JULIANE MACIEL HENSCHEL

BIOMASS ALLOCATION IN RESPONSE TO LIGHT AND NITROGEN AVAILABILITY: ROLES FOR GIBBERELLIN AND AUXIN

Thesis presented to the Universidade Federal de Viçosa as part of the requirement of the Graduate Program in Plant Physiology for the obtention of the degree of *Doctor Scientiae*.

Adviser: Dimas Mendes Ribeiro

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ABSTRACT

HENSCHEL, Juliane Maciel, D.Sc., Universidade Federal de Viçosa, November, 2020. **Biomass allocation in response to light and nitrogen availability: roles for gibberellin and auxin**. Adviser: Dimas Mendes Ribeiro.

Gibberellins (GAs) and auxins are plant hormones that regulate plant growth and development, as well as their responses to environmental conditions. GAs modulate the biomass allocation in root vegetables, and low levels of GAs induce root growth instead of shoots. Thus, it was investigated whether low levels of GAs, caused by the application of paclobutrazol (PAC), induce biomass allocation to roots of radish plants, increasing their production. In addition, it was investigated whether the increased biomass allocation to shoots in response to high-density (HD) of plants and to low light intensity (LL) can be reversed by the application of PAC. The results show that low levels of GAs (PAC treatment) inhibited the growth of radish plants, with stronger growth inhibition in shoots than roots. In addition, GAs regulated radish growth in response to LL, but not in response to HD of plants. The biomass allocation of radish in response to LL was modulated by GA levels, which regulated the expression of genes related to auxin biosynthesis and cell expansion (*EXP* and *XTH*). Still, low levels of GAs altered the biomass allocation, contributing to the nutritional quality of tuberous roots of radish. Another important aspect of root growth is the modulation of root architecture in response to nitrogen availability. As auxins are central regulators of root growth, it was investigated how auxin transport, mediated by ABCB transporters, regulates root growth of *Arabidopsis thaliana* in response to different nitrogen sources and levels. It was observed that root growth in response to nitrogen availability is a result of auxin redistribution instead of global auxin levels in roots. In addition, auxin transport mediated by ABCB1/ABCB19, regulates the inhibition of lateral root emergence and root bending in response to ammonium and glutamine, whereas ABCB6/ABCB20 regulate root bending regardless of nitrogen source. This study provides new perspectives on the role of GAs and auxins on root growth and development in response to irradiance and nitrogen availability.

Keywords: Arabidopsis. Auxin. Biomass allocation. Light. Nitrogen. Gibberellin. Radish.

RESUMO

HENSCHEL, Juliane Maciel, D.Sc., Universidade Federal de Viçosa, novembro de 2020. **Alocação de biomassa em resposta à luz e à disponibilidade de nitrogênio: ação da giberelina e auxina**. Orientador: Dimas Mendes Ribeiro.

Giberelinas (GAs) e auxinas são hormônios vegetais que regulam o crescimento e o desenvolvimento das plantas. GAs modulam a alocação de biomassa em hortícolas tuberosas, sendo que em baixos níveis induzem o crescimento de raízes em detrimento da parte aérea. Dessa forma, buscou-se investigar se baixos níveis de GAs, causados pela aplicação de paclobutrazol (PAC), induzem a alocação de biomassa para raízes de rabanete, aumentando sua produção. Além disso, investigou-se se a maior alocação de biomassa para parte aérea em resposta à alta densidade de plantas (DP) e à baixa intensidade luminosa (IL) pode ser revertida pela aplicação de PAC. Os resultados mostram que PAC inibiu o crescimento de plantas de rabanete, sendo o crescimento da parte aérea mais afetado que a raiz. Além disso, GAs regularam o crescimento de rabanete em resposta à baixa IL, mas não à alta DP. A alocação de biomassa de rabanete em resposta à IL foi modulada pelos níveis de GA, que regularam a expressão de genes da biossíntese de auxina e expansão celular (*EXP* e *XTH*). Ainda, baixos níveis de GAs alteraram a alocação de biomassa, contribuindo para a qualidade nutricional da raiz tuberosa do rabanete. Outro aspecto importante do crescimento de raízes é a modulação da arquitetura radicular em resposta à disponibilidade de nitrogênio (N). Como auxinas são reguladores centrais do crescimento das raízes, buscou-se investigar como o transporte de auxinas, mediado por transportadores ABCB, regula o crescimento de raízes em *Arabidopsis thaliana* em resposta a diferentes fontes e níveis de N. O crescimento radicular em resposta ao N resultou da redistribuição de auxina e não dos seus níveis globais na raiz. Além disso, o transporte de auxina, mediado por ABCB1/ABCB19, parece regular a inibição da emergência de raízes laterais e a curvatura da raiz em resposta ao amônio e a glutamina, enquanto ABCB6/ABCB20 regulam a curvatura da raiz independentemente da fonte de N. Este estudo fornece novas perspectivas acerca da ação das GAs e auxinas no crescimento e desenvolvimento de raízes em resposta a irradiância e disponibilidade de N.

Palavras-chave: Arabidopsis. Auxina. Alocação de biomassa. Luz. Nitrogênio. Giberelina. Rabanete.

SUMMARY

GENERAL INTRODUCTION

Under natural and agricultural conditions, plants compete for resources such as nutrients, water, and light. Among them, light is an important resource that not only provides energy for photosynthesis but also triggers developmental responses, integrating plant development with environmental conditions (Kami et al., 2010; Yang and Li, 2017). Additionally, nitrogen (N) is an important nutrient required for plant growth and development, and its availability modulates root growth and architecture and crop production (Kiba and Krapp, 2016). Altered biomass allocation among plant organs can be a response to limited resources, being governed by optimal partitioning and size-dependent allometric partitioning (Bloom et al., 1985; Huxley, 1932; Niklas, 1994). These responses result in more resources allocated to the organs by which the limiting resources are absorbed, following the genetic potential of the plant. Thus, altered biomass allocation among plant organs, such as shoots and roots for example, can be a strategy to ensure better resource uptake under limiting irradiance and nutrients (Poorter et al., 2012). The perception of environmental signals such as shade and nutrient availability, as well as the responses to cope with them are known to be controlled by plant hormones (Verma et al., 2016). Although the plant hormones abscisic acid (ABA), salicylic acid (SA), jasmonate (JA) and ethylene (ET) are mainly known to mediate defense responses against biotic and abiotic stresses, studies have shown that they interact with the growth-promoting hormones auxins, gibberellins (GAs) and cytokinins (CKs) in regulating plant defense response (Verma et al., 2016; Bechtold and Field, 2020; Devireddy et al., 2020). In this sense, the role of auxin and GAs in controlling plant growth responses to shade and nitrogen availability will be further discussed here.

Plants absorb light in the range of 280–400 nm (UV radiation), 400–700 nm (photosynthetically active radiation – PAR) and reflect far-red wavelengths (700–800 nm) (Casal, 2013). Thus, both light intensity and quality are altered in the surrounding area. The changes in light quality include reductions in blue and UV irradiance and lower red/far red (R/FR) and blue/green ratios (Casal, 2013; Pierik and de Wit, 2014). Plants perceive these light signals and translate them into biological responses through sensors called photoreceptors by modulating gene expression. These photoreceptors trigger a cascade of internal signals, including through hormonal pathways, that culminate in a spectrum of morphological and physiological changes (Yang and Li, 2017). These responses are commonly called shade avoidance responses (SARs) and include changes in biomass allocation, with increased elongation of stems, hypocotyls, and petioles instead of other organs such as leaves, fruits, seeds, and roots (Casal, 2013; Ballaré and Pierik, 2017).

The main light signal determining growth responses during shade is the reduction in the R/FR ratio, which is perceived by phytochromes (phy) (Ballaré and Pierik, 2017). There are five members in the phytochrome family in the model plant *Arabidopsis* and radish plants (*Raphanus sativus* L.) (phyA–phyE), being that phyB is the predominant phy controlling SARs, while phyD and phyE act redundantly, and phyA is active only in emerging young seedlings (Franklin and Quail, 2010; Nie et al., 2016; Ballaré and Pierik, 2017). Phytochromes are photoconvertible dimers found in a photoequilibrium between the inactive (Pr, absorbs red light) and active forms (Pfr, absorbs far red light), with the Pfr/Pr ratio reflecting the R/FR ratio (Vandenbussche et al., 2005). When activated by red light, Pfr is directed to the nucleus where it interacts with two classes of photomorphogenesis repressors, the complex COP1/SPA (CONSTITUTIVELY PHOTOMORPHOGENIC1/SUPPRESSOR OF phyA) and the PHYTOCHROME INTERACTING FACTORS (PIFs) (Xu et al., 2015). PIFs are transcription factors activated under shade conditions, binding to DNA and inducing the expression of genes related to shade-induced growth (Li et al., 2012). Overall, downstream of photoreceptors, PIFs are the key regulators of gene expression in response to shade, linking light signals to hormones (Yang and Li, 2017).

The expression of the auxin biosynthetic gene *YUCCA,* for example, is known to be regulated by PIF4, PIF5 and PIF7 (Hornitschek et al., 2012; Li et al., 2012). Besides that, PIF4 and PIF5 are known to increase plant sensitivity to auxin (Hersch et al., 2014; Iglesias et al., 2018). Auxin is a growth-promoting hormone required for shade avoidance responses such as stem, hypocotyl and petiole elongation, leaf hyponasty, and reduced leaf blade expansion (Tao et al., 2008; Kozuka et al., 2010; Muller-Moule et al., 2016; Ma and Li, 2019). This is confirmed by the fact that altered auxin biosynthesis, perception, and transport results in impaired SARs (Kozuka et al., 2007; Keuskamp et al., 2010; Li et al., 2012; Ge et al., 2017; Muller-Moulé et al., 2016). Furthermore, gibberellins (GAs) are another group of hormones involved in SARs, mainly by inducing cell elongation, stem and hypocotyl growth and increasing specific leaf area (de Lucas et al., 2008; Feng et al., 2008; Du et al., 2018). The interaction between GAs and PIFs occurs mainly through DELLA proteins, which are negative regulators of GAs responses (Davière and Achard, 2013). DELLA proteins directly bind to PIFs, preventing their interaction with DNA and, consequently, the expression of shade-related genes (de Lucas et al., 2008; Feng et al., 2008; Li et al., 2016). Therefore, under shade conditions, the level of bioactive GAs is increased, which drives to proteasomal degradation of DELLA proteins, releasing PIFs and inducing the transcription of target genes (Gommers et al., 2013; Colebrook et al., 2014; Li et al., 2016). This is confirmed by *gai* mutants containing a stable DELLA protein, which presented reduced SARs (Djakovic-Petrovic et al., 2007). Accordingly, studies have shown that GAs modulate shade-induced responses downstream of auxin pathways, confirming the role of DELLA proteins in SARs (Jiang et al., 2020). Besides that, the use of auxin and gibberellin inhibitors prevented shade-induced growth, confirming that these hormones are pivotal for SARs (Bawa et al., 2020; Jiang et al., 2020).

Considering that shade increases biomass allocation to shoots through auxin and GAs, the biomass allocated to root growth is reduced, resulting in lower root-to-shoot ratio (van Gelderen et al., 2018). Although these changes in biomass allocation ensure plant survival, they can reduce crop production of plants such as radish, in which the root is the commercial organ (Ballaré and Pierik, 2017; Yang and Li, 2017). In root vegetables, the application of the GA biosynthesis inhibitor, paclobutrazol (PAC), reduces shoot growth but increases storage root growth, while GA_3 treatment induces shoot growth, inhibiting root growth (Tekalign and Hammes, 2004; Njiti et al., 2013; Wang et al., 2015). In this context, it is important to understand the effects of GAs levels in radish growth under limiting conditions such as high-density of neighbor plants and low irradiance. Thus, it was hypothesized here that PAC can prevent SARs in radish plants, allowing increases in biomass allocation to roots (Fig. 1).

Nitrogen (N) is a limiting resource and, thus, plants developed strategies to overcome the soil heterogeneity by modulating their root morphology and physiology (Dong et al., 2018). N is mostly acquired by plants as the combination of nitrate (NO_3^-) and ammonium (NH_4^+), but N can also be assimilated as urea, amino acids and peptides (Tegeder and Rentsch, 2010; Kiba and Krapp, 2016). Studies have shown that primary and lateral root growth are affected by the source and concentration of nitrogen. N depletion impairs primary root (PR) and lateral root (LR) growth, whereas mild N limitation induces PR and LR elongation (Gruber et al., 2013; Giehl et al., 2014; Araya et al., 2015). Besides that, high NO_3^- inhibits PR elongation and stimulates LR elongation and high NH_4^+, in turn, results in shorter but highly branched lateral roots (Walch-Liu and Forde, 2008; Lima et al., 2010; Vidal et al., 2013; Forde, 2014; Giehl et al., 2014; Meier et al., 2020).

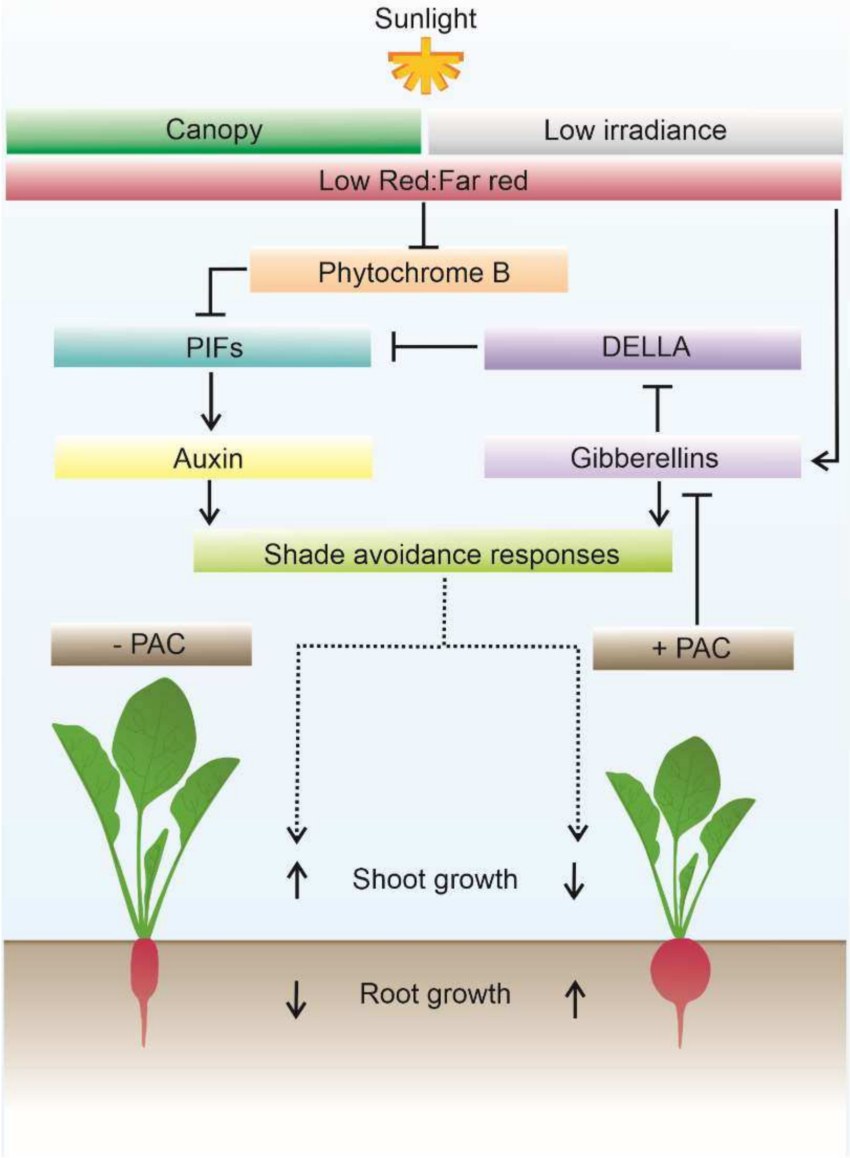

Figure 1. Simplified overview of canopy shade (under high-density of neighbor plants) and low irradiance effects, and their interaction with gibberellin and auxin, in biomass allocation of radish plants. Arrows indicate positive regulation, blunt arrows indicate negative regulation and dotted lines indicate the probable effect of PAC treatment in shade avoidance responses. Briefly, shade reduces R:FR ratio, which inactivates phyB and results in accumulation of PIFs. Besides that, low R:FR induces GAs biosynthesis, that triggers proteasomal degradation of DELLA proteins, releasing PIFs and further increasing PIFs abundance. In turn, PIFs induce the transcription of auxin-related genes which, together with GAs, trigger shade avoidance responses that culminate in increased biomass allocated to shoots instead of roots. It was hypothesized here that PAC treatment could prevent shade-induced growth in radish plants by reducing GAs levels and, consequently, preventing PIFs from accumulate and induce transcription of target genes. Thus, PAC could reduce shoot growth and increase root growth under shade conditions.

