell files in the root to modulate the gravitropic response, which is in agreement with previous findings (Buer and Muday 2004). It is also of interest to note that increased concentrations of OGs caused alterations in the gravitropic response; this could be due to specific flavonoid accumulation in the root elongation zone (Fig. 9c). Several mechanisms regulate auxin transport during root gravitropism: One is the targeting of auxin efflux PIN3 protein to unique membranes in response to gravitropic stimulation. A second mechanism is reversible protein phosphorylation, which modulates the activity, abundance, or localization of auxin transport proteins. A third mechanism is the localized synthesis of flavonoids that regulate auxin transport to modulate the rate of gravity response (Buer and others 2006). As discussed above, the reduced IAA level in primary root tips in response to OGs may occur because IAA and camalexin have a common intermediate at indole acetaldoxima (IAOx), which may eventually become limiting (Zhou and others 1999; Sugawara and others 2009), or by increased accumulation of flavonoid in the root elongation region as shown in the present work.

Based on these analyses, we conclude that OGs are capable of regulating defense responses and root morphogenetic processes in *Arabidopsis thaliana*, inducing flavonoid accumulation at the root elongation region of the primary root and likely affecting auxin distribution within the root system. The combined effects of these responses in plants may ultimately determine the adaptive capability of the plant to the challenging biotic and abiotic environment.

Acknowledgments We thank Professors Angus Murphy for *pgp1*, *pgp4*, and *pgp19* mutant seeds and Jane Glazebrook for providing camalexin standard and extraction methodology. Dr. Rosa Elva del Río Torres, Dr. Lourdes Macias Rodriguez, and LDG A. Kanek Ballesteros Coria are thanked for excellent technical support in the chemical inactivation of OGs, interpretation of MALDI TOF-TOF analysis, and photographic work, respectively. We gratefully acknowledge the Unidad de Proteómica y Metabolómica, CINVESTAV, IPN for MALDI-TOF-TOF analysis. This research was supported in part by grants from the Consejo Nacional de Ciencia y Tecnología (CONACYT, grant No. 47239 and 48712) and Universidad Michoacana de San Nicolás de Hidalgo (grant No. CIC 2.11).

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