In addition to N availability, auxin is known to regulate every aspect of root growth and development (Krouk et al., 2010; Olatunji et al., 2017). Growth of primary and lateral roots is determined by local auxin maxima and minima generated over tissues by polar and lateral auxin transport (Adamowski and Friml, 2015). Local auxin levels are modulated by three main families of auxin transporters: PIN (PIN-FORMED), AUX1/LAX (AUXIN1/LIKE AUXIN1), and ABCB (ATP Binding Cassette subfamily B) transporters (Yang and Murphy, 2009; Geisler et al., 2017; Swarup and Bhosale, 2019). Among the *Arabidopsis* ABCB transporters, the ABCB1/ABCB19, ABCB4/ABCB21 and ABCB6/ABCB20 isoforms, are known to regulate rootward and shootward auxin transport, also preventing apoplastic auxin reflux in roots (Noh et al., 2001; Blakeslee et al., 2007; Kamimoto et al., 2012; Geisler et al., 2017; Zhang et al., 2018; Jenness et al., 2019). The importance of ABCB transporters for root growth is confirmed by studies showing impaired root growth in mutants defectives in *abcb19, abcb21*, *abcb6* and *abcb20* (Wu et al., 2007; Zhang et al., 2018; Jenness et al., 2019). Although the role for nitrogen and auxin during root growth and development have been extensively studied, the interaction between them remains unclear. Considering the importance of root system architecture to provide mechanic support and water and nutrient acquisition, the relation between auxin transport through ABCBs and nitrogen sources and availability will be further discussed here in Chapter 3.

To better understand the role of gibberellins and auxin during plant growth in response to high-density of neighbor plants, shade and nitrogen availability, this thesis was organized into three independent chapters. The effect of altered levels of GAs in radish growth was analyzed under high-density of neighbor plants in Chapter 1 and under different irradiance levels in Chapter 2. In Chapter 3, the relation between auxin levels (mediated by ABCB transporters) and nitrogen availability was evaluated in root growth of *Arabidopsis thaliana*.

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CHAPTER 1

Reduced gibberellins levels affect differentially the growth of shoots and roots of *Raphanus sativus* L. but not their response to high-density of neighbor plants

Abstract

Plant growth is regulated by environmental factors and their relation to internal signals such as plant hormones. Gibberellins (GAs) are known to regulate shoot growth in response to shade, however, their effect on roots remains unclear, especially in root vegetables like radish. Thus, understanding the radish growth in response to GAs levels and light conditions is an important way to increase productivity. In this sense, radish growth was evaluated in response to treatments with gibberellic acid (GA_3) and/or paclobutrazol (PAC), a gibberellin biosynthesis inhibitor. These treatments were also evaluated in plants growing under shade caused by neighbor plants. In general, PAC reduced radish growth in a dose-dependent manner, and, although PAC affected growth of shoots and roots, the reduction was more drastic in shoots. Radish plants exhibited higher specific leaf area under high-density (HD) of neighbors, being that HD seem to induce leaf expansion through GA-independent pathways. Taken together, these results indicate that PAC treatment did not prevent shade avoidance responses or promote storage root growth of radish plants in the conditions investigated here. These results provide new insights about the manipulation of GA levels and the effects of shading on the biomass allocation and growth of storage roots in radish.

Keywords: Biomass allocation · Paclobutrazol · Plant density · Radish · Shade avoidance responses · Root growth

1 Introduction

Plant growth and development are related to cell division, expansion and differentiation, that are affected by environmental factors and endogenous signals like plant hormones (Gonzalez et al., 2012; Wang and Ruan, 2013). Gibberellins (GAs) are plant hormones that control cell division and cell expansion and regulate physiological processes such as phenological transition from vegetative to floral stages, seed germination, leaf expansion and stem elongation (Ueguchi-Tanaka et al., 2007; Tenreira et al., 2017). Although the effects of GAs on shoot growth are well known, their effect on roots remains unclear. It is known that GAs are essential for root development (Achard et al., 2009), and studies had shown that changes in GAs availability affect root growth (Tekalign and Hammes, 2005; Wang et al., 2015; Wang et al., 2017). Thus, it is important to understand the relation between GAs and root growth, especially on plants with storage roots such as radish, carrot, turnip and sugar-beet.

Radish (*Raphanus sativus* L.) is a root vegetable cultivated worldwide and considered a functional food (Goyeneche et al., 2015). Two distinct anatomical portions compose its tuberous root: the upper part is originated from hypocotyl and the bottom part is originated from true root tissue (Tsuro et al., 2008). The radial growth of roots is a result of the cambial activity that originates secondary xylem and phloem and the outer layer, in its turn, is a secondary cortex derived from the pericycle (Webster et al., 1972). The developmental stages of the tuberous root are markedly distinct, occurring in a short life cycle. Overall, the storage root development occurs in three steps after germination: pre-cortex splitting stage, cortex splitting stage, and cortex expansion. These steps occur approximately at 10, 22, and 30 days after sowing (DAS), respectively. During the pre-cortex splitting stage (about 10 DAS) occurs mainly the root axial elongation. Starting from 10 DAS the plant initiates the cortex splitting, which characterizes the radial growth. Then, the cortex cells stop the division and start the expansion (Mitsui et al., 2015; Yu et al., 2016). These characteristics make radish plant a good model for the study of formation and development of sink organs and their relation with GAs levels and environment conditions.

Endogenous levels of GAs have been manipulated since the so-called “green revolution”, in which the gene manipulation led to great increases in rice and wheat production (Hedden and Sponsel, 2015). However, GAs levels in plants can also be changed by treatment with gibberellic acid (GA_3) and paclobutrazol (PAC). PAC is a GAs biosynthesis inhibitor, which acts on the enzyme ent-kaurene oxidase that catalyzes the ent-kaurene conversion to ent-kaurenoic acid, reducing the endogenous levels of GAs (Hedden and Thomas, 2012). Changes in endogenous GAs levels are known for modifying biomass allocation among plant organs, being that high levels of GAs favors biomass allocation to shoots (leaves, petioles and stems), resulting in higher shoot-to-root ratio (Nagel et al., 2001; Ribeiro et al., 2012; Tanimoto, 2012; Sugiura et al., 2016). In root vegetables, inhibition of GAs biosynthesis by PAC reduces shoot growth but increases storage root growth, while GA_3 treatment induces shoot growth, inhibiting root growth (Tekalign and Hammes, 2004; Njiti et al., 2013; Wang et al., 2015). The fact that drastic reductions in photosynthetic area caused by PAC do not reflect in proportional reduction of sink organs is intriguing, and the process is still not understood. However, the modulation of biomass allocation is essential for plants to ensure their survival under natural and agricultural conditions, where plants compete with neighbor plants for resources, such as light and nutrients.

Alterations in light supply are recognized by plants through reductions in both, total irradiance and red:far red ratio (R:FR) (Pierik and de Wit, 2014). Plants perceive these light signals through photoreceptors, such as the phytochromes, cryptochromes and phototropins that trigger shade avoidance responses (SARs) (Casal, 2013; Ballaré and Pierik, 2017). SARs are a set of responses closely related to GAs that allows plants to better intercept light, and includes hypocotyl, stem and petiole elongation, upward leaf movement (hyponasty), increased specific leaf area, inhibition of branching and early flowering (Ballaré and Pierik, 2017; Yang and Li, 2017). In addition, SARs can impair root growth as a consequence of the increased biomass allocation to shoots (van Gelderen et al., 2018b). If, on the one hand, SARs ensure plant survival, on the other hand, they can culminate in production losses in crops like radish (van Gelderen et al., 2018a).

Considering that shade can induce SARs through increases in GAs levels, resulting in reduced root growth (van Gelderen et al., 2018b), and that PAC can alters the biomass allocation in root vegetables, possibly favoring root growth (Wang et al., 2015), it was hypothesized that GAs depletion by PAC could increase root growth of radish plants and, also, mitigate SARs in radish plants growing in high-density conditions, allowing the storage root growth under limiting conditions.

2 Materials and methods

2.1 Plant material and growth conditions

The experiments were conducted in a greenhouse at the Federal University of Viçosa (20º 45 'S, 42º 15' W), in Viçosa, Minas Gerais. The radish seeds (*Raphanus sativus*, Margaret Queen Kobayashi hybrid) were sown in polyethylene pots of 3.5 L of capacity, containing commercial substrate fertilized with NPK (4-14-8) (5 g per pot). Eight days after sowing were maintained two plants per pot (74 plants m^{-2}).

The treatments with paclobutrazol (PAC) were performed by applying 20 mL of deionized water (control) or PAC solutions (5, 25, 50, and 150 μM) above the substrate with a pipette. A single application was performed ten days after sowing (10 DAS). For GA_3 treatment, leaves were sprayed with 50 μM GA_3 containing 0.05% Tween 20 (v/v) every week starting at 13 DAS. The reversion of PAC effect was investigated by applying GA_3 in plants previously treated with PAC. Irrigation was performed daily, maintaining the moisture of the pots near the field capacity.

2.2 *Experimental procedures*

Four experiments were performed to determine the concentration of PAC to be used and its effect on radish growth. Each experiment was composed by four treatments: control (deionized water); gibberellin (GA_3 – 50 μM); paclobutrazol (PAC – 5, 25, 50 or 150 μM); and the combination of PAC and GA_3 (PAC+GA_3). For the concentration tests, at least six plants of each treatment were collected every five days (from 15 to 35 DAS) to determine morphological and biomass parameters of roots and shoots. Next, one experiment was performed with 25 μM PAC to evaluate its effect on radish growth at the harvest point (30 DAS).

To further investigate the relation between GA levels and the radish responses to the surrounding environment, radish plants treated with PAC were evaluated under high density of plants. For this, control (water) and PAC-treated plants (5, 50 and 150 μM) were potted in a "low-density" stand setup (LD stand; 2 plants per pot; 74 plants m^{-2}) or in a "high-density" stand setup (HD stand; 6 plants per pot; 223 plants m^{-2}). At the end of the experiment (30 DAS), morphological parameters and biomass production of shoots and roots were evaluated.

2.3 *Morphological parameters*

For morphological parameters determination, plants were separated in shoots and roots using a scalpel. The diameter, length and volume of roots were determined. For shoots, the total leaf area and number were determined. The root diameter and length were determined with a caliper and the root volume with a graduated cylinder. The total leaf area was measured using a Li-Cor

3100 area meter (LI-COR Model 3100, Lincoln, NE, USA). Then, the shoots and roots were dried at 65 °C until a constant weight to determine their dry weights. The relative growth rate (RGR) was calculated between the 5th and 30th days using the procedure as described by Hunt (1982). The mass fraction for leaf, petiole, and tuber was measured as the mass of leaf, petiole and tuber divided by the plant total mass. The specific leaf area (SLA) was calculated as total leaf area of the radish plant divided by their dry mass.

2.4 *Statistical analysis*

All experiments were conducted in a completely randomized design, with at least six replicates. The experiments were repeated at least twice. For the experiments with 5, 50, and 150 μM of PAC, treatments were contrasted with control by the *t*-Student test ($P \leq 0.05$). The data of density of neighbor plants experiments were subjected to analysis of variance ($P \leq 0.05$) using SPSS 11.0 software (Statistical Software Package, Inc., Chicago, IL, USA), and the mean values were compared through Tukey's test ($P \leq 0.05$).

3 Results

3.1 *Gibberellin level differentially affects shoot and root growth of radish plants*

In order to evaluate the effect of GAs levels in radish growth, three experiments with GA_3 and/or different PAC concentrations (5, 50 and 150 μM) were performed. In these experiments, the growth and morphology of radish plants were analyzed throughout their development. Thereafter, another experiment with an intermediary PAC concentration (25 μM) was performed, in which radish growth was evaluated only at 30 days after sowing (30 DAS). In general, treatments with increasing concentrations of PAC (range 5-150 μM) reduced the growth of radish plants, at least in some of the developmental stages (Figs. 1-5). The root growth was reduced by PAC treatment mainly at late stages of development, as seen for root volume and dry weight at 30 and 35 DAS (Fig. 2P-U). However, the root volume and dry weight at early stages (15 and 20 DAS) was reduced only by 150 μM PAC, being that 5 μM PAC even increased the root growth at 15 and 20 DAS compared to control (Fig. 2P and 3D).

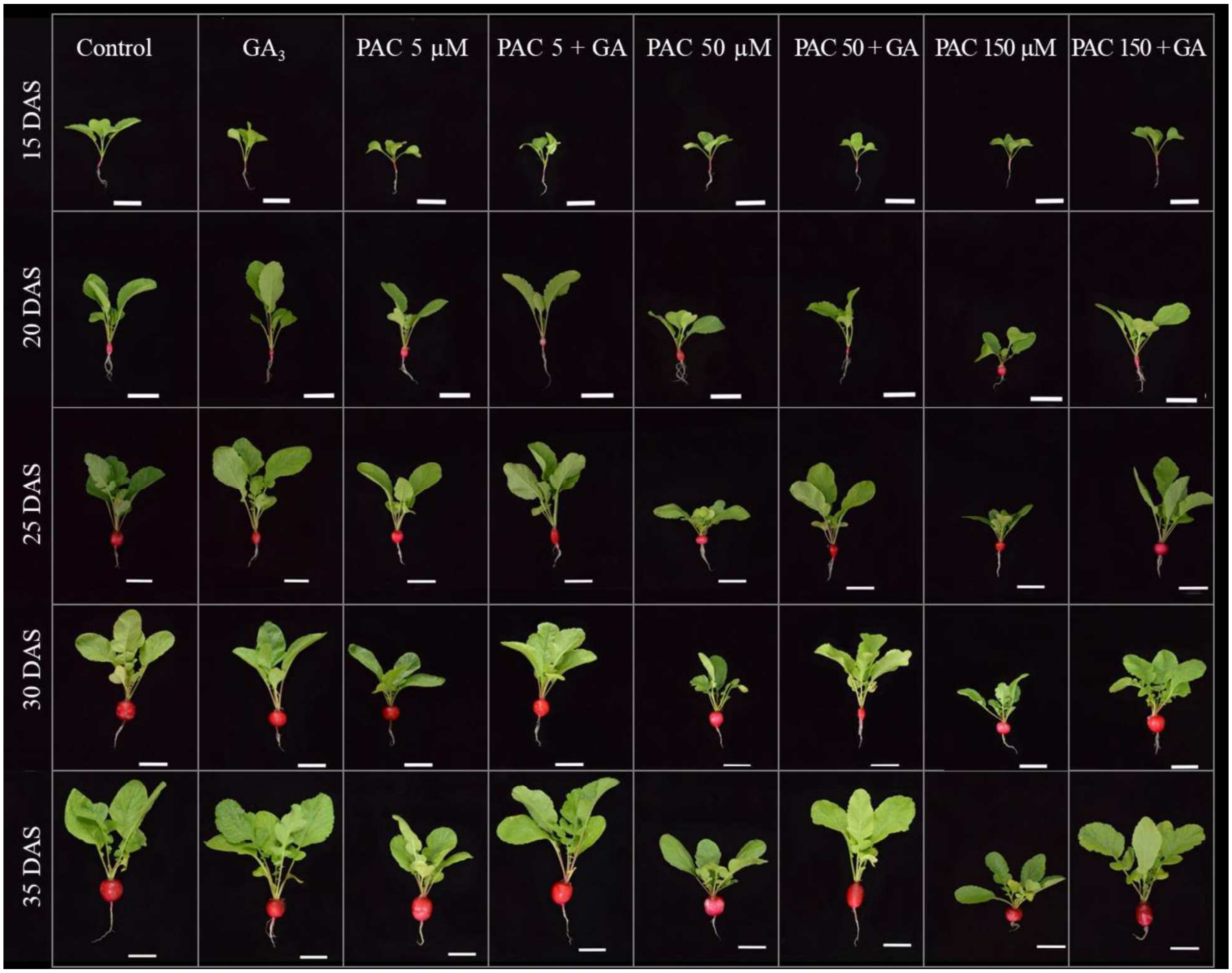

Figure 1. Effects of different PAC concentrations and/or gibberellin (GA$_3$, 50 µM) treatments on radish plants growth. Pictures were obtained from representative individuals at 15, 20, 25, 30 and 35 days after sowing (DAS). White bars at the bottom correspond to 5 cm.

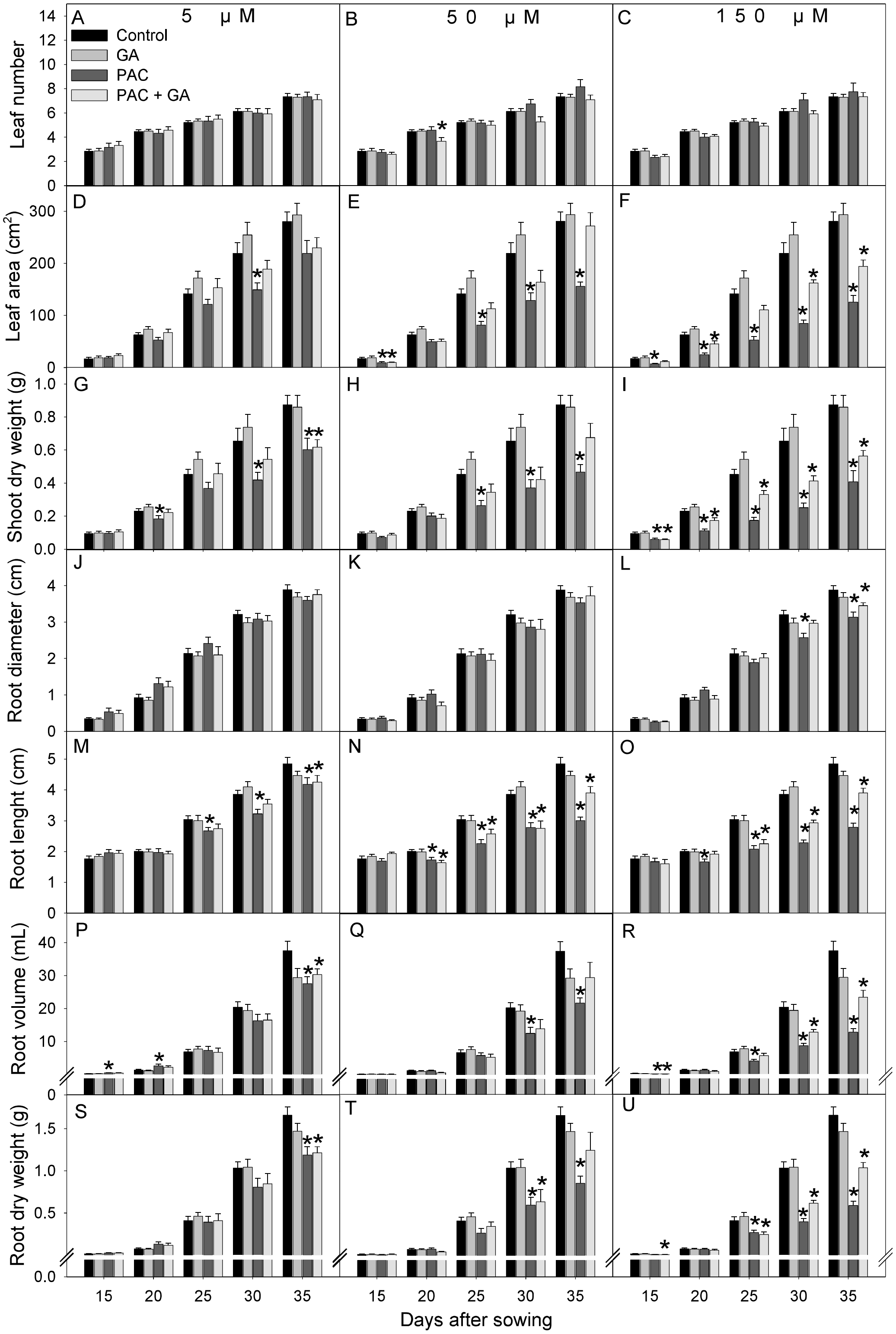

Figure 2. Effects of different PAC concentrations and/or gibberellin (GA_3, 50 µM) in radish plants growth. Bars indicated by asterisks differ statistically from the control (water) at 5% level by the *t* test. Averages were obtained of 6 replicates ± standard error.

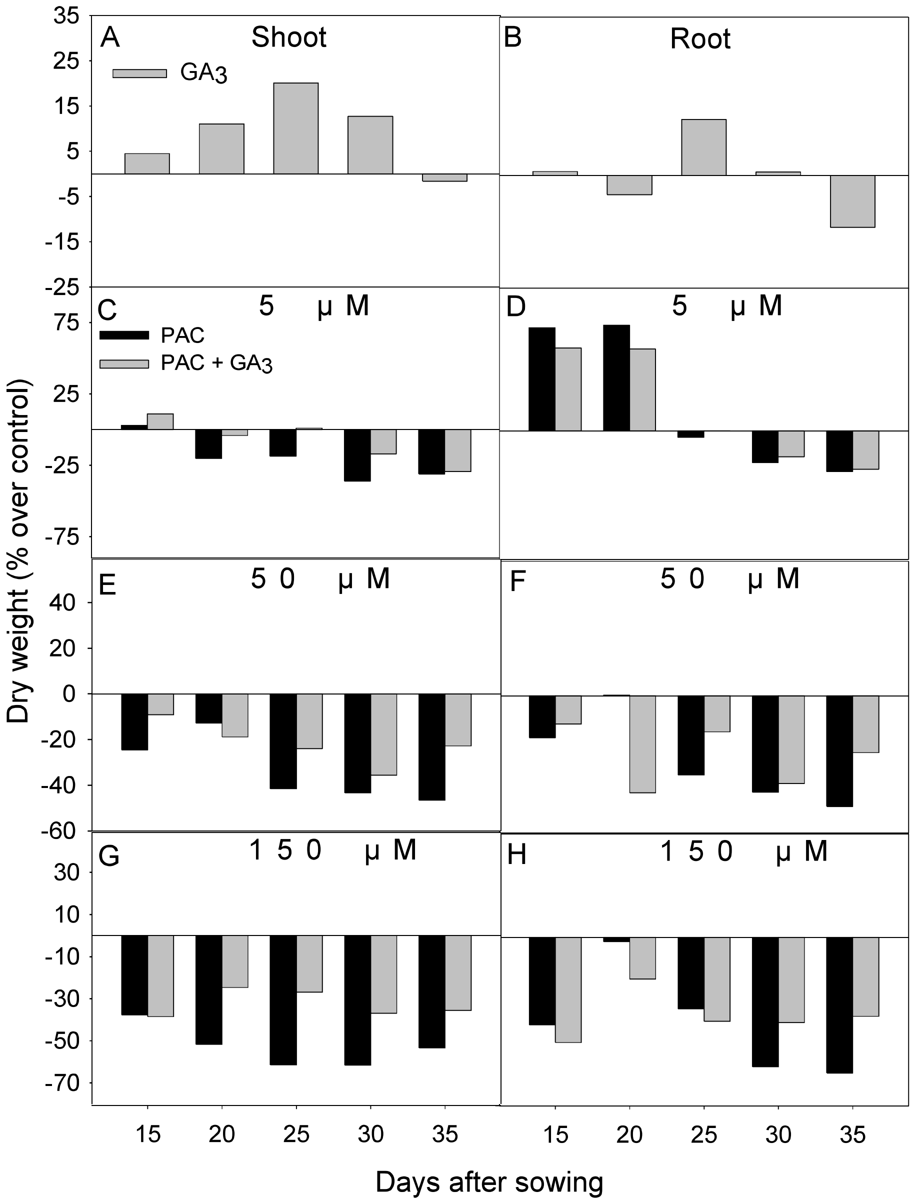

Figure 3. Biomass allocation in radish plants treated with PAC (5, 50 and 150 μM) and/or GA_3 (50 μM) in relation to control plants. Shoot dry weight (A, C, E, F and G); root dry weight (B, D, F and H). The percentages represent differences over the control. Averages were obtained from 5 replicates.

On the other hand, PAC treatment reduced the root length in almost all evaluations, but the root diameter was reduced only by 150 µM PAC at late stages (Fig. 2 J-O). In turn, the shoot DW was reduced with increasing concentration of PAC except with 5 µM (15 and 25 DAS) and 50 µM (15 and 20 DAS) (Fig. 2G-I). Similar results were found for leaf area, which was reduced mainly by 50 and 150 µM PAC, being less affected by 5 µM PAC (Fig. 2 D-F). In general, the leaf number was not affected by PAC treatment through the development (15-35 DAS) in comparison with control (Fig. 2 A-C). Overall, the treatment with GA_3 did not affect the radish development (Fig. 2). Although GA_3 increased the percentage of shoot DW over the control at 15, 20, 25 and 30 DAS (Fig. 3A), there were no statistical differences (Fig. 2 D-I). In addition, GA_3 treatment of plants previously treated with PAC, partially reversed the inhibitory effect (Figs. 2 and 3). It is important to note that PAC treatment resulted in stronger reduction of shoot growth compared to roots, mainly at early stages (Fig. 3).

This biomass allocation pattern resulted in lower shoot-to-root ratio for most PAC concentrations at early stages (15, 20 and 25 DAS) and unaffected or even increased the ratio at 35 DAS (Fig. 4). Furthermore, the specific leaf area (SLA), was reduced by 150 µM PAC, while the other concentrations only affected SLA at 15 and 20 DAS (Fig. 4). The relative growth rate was reduced by 50 and 150 µM PAC compared to control, while GA_3 did not affect RGR. Considering that high PAC concentrations (50 and 150 µM) strongly reduced the growth of radish plants and that 5 µM PAC did not affect some of the growth parameters, an intermediary concentration (25 µM) was used to better evaluate the GA inhibition on radish growth. In general, altered GAs level mainly affected shoot growth (Fig. 5). Decreased GAs level reduced petiole length, but not the petiole dry weight (PDW) compared to control (Fig. 5F and H). In contrast, PDW was increased by GA_3 treatment in comparison with the other treatments, but petiole length did not differ from control (Fig. 5F and H). PAC treatment reduced leaf dry weight (LDW) and leaf area (LA) compared to GA_3-treated plants but not compared to control plants (Fig. 5D, I). On the other hand, root length, root volume and root dry weight (RDW) (Fig. 5B, C and G) were not affected by treatments, while the root diameter was reduced by PAC treatment compared to control (Fig. 5A). The organ mass fractions were not altered by 25 µM PAC compared to control at 30 DAS (Fig. 5K). However, GA_3 treatment resulted in higher biomass allocation to leaves and petioles and, consequently, lower biomass allocated to the tubers. These results also reflected in increased shoot-to-root ratio of plants treated with GA_3 compared to control plants (Fig. 5J).

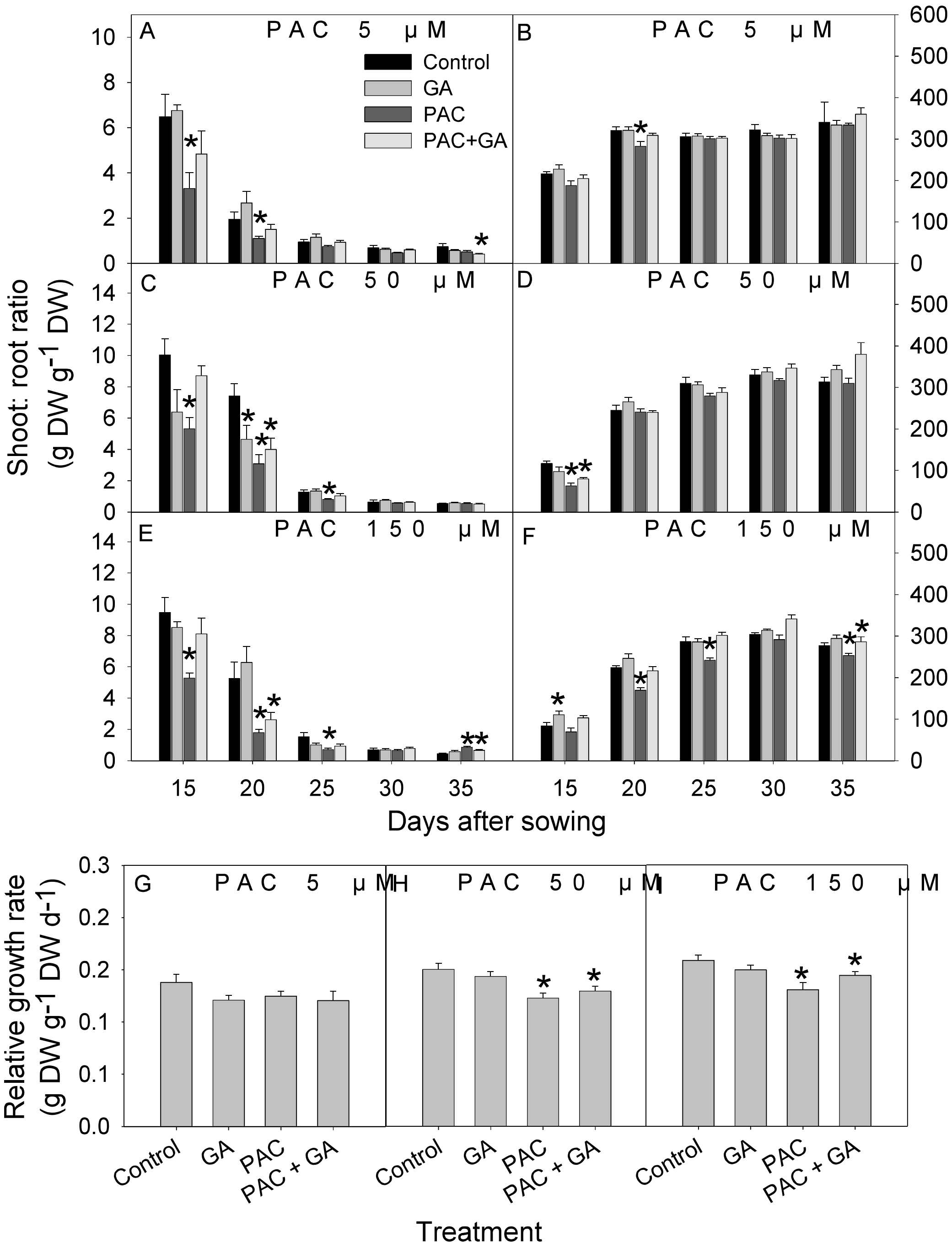

Figure 4. Effects of PAC and/or GA_3 on radish growth parameters. Values represent the average + SE of 6 individual plants. The asterisks indicate statistical differences in relation to control (water) by the *t* test at 5% of probability.

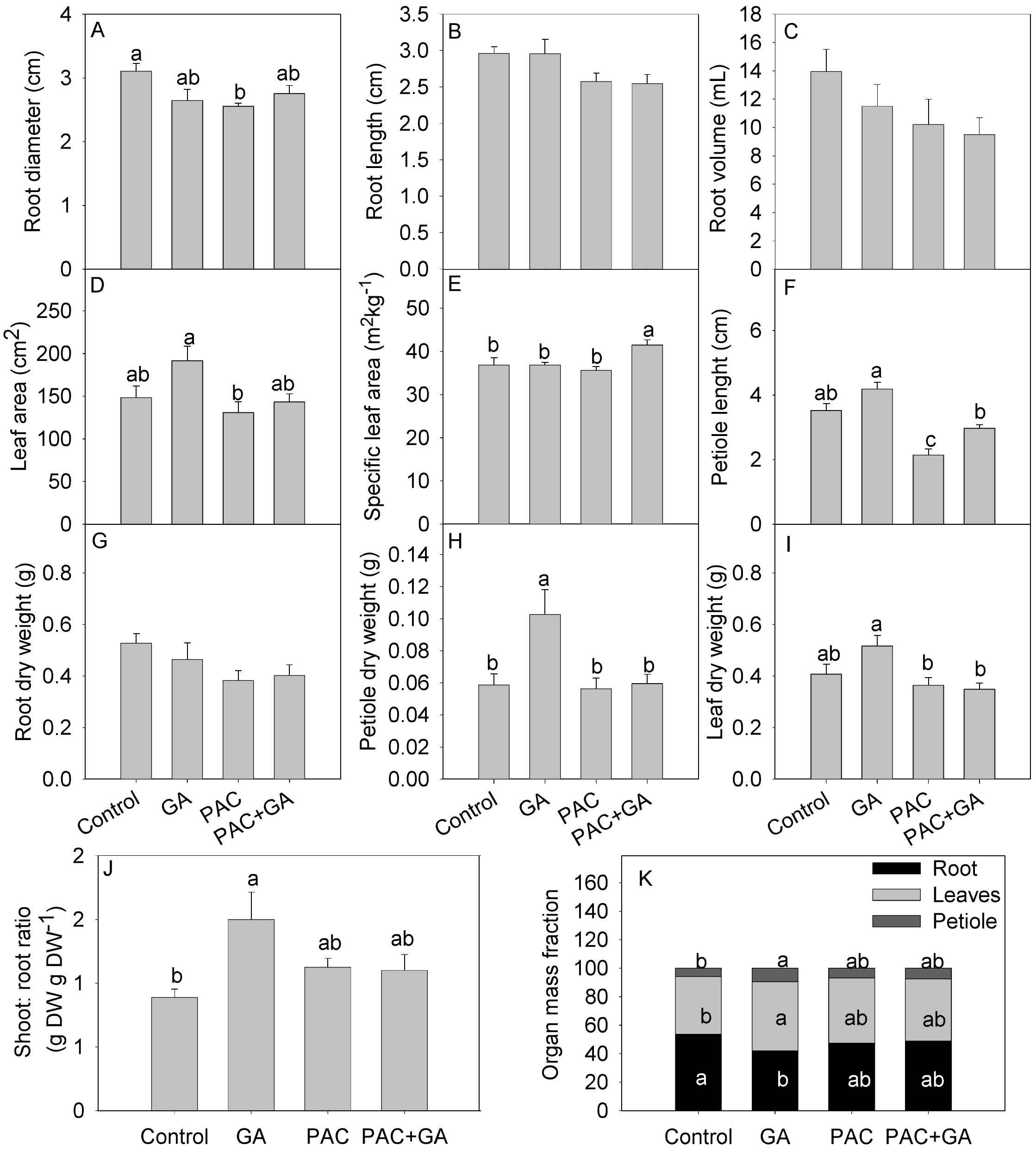

Figure 5. Effect of PAC (25 µM) and/or GA₃ (50 µM) treatments on radish growth parameters and organ mass fractions (A-K). The values were compared by Tukey test at the 5% level. Values are presented as means + SE of 10 individual plants. Different letters indicate significant differences.

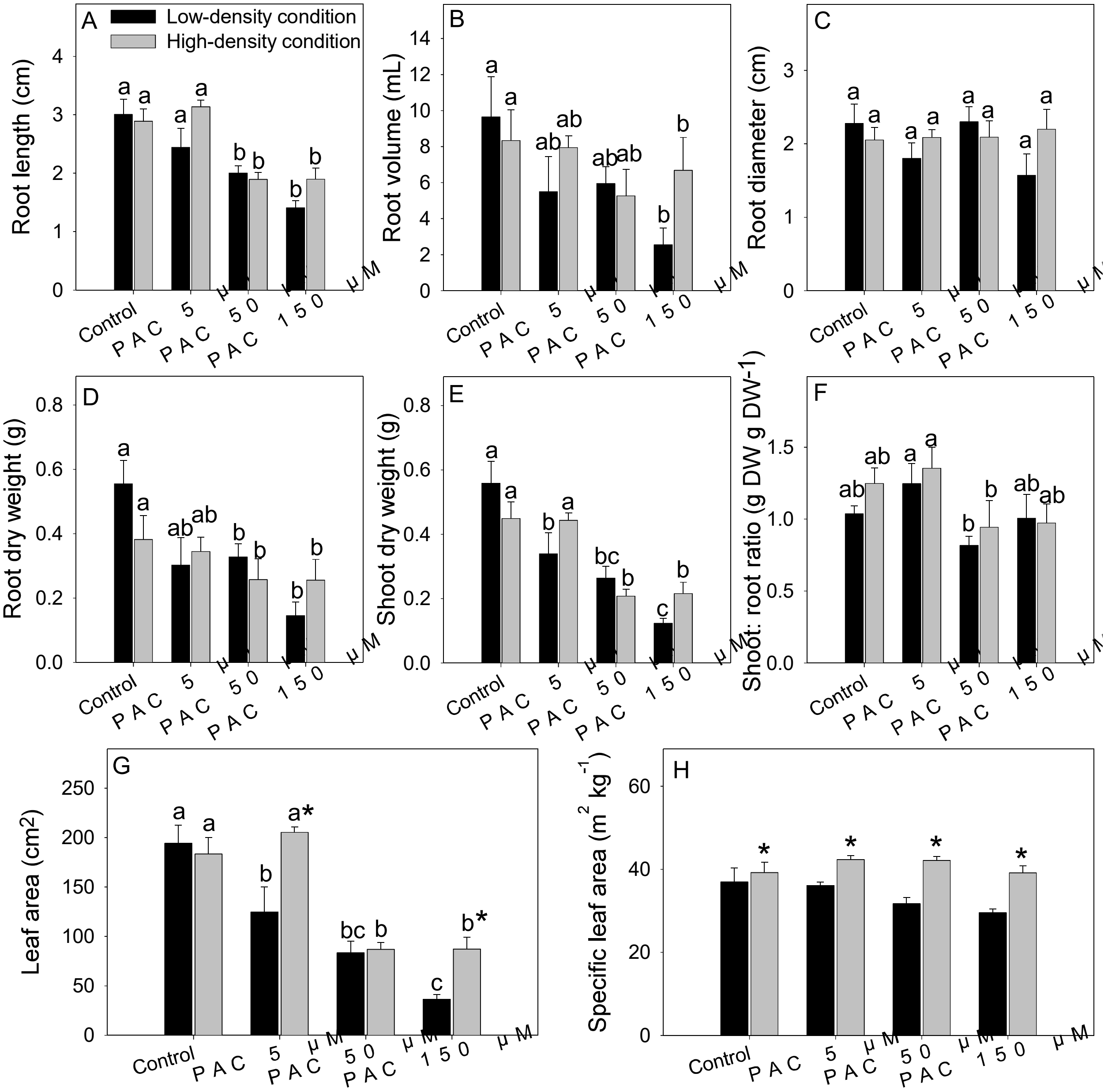

Figure 6. Effects of PAC treatment on the growth of radish plants cultivated at low-density stands (LD; 2 plants per pot) or high-density stands (HD; 6 plants per pot). Different letters indicate statistical differences among treatments and asterisks indicate differences between stand density conditions by the Tukey test at 5 % probability. Values were obtained by the mean + SE of 6 individual plants.

3.2 *Effects of reduced GAs levels on the radish growth in response to high-density of neighbor plants*

There was no interaction between GAs level and high-density of neighbor plants in growth parameters of radish. Shoot growth was affected individually by PAC treatments and plant density, whereas root growth was affected only by PAC (Fig. 6). Root length, root volume and root dry weight were reduced with the increase of PAC concentration (Fig. 6A, B and D). The same was observed for shoot dry weight and leaf area of plants grown in low-density conditions (LD), that were reduced as the PAC concentration increased (Fig. 6E and G). However, when PAC-treated plants (5 and 150 µM) were grown under high-density (HD) of plants they increased their leaf area in relation to those grown at low density (LD) of plants (Fig. 6G). In addition, the shoot DW of PAC-treated plants (5 µM) on HD conditions did not differ from the control plants. The specific leaf area (SLA) was increased under HD conditions independently of PAC treatment (Fig. 6H).

4 Discussion

Gibberellins are plant hormones involved in cell division and cell expansion and its inhibition lead to reductions in plant growth (Tenreira et al., 2017). However, there is evidence that in root vegetables, GAs depletion could promote the growth of storage roots instead of shoots (Balamani and Poovaiah, 1985; Wang et al., 2015). Increased GA_3 levels, in turn, direct the partitioning of assimilates to growth of shoots rather than to roots (Ribeiro et al., 2012; Wang et al., 2015), as shown by the high shoot-to-root ratio observed here (Fig. 5J). It was observed here that GA biosynthesis inhibitor PAC reduced the radish growth, but the growth inhibition seems to be more drastic on shoots than on roots, mainly in the early developmental stages. The shoot growth inhibition of PAC-treated plants was also observed in other root vegetables such as carrot (Wang et al., 2015), sweet potato (Njiti et al., 2013), cassava (Panyapruek et al., 2016) and potato (Tekalign and Hammes, 2004; 2005). However, these studies found increases on tuberous roots growth, which was not observed here. These results may be related to the fact that shoots and roots have differential sensibility to GAs, with roots requiring lower GA levels to trigger biological responses (Tanimoto, 2012). In late developmental stages, the growth inhibition was pronounced in both shoots and roots. These responses are in accordance with studies showing that disturbances on levels or perception of GAs culminate in plant dwarfism or semi-dwarfism (Hedden and Phillips, 2000; Olszewski et al., 2002; Zhu et al., 2016) as shown for the “green revolution wheat” (Peng et al., 1999). Moreover, the semi-dwarfism increases the harvest index by directing the partitioning of assimilates into reproductive organs rather than into the stem or leaves (Hedden, 2003). PAC treatment reduces plant growth by uncoupling growth from carbon availability, and by increasing stability of DELLA proteins, which are repressors of GAs responses (Hedden and Thomas, 2012; Ribeiro et al., 2012; Blanco-Touriñán et al., 2020). Considering that DELLA proteins modulate the activity of more than 300 transcription factors and transcriptional regulators, they are main regulators of plant growth and responses to environmental conditions (Van de Velde et al., 2017; Blanco-Touriñán et al., 2020; Lantzouni et al., 2020).

Another aspect of GA biosynthesis inhibition induced by PAC is the apparent change in the root shape, resulting in slightly shorter roots with larger or equal diameters (Fig. 2 J-O), although that was not observed in roots of plants treated with 25 μM PAC (Fig. 5). This could be related to the cortical microtubule arrangement, considering that GAs stimulate the axial cell expansion, which is driven by transversal microtubule orientation. Thus, the GAs depletion can result in random microtubule orientation, allowing pronounced radial growth (Locascio et al., 2013), which was also observed in potato tubers that were unable to grow radially when treated with GA_3 due to microtubule orientation (Vreugdenhil and Sergeeva, 1999). Besides that, the root growth seems to be primarily influenced in the early developmental stages, when GAs depletion could influence the hypocotyl axial growth. However, after its onset, the root growth rate remains constant, unaffecting the root diameter (Craker et al., 1983). It could be a result of changes in the switch point from the primary (pre-cortex splitting stage and cortex-splitting stage – until 25 DAS) to the secondary root growth (cortex expanding stage – starting on 25 DAS).

Under high-density of neighbor plants (HD), the light is filtrated through the neighbor canopy, reducing the red:far red ratio (low R:FR) (Gommers et al., 2013). Under low R:FR, the phytochrome B is converted to its inactive form, which causes the accumulation of phytochrome-interacting factors (PIFs). PIFs are transcription factors that modulate the transcription of genes related to shade-induced responses such as elongation of hypocotyl, stems and petioles, responses also called shade avoidance responses (SARs) (Gommers et al., 2013; Gruntman et al., 2017). GAs are known to be involved in SARs, mainly by inducing cell elongation, stem and hypocotyl growth and increasing specific leaf area (de Lucas et al., 2008; Feng et al., 2008; Du et al., 2018). GAs trigger SARs by modulating the stability of DELLA proteins, which directly interact with PIFs, controlling gene expression (de Lucas et al., 2008; Li et al., 2016). In the conditions tested here, HD of plants only affected leaf area and SLA, not affecting other root and shoot growth parameters (Fig. 6). Besides that, PAC treatment inhibited growth of radish plants in a dose-dependent manner. Although increased SLA is not considered a SAR, it is a phenotypic trait often observed in shaded plants (Evans and Poorter, 2001; Ballaré and Pierik, 2017). Considering that the increase in SLA occurred independently of GAs, it suggests that the increased SLA could be triggered by other signaling pathways such as auxin-, ethylene- and brassinosteroids-related (Djakovic-Petrovic et al., 2007; Hornitschek et al., 2012; Yang and Li, 2017). This is in accordance with Pierik et al. (2009), who showed that SARs mainly depend on auxin activity, being independent of GAs. Consistent with our results, sugar beet plants grown under simulated canopy shade also presented high SLA (Artru et al., 2018). Leaf area, in turn, was affected by both, PAC and density of plants, being that the reduction in LA caused by PAC was reversed when these plants were grown under HD of plants (Fig. 6G). It is known that shade stress increases endogenous levels and the perception of GAs and auxins (Martínezgarcía et al., 2010; Bou-Torrent et al., 2014; Ma and Li, 2019). In this context, the reversion of PAC-reduced LA observed under HD of plants, could be a result of increased auxin levels, suggesting that HD of plants could induce leaf expansion through GA-independent pathways. It is known that SARs can impair root growth because of sugar availability and biomass allocated preferentially to the shoot (van Gelderen et al., 2018b). Although there is evidence that plant density can reduce the storage root growth of radish (El-Desuki et al., 2005), it was not observed here, where root growth was only affected by PAC treatment.

5 Conclusions

Overall, the inhibition of GAs by PAC reduced shoot and root growth of radish plants, but the inhibition was stronger in shoots. High-density of plants only affected leaf area and specific leaf area, being that, HD seem to induce leaf expansion through GA-independent pathways. These results indicate that PAC treatment inhibit radish growth and does not prevent shade avoidance responses or promote storage root growth of radish plants in the conditions investigated here. This study brings new perspectives about the manipulation of GA levels and the effects of shading on the biomass allocation and growth of storage roots in radish, which can be used to optimize large-scale cultivation of this important vegetable crop.

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CHAPTER 2

Gibberellin-regulated biomass allocation in *Raphanus sativus* L. plants is dependent on irradiance levels

Abstract

Gibberellin has been proposed to increase leaf elongation in radish (*Raphanus sativus* L.) plants, which is associated with decreased tuber growth. Since light intensity can control growth through interaction with gibberellin, investigation of the effect of gibberellin levels on the growth of radish plants would be a step forward towards unraveling factors that underlie biomass accumulation and allocation in response to irradiance levels. Here, it is reported that the gibberellin biosynthesis inhibitor paclobutrazol (PAC) decreased petiole elongation, but not lamina growth of radish plants grown under full sunlight. However, the perception of shade by the plant drives shoot elongation, while in plants treated with PAC the petiole and leaf lamina fail to elongate. Plants treated with PAC allocated more biomass to their tubers and less to shoot compared to control under shade. Moreover, PAC decreased the abundance of transcripts encoding cell wall expansion proteins in leaf lamina and petiole of plants grown under shade, which was positively correlated with sugar consumption by the tuber, thereby increasing the mass fraction and concentrations of minerals for tuber. Thus, allocation of biomass during the growth of radish plants and nutritional quality of tubers depend on the interaction between gibberellin levels and light intensity.

Keywords: Biomass allocation · Carbon metabolism · Cell expansion · Paclobutrazol · Petiole elongation

1 Introduction

Biomass allocation within the plant is regulated by multiple environmental factors such as temperature, carbon dioxide levels, light intensity, availability of water and mineral nutrient (Poorter et al., 2012; Sugiura et al., 2017). In this context, irradiance levels have a strong impact on accumulation and biomass allocation patterns, although the scale and details of the response may vary among species (Poorter et al., 2019). Interspecies comparison have found that shoot weight increased more than root weight when plants were grown under low irradiance conditions (Poorter et al., 2012). The changes in biomass allocation are also known to be strongly influenced by plant hormones (Dias et al., 2010; Sugiura et al., 2016). Among the plant hormones, gibberellin usually increases shoot-to-root ratio (Ribeiro et al., 2012a; Voorend et al., 2016). Additionally, the gibberellin biosynthesis inhibitor paclobutrazol (PAC) decreases shoot growth, but increases root growth of tomato and carrot plants (Nagel et al., 2001; Wang et al., 2015). In contrast, PAC reduces rosette growth in tobacco, but does not affect the root growth (Falcioni et al., 2018a). These results show that although low gibberellin levels inhibits shoot growth, the effects of gibberellin on root growth are hitherto unclear. Since light specifically alters the expression of genes encoding proteins involved in gibberellin biosynthesis, catabolism and signaling (Arana et al., 2011; Weller et al., 2009), it is possible that the ability of gibberellin to regulate biomass allocation in plants is dependent on the irradiance levels.

Growth analyses of radish (*Raphanus sativus*) plants indicate that biomass fraction increases in leaves under low irradiance, combined with a large leaf area per unit leaf biomass (Marcelis et al., 1997). The mechanisms involved in cell-wall modification are important to control acclimation growth response of the plants to different irradiance levels (Falcioni et al., 2018b). Light induces changes in the expression of *EXPANSIN* (*EXP*) and *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/ENDOHYDROLASES* (*XTH*) genes, which control cell wall loosening and thus allow cell expansion (Cosgrove, 2016). Loss-of-function knockout mutants for the *XTH* genes show reduced petiole elongation in response to shade (Sasidharan et al., 2010). On the other hand, the expression of *EXP* genes is upregulated in old leaves of plants under shade compared to plants grown under full sunlight (Wu et al., 2017). Moreover, gibberellin and auxin are implicated in the regulation of *EXP* and *XTH* genes in a dose-dependent manner (Majda and Robert, 2018; Voegele et al., 2011). A decrease in gibberellin levels by the application of PAC results in inhibited leaf growth, which is associated with decreased expression of *EXP* and *XTH* genes (Ribeiro et al., 2012b). Shade-induced auxin biosynthesis mediates petiole elongation by activating *XTH* (de Wit et al., 2015). Taken together, these studies suggest that the light environment is effective in modulating leaf structure by activating hormones and cell-wall extensibility in a spatio-temporal manner. In radish plants, although low irradiance triggers the elongation of leaves, it reduces growth of the tuberous roots, resulting in reduced crop yield (Craker et al., 1983; Hole and Dearman, 1993). Thus, the interaction between hormones and their regulation of cell-wall remodeling is likely an important aspect of the growth response of radish plants to irradiance levels.

Light influences the levels of compounds associated with primary cell metabolism, which is directly related to the photosynthetic performance (Annunziata et al., 2018). It has been described that radish plants readjust partitioning of biomass to maintain a functional equilibrium between shoot and root growth in response to different irradiance levels (Zha and Liu, 2018). These results suggest that radish plants show sufficient plasticity to acclimate to different irradiance levels. In this context, growth and production of tuberous root of radish plants could be optimized by modulating light intensity. However, little is known about how radish plants cope with variation in irradiance levels to improve their patterns of biomass accumulation and allocation. The increase in leaf elongation of radish plants that occurs in response to a reduced irradiance is associated with an arrest of root growth and with higher gibberellin levels (Craker et al., 1983; Jabir et al., 2017). Thus, it was hypothesized that low gibberellin levels could inhibit petiole and lamina growth by altering the expression of genes encoding for XTH and EXP and, thus, maintain investment of tuber biomass in response to low light intensity.

2 Materials and methods

2.1 *Plant material and growth conditions*

Seeds of radish (*Raphanus sativus* L.) cv. Margaret Queen Kobayashi were germinated on commercial substrate (Tropstrato HT©) in plastic pots with 3.5 L capacity and the plants were grown in a greenhouse in Viçosa (20º45'S; 42º51'S; 650 m in altitude), Minas Gerais, Brazil, between March 2017 and October 2019. The air temperature in the greenhouse was 26/21 °C day/night cycle with 60/70% relative humidity. Five days after sowing, radish plants growing singly in pots were watered with 20 mL of deionized (control) water or PAC solution (25 µM). For gibberellin treatment, plants were foliar sprayed with 50 µM GA_3 containing 0.05 % (v/v) Tween 20 every week. After treatments, plants were exposed to two different light regimes in greenhouse, each with a 13/11 h day/night photoperiod, as follows: (I) "Sun" treatment. Plants were exposed to sunlight, with the midday irradiance of ~900 µmol m^{-2} s^{-1}. (II) "Shade" treatment. Plants were maintained on separate bench covered with neutral density black nylon nettings, with the midday irradiance of ~400 μmol m^{-2} s^{-1}. Irradiance was measured at intervals of 20 min using LI-190SA quantum sensor (LI-COR, Lincoln, NE, USA) coupled to a data logger (LI-1400, LI-COR, Lincoln, NE, USA). The light quality was measured at dawn, midday and dusk using LI-1800 spectroradiometer (LI-COR, Lincoln, NE, USA) under sunlight and shade conditions. The positions of the pots were rotated twice a week to minimize any variation within each light environment. Radish plants were harvested at the age of 30 days.

2.2 *Plant morphology determinations*

The shoot and tuber of 10 radish plants per treatment were separated using a scalpel and total leaf area was determined by using a Li-Cor 3100 area meter (LI-COR, Lincoln, NE, USA). In addition, petiole-lamina junction was separated using a scalpel and leaf lamina was immediately scanned with an area meter to obtain their surface area. Measurements of petiole length were performed using a Vernier caliper. Afterwards, tuber, petiole and leaf lamina of each radish plant were oven-dried at 65 °C until constant mass to determine their dry masses. The specific leaf area (SLA) was calculated as total leaf area of the radish plant divided by their dry mass.

2.3 *Metabolite measurements*

To quantify metabolites, the radish plants were harvested at the end of the photoperiod washed with deionized water and paper-dried. Shoot of radish plants were immediately separated from tuber using a scalpel, frozen in liquid nitrogen and stored at –80 °C until analysis. The analysis of starch, sucrose, hexoses, total amino acids and total protein was performed using 100 mg of freeze-dried samples of shoot and tuber as described by Cross et al. (2006).

2.4 *Real-time quantitative PCR (RT-qPCR)*

Total RNA was isolated from petiole and leaf lamina of radish plants using an RNeasy Plant Mini Kit (Qiagen) and cDNA was synthesized from 2 μg total RNAs using SuperScript TM III reverse transcriptase (Invitrogen), following the manufacturer's protocol. Quantitative real-time reactions were performed with Power SYBR Green PCR Master Mix using gene-specific primers

(Table S1) and the radish *ACTIN* gene as an internal reference for normalization (Wang et al., 2013; Xu et al., 2012). Candidate genes were selected from the literature that have been shown to regulate cell expansion, auxin biosynthesis and gibberellin metabolism in radish plants (Yu et al., 2016; Xie et al., 2018).

2.5 *Mineral analysis*

Mineral elements were determined in whole tubers from the plants at the age of 30 days. Five tubers from each sample were dried at 65 °C until a constant weight reduced to powder using a mill (CIENLAB CE-430) and then digested with HNO_3:$HClO_4$ (10:1, v/v). Concentrations of P, K, Ca, Mg, S, Cu, B, Fe, Mn, Zn and Mo were analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES, Perkin-Elmer, Shelton, CT) as described by Wheal et al. (2011). Concentrations of N in tuber of radish plants were determined by the Dumas combustion method (Jung et al., 2003).

2.6 *Statistical analysis*

The statistical design was completely randomized distribution. Five independent samples of five rosettes or five tubers per sample were collected per treatment for metabolite measurements. For real-time qPCR analysis, three independent samples containing leaf lamina or petiole of five rosettes per samples were assayed. The data were subjected to analysis of variance ($P \leq 0.05$) using the SPSS (Statistical Package for the Social Sciences) 11.0 version, and then mean values were compared through Tukey test at the 5% level of significance. The differences between sun plants and shade plants were analyzed by the Student's *t*-test at $P \leq 0.05$.

3 Results

3.1 *Gibberellin level alters growth differentially in low- and high-light conditions*

To characterize the functional link between gibberellin and growth under two contrasting irradiance levels, radish plants were treated with GA_3 and/or PAC and grown in two irradiance levels, sun and shade, under average daily light integral (DLI) of 29 mol m^{-2} d^{-1} and 15 mol m^{-2} d^{-1}, respectively (Fig. 1a and Fig. S1a). The neutral shade cloth did not alter the ratio of red:far red (R:FR) light compared to direct sunlight (Fig. S1b). In this context, plants treated with PAC and GA_3+PAC under sun conditions had a similar shoot:tuber ratio to the control (Fig. 1b). The increase in shoot:tuber ratio in GA_3-treated plants under full sunlight was due to a stimulation of shoot growth and an inhibition of tuber growth. Under shade conditions, the shoot:tuber ratio was decreased by 47% in plants treated with PAC compared with control, showing that the tuber dry weight increased more than shoot dry weight in PAC treated plants (Fig. 1b).

When plants grown in sun and shade conditions are compared, the shoot:tuber ratio was higher in untreated control and GA_3+PAC treatment and lower in PAC-treated plants. Interestingly, shading increased petiole length and lamina area in untreated control and in plants treated with GA_3 and GA_3+PAC but not in plants treated with PAC alone, when compared with plants in the sun conditions (Fig. 1c, d). Although no statistical differences were found in the lamina area of plants treated with GA_3 and/or PAC compared with control within the high irradiance conditions (Fig. 1d), the petiole length was shorter in plants treated with PAC than in the other treatments (Fig. 1c). In our experimental setup, total SLA was not affected by GA_3 and/or PAC treatment under sun conditions (Fig. 1e). On the other hand, shading increased SLA in untreated control and plants treated with GA_3 and GA_3+PAC compared with plants in the sun conditions. In contrast, shading did not lead to significant changes in SLA of radish plants treated with PAC compared with PAC-treated plants grown under full sunlight (Fig. 1e). No differences were found for A, g_s, ETR and R_d (Fig. S2a-d).

3.2 *Transcriptional responses to growth irradiance are gibberellin dependent*

To investigate how gibberellin and irradiance levels affect leaf growth in radish plants, the expression of genes involved in cell expansion was analyzed, auxin biosynthesis and gibberellin signaling in petiole and leaf lamina of plants treated with GA_3 and/or PAC under contrasting levels of irradiance. In general, shading led to increase in the expression of genes associated with cell wall expansion and auxin biosynthesis in both petiole and leaf lamina of the untreated control and plants treated with GA_3 and GA_3+PAC, but not in plants treated with PAC compared with plants in the sun conditions (Fig. 2a-l).

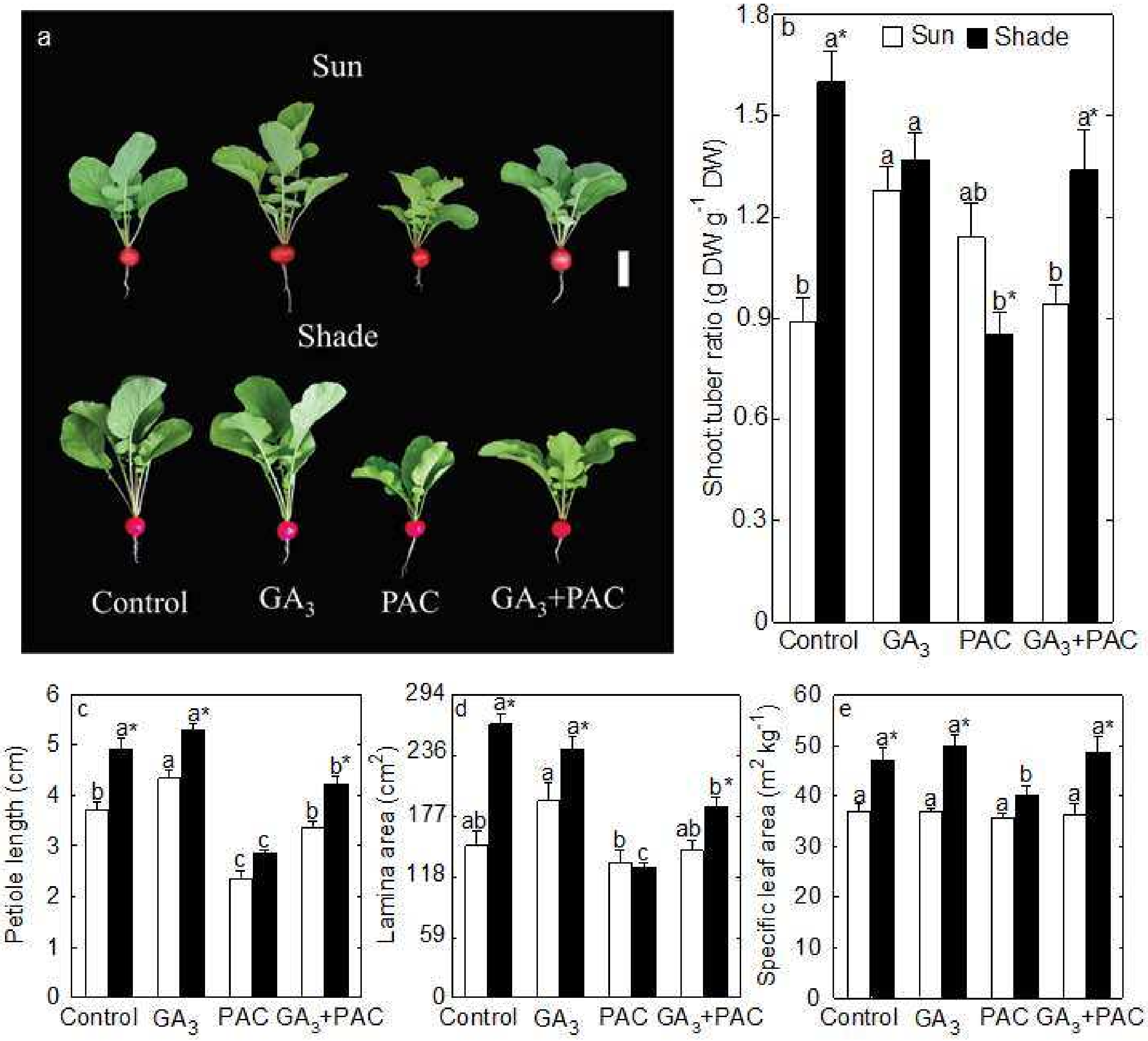

Figure 1. Phenotypic changes of radish plants caused by treatment with GA_3 and/or PAC grown in either sun or shade conditions. (a) Photography of representative radish plants at the age of 30 days. The scale bar represents 5 cm. (b) Shoot:tuber ratio. (c) Petiole length. (d) Lamina area. (e) Specific leaf area. Bars labeled with the different letters indicate differences between treatments within the same irradiance condition by Tukey's test ($P \leq 0.05$). Asterisks indicate values determined by the Student's *t*-test to be significantly different in shade plants with respect to sun plants ($P \leq 0.05$). Data are mean + standard error of three independent experiments.

Next to that, genes related to cell expansion (i.e. *EXPA1*, *EXPB1*, *XTH15* and *XTH24*) were induced by GA_3 in petiole under full sunlight, but repressed by PAC treatment compared with control (Fig. 3a, b, e, f). On the other hand, there was no significant change in the expression of *EXPA1*, *EXPB1*, *XTH15* and *XTH24* genes in leaf lamina of radish plants treated with GA_3 and/or PAC compared with control under sun conditions (Fig. 3c, d, g, h). Moreover, the expression of *YUCCA1* (*INDOLE-3-PYRUVATE MONOOXYGENASE YUCCA1*) and *YUCCA2* (*INDOLE-3-PYRUVATE MONOOXYGENASE YUCCA2*) genes in petiole and leaf lamina did not differ between treatments within sun conditions (Fig. 3i-l). Overall, irradiance conditions affected the response of genes encoding proteins involved in gibberellin signaling (RGA1, REPRESSOR OF GA1; and GID1b, GA INSENSITIVE DWARF1B) in both petiole and leaf lamina towards application of GA_3 and/or PAC (Fig. 2m-p). Shading increased the expression of *RGA1* in petiole and leaf lamina of the untreated control and plants treated with GA_3 and/or PAC, when compared with plants in the sun conditions (Fig. 2m, o). The expression of *RGA1* was increased in petiole and leaf lamina of plants treated with GA_3 compared with control under both irradiance conditions, whereas PAC decreased the expression of this gene (Fig. 2m, o).

Importantly, inhibition of *RGA1* expression induced by PAC was reversed by application of GA_3 under both irradiance conditions. In our experiment, *GID1b* transcript abundance increased in petiole and leaf lamina of plants treated with PAC or GA_3+PAC compared with control under both irradiance conditions, but GA_3 did not affect the expression of this gene (Fig. 2n, p). Interestingly, shading decreased the expression of *GID1b* in petiole and leaf lamina in untreated control and in plants treated with GA_3 and PAC but not in plants treated with GA_3+PAC, when compared with plants in the sun conditions (Fig. 2n, p). Treatment with GA_3 repressed the expression of *GA20ox1* (*GIBBERELLIN 20 OXIDASE1*) and *GA3ox1* (*GIBBERELLIN 3-BETA-DIOXYGENASE1*) in both petiole and leaf lamina under both irradiance levels, while PAC induced the expression of these genes compared with control (Fig. S3a-d). In addition, the upregulation of *GA20ox1* and *GA3ox1* genes by PAC treatment in petiole and lamina under both irradiance conditions was inhibited by application of GA_3, suggesting that PAC had a specific effect on gibberellin biosynthesis in radish plants.

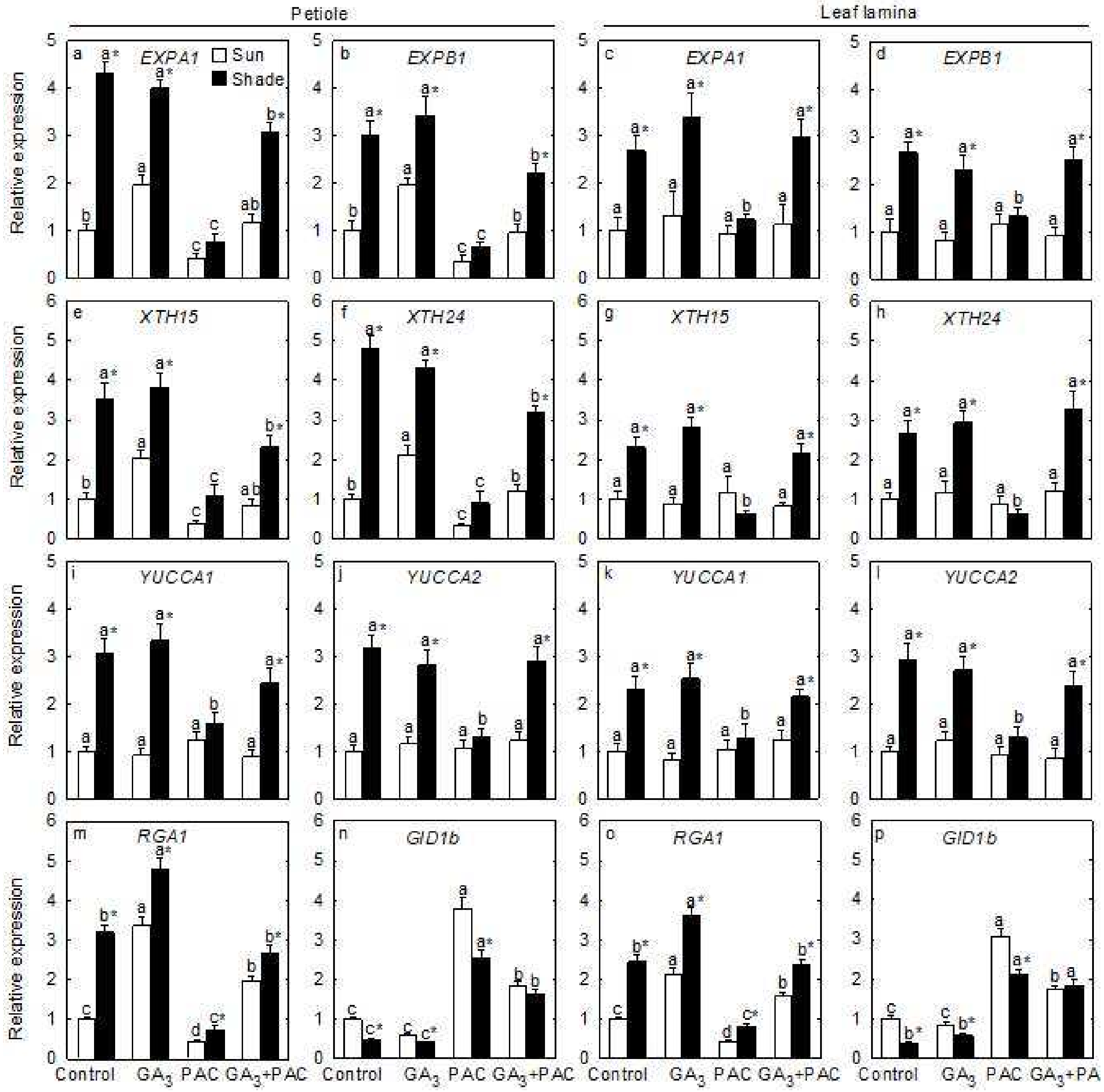

Figure 2. Change in gene expression in leaf petiole and lamina of radish plants treated with GA_3 and/or PAC grown in either sun or shade conditions. Relative expression levels of genes involved in (a-h) cell expansion, (i-l) auxin and (m-p) GA signaling. Bars labeled with the different letters indicate differences between treatments within the same irradiance condition by Tukey's test ($P \leq 0.05$). Asterisks indicate values determined by the Student's *t*-test to be significantly different in shade plants with respect to sun plants ($P \leq 0.05$). Data are mean + standard error of three independent experiments.

3.3 *Gibberellin induces metabolites changes in shoot and tuber in response to irradiance levels*

As changes in carbon allocation in plants could modulate shoot:root ratio (Quick et al., 1991), the concentrations of starch, sucrose, hexoses, and amino acids were analyzed in both shoot and tuber of radish plants treated with GA_3 and/or PAC grown under sun and shade conditions (Fig. 3a-d). Concentrations of starch in shoot were slightly decreased in the shade, with no differences between treatments (Fig. 3a). Analysis in the tuber revealed that a significant decrease in starch concentration was only observed in PAC-treated plants under shade conditions (Fig. 3a). Concentrations of sucrose and hexoses in both shoot and tuber of plants treated with GA_3 and/or PAC grown under sun conditions remained at the same level as in the control (Fig. 3b, c). Moreover, shoot of plants treated with PAC and GA_3+PAC showed lower concentration of sucrose than the control under low irradiance (Fig. 3b). Hexoses were reduced by shading in shoot of plants treated with GA_3 and/or PAC but more strongly in PAC-treatment than in control (Fig. 3c). There was also a decrease in concentrations of sucrose and hexoses in the tuber of plants treated with PAC compared with control under shade conditions (Fig. 3b, c). Amino acids were increased in shoot of plants treated with PAC under full sunlight, while GA_3 and GA_3+PAC did not change the concentration of amino acids compared with control (Fig. 3d). Under shade conditions, there was also a decrease in amino acids in shoot of plants treated with GA_3 compared with control. On the other hand, amino acids did not vary in tuber between treatments under full sun light (Fig. 3d). There was a decrease in concentration of amino acids in tuber of untreated plants and plants treated with GA_3 and/or PAC under shade compared with sun plants. Moreover, the reduction of amino acids by shading was steeper in tuber of plants treated with PAC compared to control (Fig. 3d). Concentrations of protein in both shoot and tuber were not affected by treatments or irradiance levels (Fig. S5).

3.4 *Tuber mineral composition*

The tuber concentrations of N and P were not affected by GA_3 and/or PAC or irradiance levels (Fig. 4a, b). A significant increase in concentration of K was observed in tuber of plants treated with PAC compared with control under both light conditions (Fig. 4c).

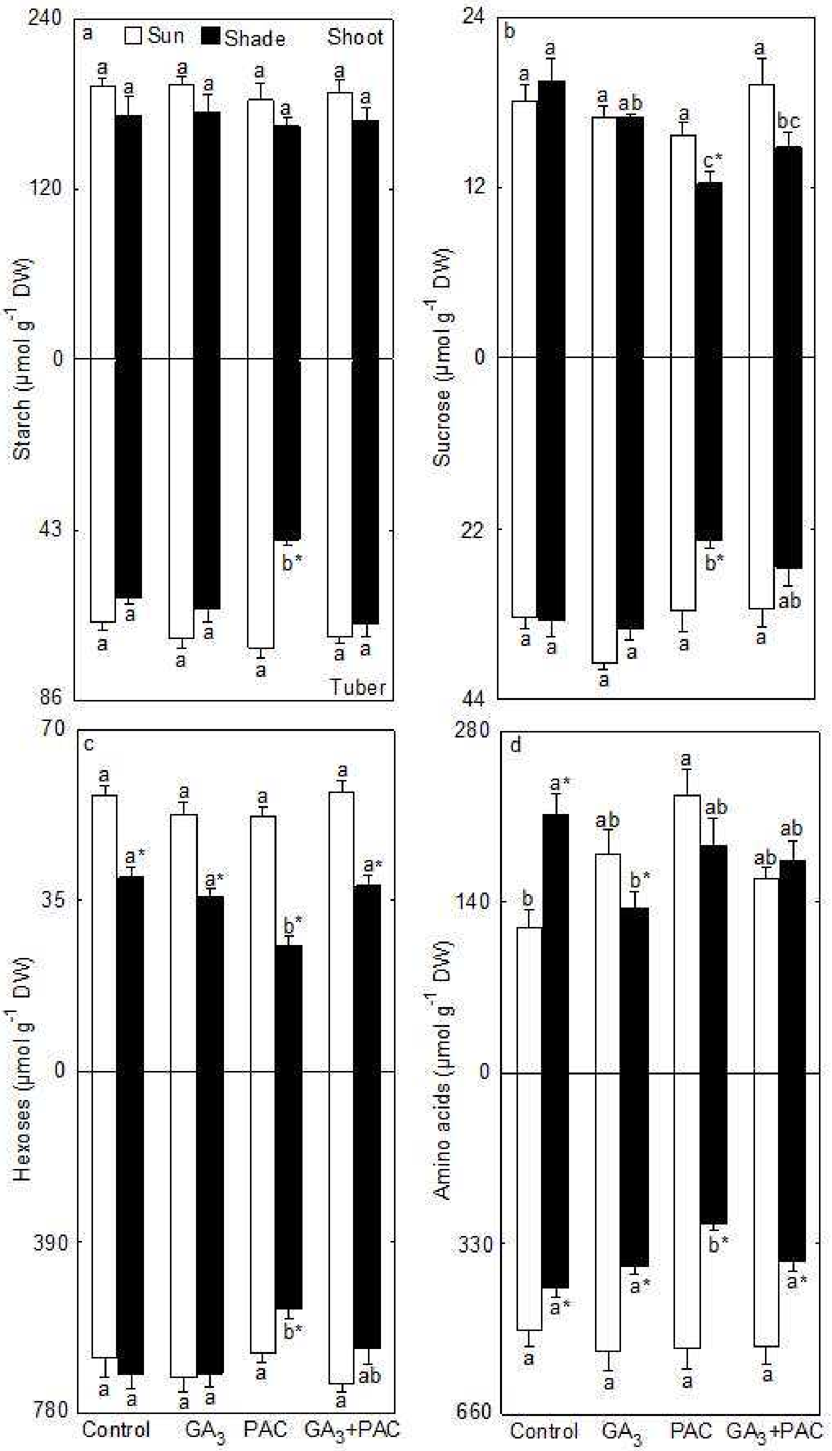

Figure 3. Concentrations of metabolites in shoot and tuber of radish plants treated with GA_3 and/or PAC grown in either sun or shade conditions. (a) Starch. (b) Sucrose. (c) Hexoses. (d) Total amino acids. Bars labeled with the different letters indicate differences between treatments within the same irradiance condition by Tukey's test ($P \leq 0.05$). Asterisks indicate values determined by the Student's *t*-test to be significantly different in shade plants with respect to sun plants ($P \leq 0.05$). Data are mean + standard error of three independent experiments.

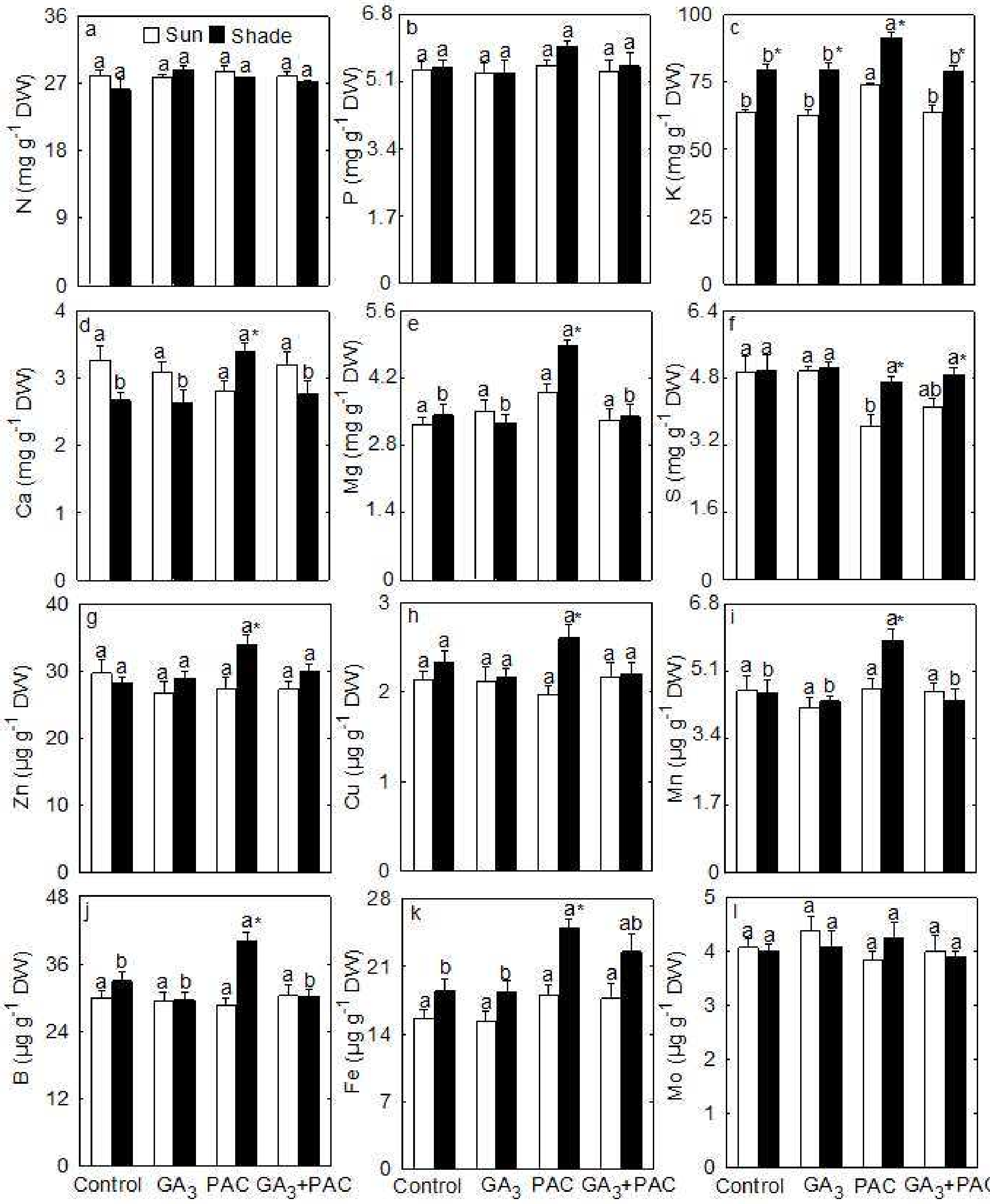

Figure 4. Macro and microelement concentrations in tubers of radish plants treated with GA$_3$ and/or PAC grown in either sun or shade conditions. (a) Total nitrogen. (b) Phosphorus. (c) Potassium. (d) Calcium. (e) Magnesium. (f) Sulphur. (g) Zinc. (h) Copper. (i) Manganese. (j) Boron. (k) Iron. (l) Molybdenum. Bars labeled with the different letters indicate differences between treatments within the same irradiance condition by Tukey's test ($P \leq 0.05$). Asterisks indicate values determined by the Student's *t*-test to be significantly different in shade plants with respect to sun plants ($P \leq 0.05$). Data are mean + standard error of three independent experiments.

Higher K concentration was observed in tuber of untreated control and plants treated with GA_3 and/or PAC under shade compared with sun conditions. Treatment with GA_3 and/or PAC did not significantly affect the tuber concentrations of Ca and Mg under sun condition (Fig. 4d, e). In contrast to sun condition, concentrations of those elements were significantly increased in tuber of plants treated with PAC compared with control under shade conditions. Moreover, treatment with PAC increased Ca (21%) and Mg (25%) concentrations in tuber of plants under shade compared with sun conditions (Fig. 4d, e). Treatment with PAC reduced the tuber concentration of S under full sunlight compared to control (Fig. 4f). There was an increase in concentration of S in tuber of plants treated with PAC and GA_3+PAC grown under shade compared with sun plants. The tuber concentrations of Zn and Cu did not differ between treatments within each irradiance condition (Fig. 4g, h). However, the results showed increased concentration of Cu (32%) and Zn (23%) in tuber of plants treated with PAC grown under shade compared with sun plants.

Treatment with GA_3 and/or PAC did not significantly affect concentrations of Mn and B under sun condition (Fig. 4i, j). In contrast to sun condition, concentrations of Mn and B were increased in tuber of plants treated with PAC compared with control under shade conditions. Moreover, treatment with PAC increased Mn (26%) and B (48%) concentrations in tuber of plants under shade compared with sun conditions (Fig. 4i, j). No differences between treatments were found in tuber concentration of Fe under sun conditions (Fig. 4k). On the other hand, the tuber concentration of Fe was increased by PAC treatment under shade conditions, with no change in GA_3 and GA_3+PAC treatment compared with control. Our results also revealed that PAC increased tuber concentration of Fe by 38% under shade compared with sun conditions (Fig. 4k). The tuber concentration of Mo was not affected by GA_3 and/or PAC or irradiance levels (Fig. 4l).

4 Discussion

In response to low light intensity some plants species prioritize growth of stems and leaves to maximize light exposure, which causes inhibition of root growth (Niinemets, 2010; van Gelderen et al., 2018). Light-dependent changes in plant growth and development are largely dependent on hormones, including auxin and gibberellin (Yang et al., 2018). The gibberellin biosynthesis inhibitor PAC suppressed petiole elongation in *Cyclamen persicum* grown in low-light intensity (Oh and Kim, 2014). However, treatment of *Arabidopsis* plants with gibberellin does not necessarily increase petiole and lamina elongation, suggesting that gibberellin regulation is modulated flexibly to achieve leaf growth (Ribeiro et al., 2012a). The results of the present study have revealed that a decrease in gibberellin levels increased biomass partitioning to tuber growth in low light intensity conditions, which increased the concentrations of several minerals in tuber. The decrease in shoot growth and increased biomass allocation to tubers of PAC-treated plants grown under low irradiance was caused by a tissue-specific growth response of petiole and lamina through alteration of *EXP* and *XTH* expression patterns.

Under full sunlight, treatment of radish plants with PAC decreased petiole elongation, but not growth of the lamina compared to control (Fig. 1). The different responses of lamina and petiole of radish plants to the gibberellin biosynthesis inhibitor PAC may be caused by a difference in the expression *EXP* and *XTH* genes (Fig. 2), which could be under the regulation of light signals. In this context, the expression of *EXP* and *XTH* genes were repressed by PAC in petiole under full sunlight, but no effect of PAC on the expression of these genes was observed in leaf lamina. On the other hand, the fact that the expression of *EXP* and *XTH* genes decreased in petiole and lamina when PAC inhibited leaf expansion of plants grown under shade conditions strengthens the idea that gibberellin differentially tunes the relationship between petiole and lamina growth in response to light intensity (Figs. 1 and 2). These findings are further supported by the fact that PAC decreased the petiole mass fraction and leaf mass fraction under shade conditions, with no changes in plants grown under full sunlight (Fig. S4). In addition, shade perception by the leaf alters the dynamics in the partitioning of biomass to shoot and tuber. Under shade, plants treated with PAC allocated proportionally more biomass to their tuber and less to shoot compared to untreated plants (Figs. 1 and S4). In carrot, PAC increased root weight but decreased shoot weight of plants grown with a constant irradiance of 300 μmol m^{-2} s^{-1} (DLI = 17 mol m^{-2} d^{-1}) (Wang et al., 2015). Our data from radish plants grown under natural light indicate that gibberellin level is not the only factor determining biomass partitioning between shoot and tuber, as the pattern of biomass partitioning was altered in PAC-treated plants grown under shade conditions (15 mol m^{-2} d^{-1}), but not under full sunlight (29 mol m^{-2} d^{-1}), when compared with their respective controls (Figs. 1 and 5). Together, our results indicate that low gibberellin level acts as a signal in controlling biomass allocation to tuber over shoot in radish plants grown under low light intensity, and this is reversed by high light conditions. In fact, the increased shoot proportion and decreased tuber proportion were associated with plants treated with PAC under sun compared with shade conditions (Fig. 5), supporting the hypothesis that gibberellin-regulated biomass allocation in radish plants is dependent on irradiance levels.

Gibberellin and auxin are known to induce expression of cell-wall-modifying genes including *XTH* and *EXP* (Ma and Li, 2019; Ribeiro et al., 2012b). Our results imply that leaf development was result of a mechanism by which the low irradiance regulates the expression of genes associated with gibberellin signaling and auxin biosynthesis which in turn differentially affects the expression pattern of *XTH* and *EXP* genes in lamina and petiole (Figs. 2 and 5). The upregulation of *XTH* and *EXP* genes was accompanied by the expansion of petiole and lamina in untreated control plants grown under shade conditions (Figs. 1, 2 and 5). However, treatment of shade-grown plants with PAC impaired the induction of these genes and thus growth of petiole and lamina was decreased compared to the control.

This result shows that gibberellin is necessary to induce cell wall expansion genes in the shade. It is noteworthy here that the changes in expression of *XTH* and *EXP* genes in leaf lamina are not evident when the plants are exposed to full sunlight, irrespective of the treatment with GA_3 and/or PAC (Fig. 2). These data suggest that high irradiance does not affect the responsiveness of the leaf lamina towards the gibberellin. In *Arabidopsis*, petiole elongation and lamina growth inhibition regulated by low irradiance are dependent on auxin through alteration on the expression of *XTH* genes (de Wit et al., 2015). The expression of *YUCCA* auxin biosynthesis genes (Zhao et al., 2012) was upregulated by low irradiance in petiole and lamina of control plants (Fig. 2). However, *YUCCA* expression increases in the shade were impaired by PAC treatment (Fig. 2). Thus, low irradiance appears to require an integration of gibberellin and auxin modulation to increase growth of petiole and lamina, which is likely to decrease tuber proportion (Figs. 5 and S4). These findings are supported by the fact that the effects of shading on petiole length, lamina area, tuber growth, and expression of genes involved in auxin biosynthesis and cell-wall remodeling mimicked GA_3 treatment under low irradiance (Figs. 1, 2 and 5). Moreover, *YUCCA* genes were not responsive to treatment with GA_3 and/or PAC under sun conditions, suggesting that full sunlight treatment may alter the interplay between auxin and gibberellin in radish plants.

Plant acclimation to low irradiance is usually associated with the increase in shoot:root ratio and specific leaf area (Poorter et al., 2012). In our experimental setup, the decrease in shoot:tuber ratio in plants treated with PAC grown under shade, compared with sun-grown plants, included increased biomass allocation to the tuber (Figs. 1 and 5).

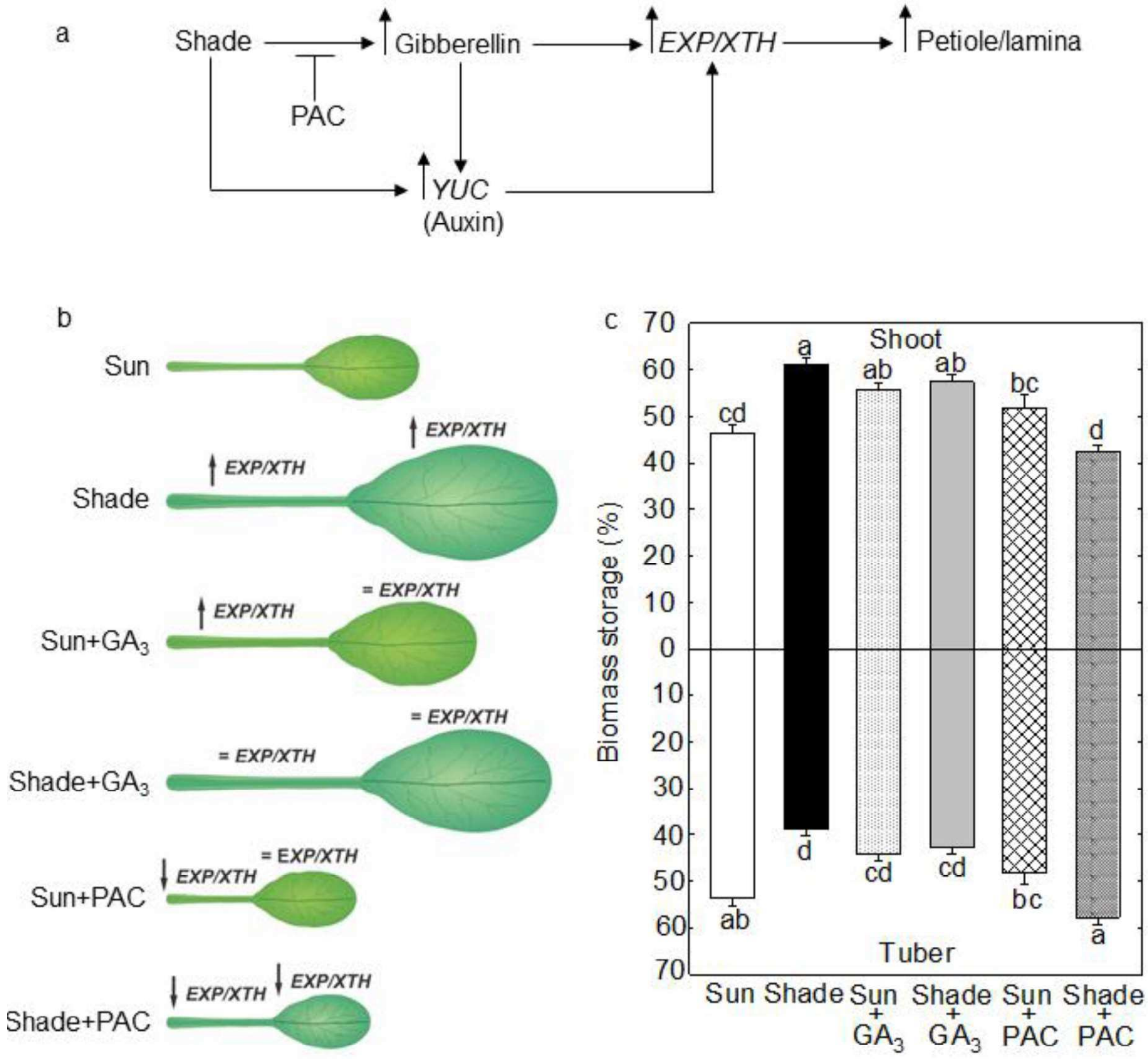

Figure 5. Proposed model of radish plants responses to GA_3 and/or PAC treatments in two irradiance levels. (a) Shading increases gibberellin and auxin metabolism in untreated control plants, which may upregulates the expression of *EXP* and *XTH* genes in petiole and leaf lamina. These combined effects might result in the stimulation of leaf elongation in radish plants. On the other hand, PAC inhibits gibberellin metabolism, thereby decreasing leaf growth of radish plants under shade conditions. (b) Mean leaf shapes for plants treated with GA_3 or PAC grown under sun and shade conditions. Note the expression of *EXP* and *XTH* genes in both petiole and leaf lamina of radish plants grown under shade compared with control under sun conditions and the expression of these genes in leaf of radish plants treated with GA_3 or PAC compared with control within each light treatment. (c) Biomass storage in shoot and tuber of radish plants treated with GA_3 or PAC grown under sun or shade conditions. Bars labeled with the different letters indicate significant differences by Tukey's test at 5% probability. Data are mean + standard error of three independent experiments.

These results provide evidence that gibberellin levels are involved in the regulation of biomass allocation and development in radish plants in response to irradiance. In agreement with these observations, a larger proportion of the biomass was allocated to growth of tuber in plants treated with PAC under low irradiance, when compared with plants in the sun conditions (Fig. S4). Moreover, SLA was increased by shading but did not vary in plants treated with PAC between irradiance levels (Fig. 1). In radish plants, petiole represents a major sink for carbohydrates (Sugiura et al., 2017). The decrease in sucrose and hexoses in leaves of radish plants treated with PAC under shade (Fig. 3) indicates that changes in biomass allocation patterns could allow the carbohydrates to be invested more efficiently, favoring tuber growth (Fig. 5). These observations indicate that the shoot developmental program itself determines how efficiently carbohydrates are converted into tuber biomass in radish plants. This is supported by the demonstration that GA_3 reversed the morphological and physiological alterations caused by PAC in radish plants (Figs. 1 and 3). Sucrose synthase plays an important role in the development of storage root in radish plants (Rouhier and Usuda, 2001; Usuda and Demura, 1999). In this context, the increase of sucrose synthase activity is accompanied by a decrease of the sucrose concentration in the root zone showing rapid expansion (Stein and Granot, 2019). In this sense, the reduced concentration of sucrose in tubers of radish plants treated with PAC grown under low irradiance indicates that sucrose is being used faster when biomass allocated to tuber growth is stimulated by PAC. In agreement with this model, GA_3 decreased tuber mass fraction of plants treated with PAC under shade and this was accompanied by an increase in sucrose concentration (Figs. S4 and 3). Moreover, amino acids concentrations in tubers were decreased by PAC treatment under shade compared to control, with no changes in total protein (Figs. 3 and S5). These results suggest that changes in the concentrations of sugars and amino acids in tubers of radish plants treated with PAC are responsible for the stimulation of tuber growth relative to shoot growth under low irradiance conditions.

It has been known for long time that the uptake by roots of adequate amounts of essential mineral nutrients as well as distribution among organs are indispensable for growing and crop production (White and Brown, 2010). In general, treatment with GA_3 and/or PAC had no effect on tuber mineral concentrations in sun conditions (Fig. 4). However, shading increased the concentration of K in untreated control compared with plants in the sun conditions. Moreover, PAC treatment increased the concentration of K and a number of minerals (e.g., Fe, Ca, Mg, S, Mn, Zn, Cu and B) not affected in untreated control plants under low irradiance conditions. These changes suggest that there is a link between biomass allocation to the tuber and increased tuber mineral concentrations in plants treated with PAC under low irradiance, and that high irradiance reverses both effects. The increase in mineral contents found here is noteworthy, considering that root vegetables such as radish and carrot are a good source of important minerals for human health (Banihani, 2017; Goyeneche et al., 2015). In this context, our study indicates that low irradiance in combination with PAC treatment may increase soil nutrient uptake and thus the nutritional quality of radish tuber.

5 Conclusions

In summary, this study shows that shade acts as a regulator of gibberellin and auxin metabolism, which in turn increases the expression of cell-wall remodeling genes in petiole and leaf lamina of radish plants (Fig. 5a, b). The upregulation of *EXP* and *XTH* genes increases petiole length and lamina area of radish plants grown under low light intensity (Fig. 5a, b). However, treatment of shade-grown plants with PAC inhibits the expression of these genes, leading to decreased petiole length and lamina area (Fig. 5a, b). The consequence of growth inhibition of petiole and lamina is increased biomass allocation to tuber over shoot in plants treated with PAC compared to untreated plants under low irradiance (Fig. 5c). Interestingly, treatment of plants grown under shade with GA_3 produced the same expression of *EXP* and *XTH* genes in petiole and lamina as shade alone, indicating that low irradiance results in a saturation of the gibberellin response (Fig. 5b). In agreement with this model, reduced tuber growth and increased shoot growth under shade are maintained in plants treated with GA_3 (Fig. 5c). On the other hand, treatment with GA_3 led to an increase in the expression of *EXP* and *XTH* genes in petiole, but not in lamina of plants grown under full sunlight (Fig. 5b). Thus, the increased shoot growth relative to tuber growth of plants treated with GA_3 under sun conditions might be driven by a direct effect on the expression of cell-wall remodeling genes in petiole rather than leaf lamina. Overall, our results indicate that gibberellin regulates biomass allocation in response to irradiance in radish. Furthermore, inhibition of gibberellin biosynthesis under low light intensity leads to changes in biomass allocation that could contribute to the nutritional quality of radish tuber with no yield penalty.

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Supplementary data

Table S1. Primers sequences used for qRT- PCR analysis

Name	Primer sequences	
	Forward	Reverse
EXPA1	5'-TCATTACTCCGACAATAACTA-3'	5'-CATCTCTCTATCTTTCCTCTT-3'
EXPB1	5'-ATTACAGTTGCCATCCTAC-3'	5'-GCCTCATATACCTATTCCTAC-3'
XTH15	5'-TGGTGTTCCGTTCCCAAAGA-3'	5'-CCTGAAGAAACAGTGCAAGCC-3'
XTH24	5'-CGAGCCAAGCTAGACTCAGAT-3'	5'-TGACAAAAGAGGAAGGAGAGCAT-3'
YUCCA1	5'-GCGGGGAGATTATTCACGCTA-3'	5'-ACATGGACTGTGTTTCGGACA-3'
YUCCA2	5'-TGTGGCTAAAGGGAATGGACA-3'	5'-TGCTGGGGACGAGAAAATGA-3'
GA20ox1	5'-GGATTCAGAGAAAAGCAGGCG-3'	5'-GATACACTTTCCCAAATGGCTCA-3'
GA3ox1	5'-GGCGTCGCTCGTATCTCAT-3'	5'-CAACGATGTCGCAGTAGTTCTTA-3'
RGA1	5'-ACCGCTCATAACAACCTCCG-3'	5'-GGTCCACTGATTTCAGCCCA-3'
GID1B	5'-CTAGTTATGATGGGCGGGGG-3'	5'-TTAACCGGTGGTAGATGCCG-3'
Actin	5'-GCATCACACTTTCTACAAC-3'	5'-CCTGGATAGCAACATACAT-3'

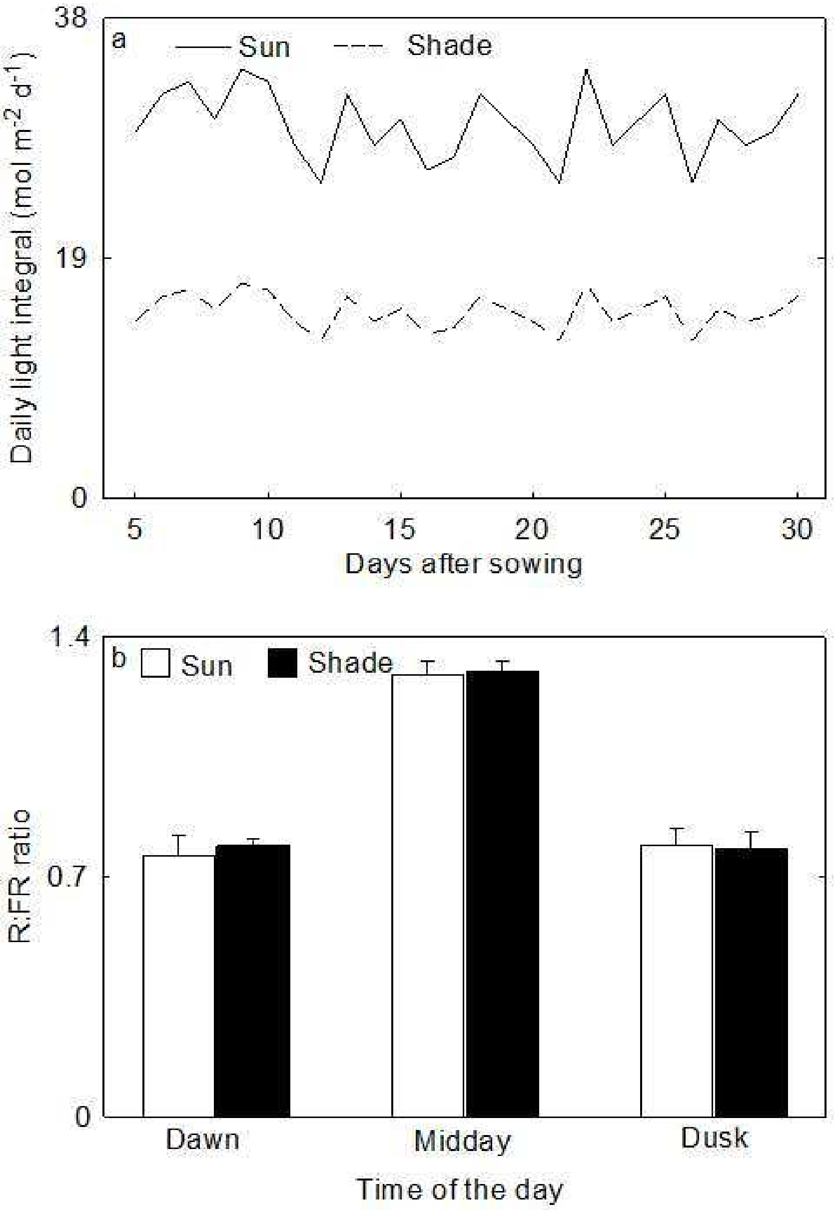

Figure S1. (a) Time course of daily light integrals (DLIs) over the radish plants grown under two irradiance levels. (b) Red:far red ratio measured in both sun and shade conditions. Data are mean + standard error of three independent experiments.

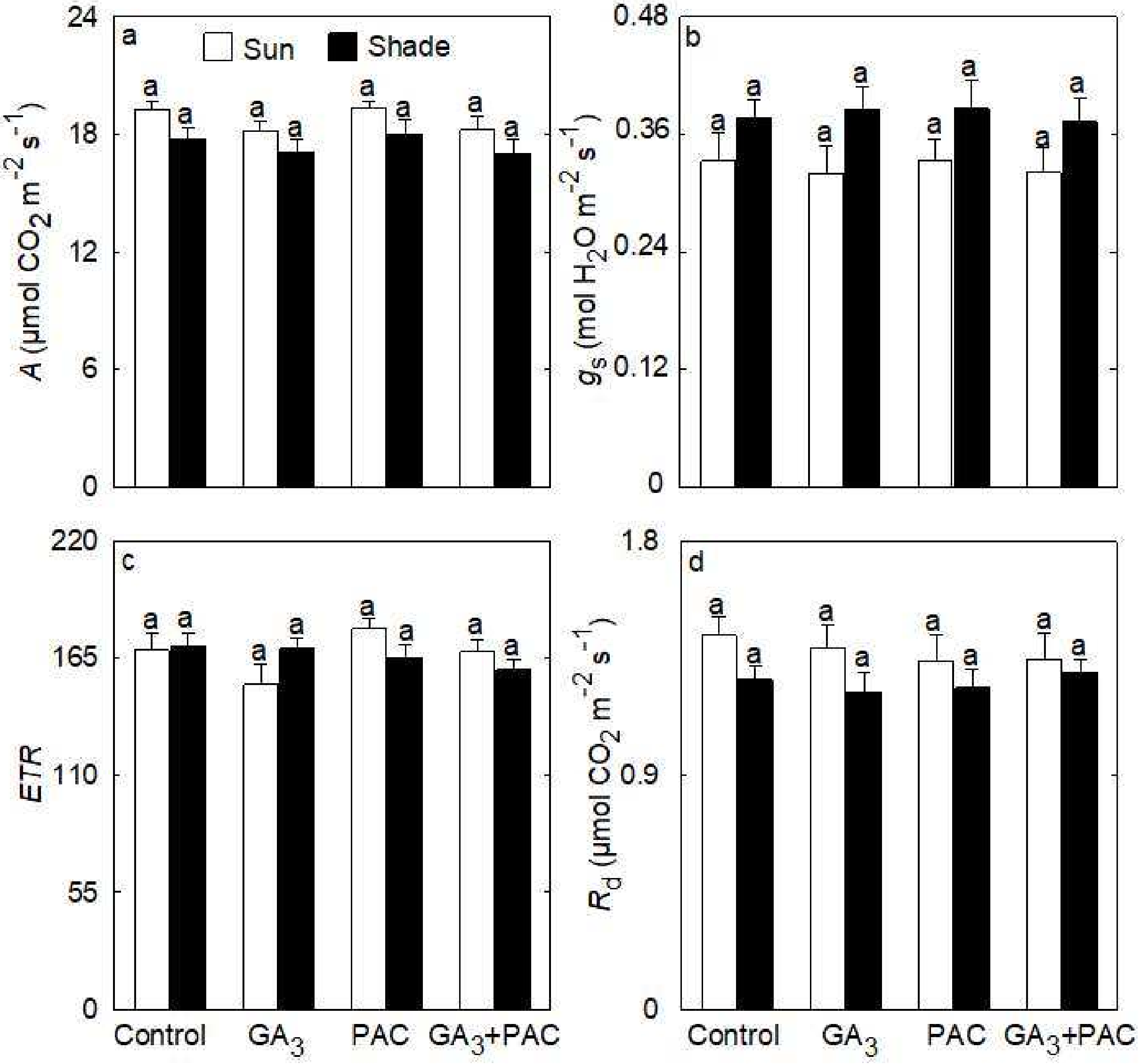

Figure S2. (a) Net CO$_2$ assimilation rate, (b) stomatal conductance, (c) electron transport rate and (d) mitochondrial respiration rate in the dark in plants treated with GA$_3$ and/or PAC grown under sun or shade conditions. Gas exchange measurements were performed using an open-flow gas exchange system infrared gas analyzer (LI-6400XT, LI-COR, Lincoln, NE, USA). The analyses were made on the third fully expanded leaf of 10 plants per treatment between 9:00 and 11:00 h in plants at the age of 30 days. Measurements of gas exchanges were conducted under artificial photosynthetically active radiation, i.e., 1000 μmol photons m^{-2} s^{-1} at the leaf level. Moreover, the conditions in the leaf chamber consisted of an air temperature of 25 °C, a vapour pressure deficit of 1.0 kPa and a reference CO$_2$ concentration of 400 μmol CO$_2$ mol^{-1} air. Bars labeled with the different letters indicate differences between treatments within the same irradiance condition by Tukey's test ($P \leq 0.05$). Data are mean + standard error of three independent experiments.

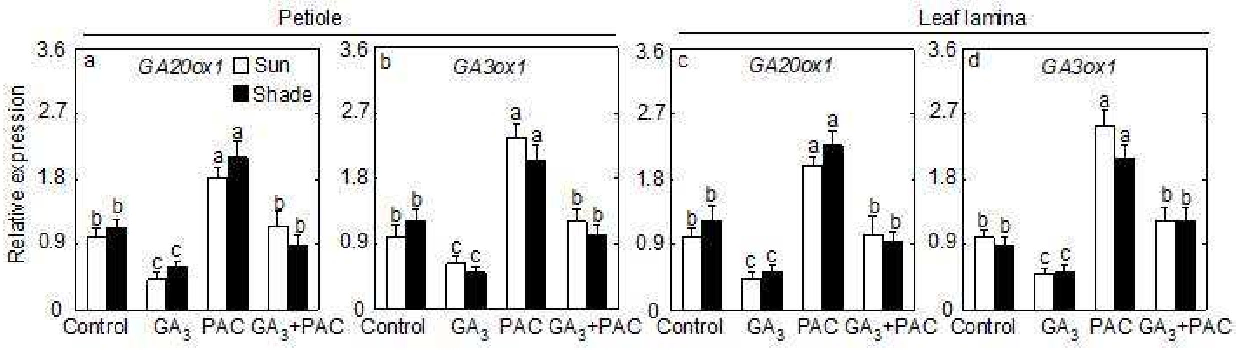

Figure S3. (a-d) Relative expression levels of genes involved in gibberellin biosynthesis in petiole and leaf lamina of radish plants treated with GA$_3$ and/or PAC grown under sun or shade conditions. Bars labeled with the different letters indicate differences between treatments within the same irradiance condition by Tukey's test ($P \leq 0.05$). Data are mean + standard error of three independent experiments.

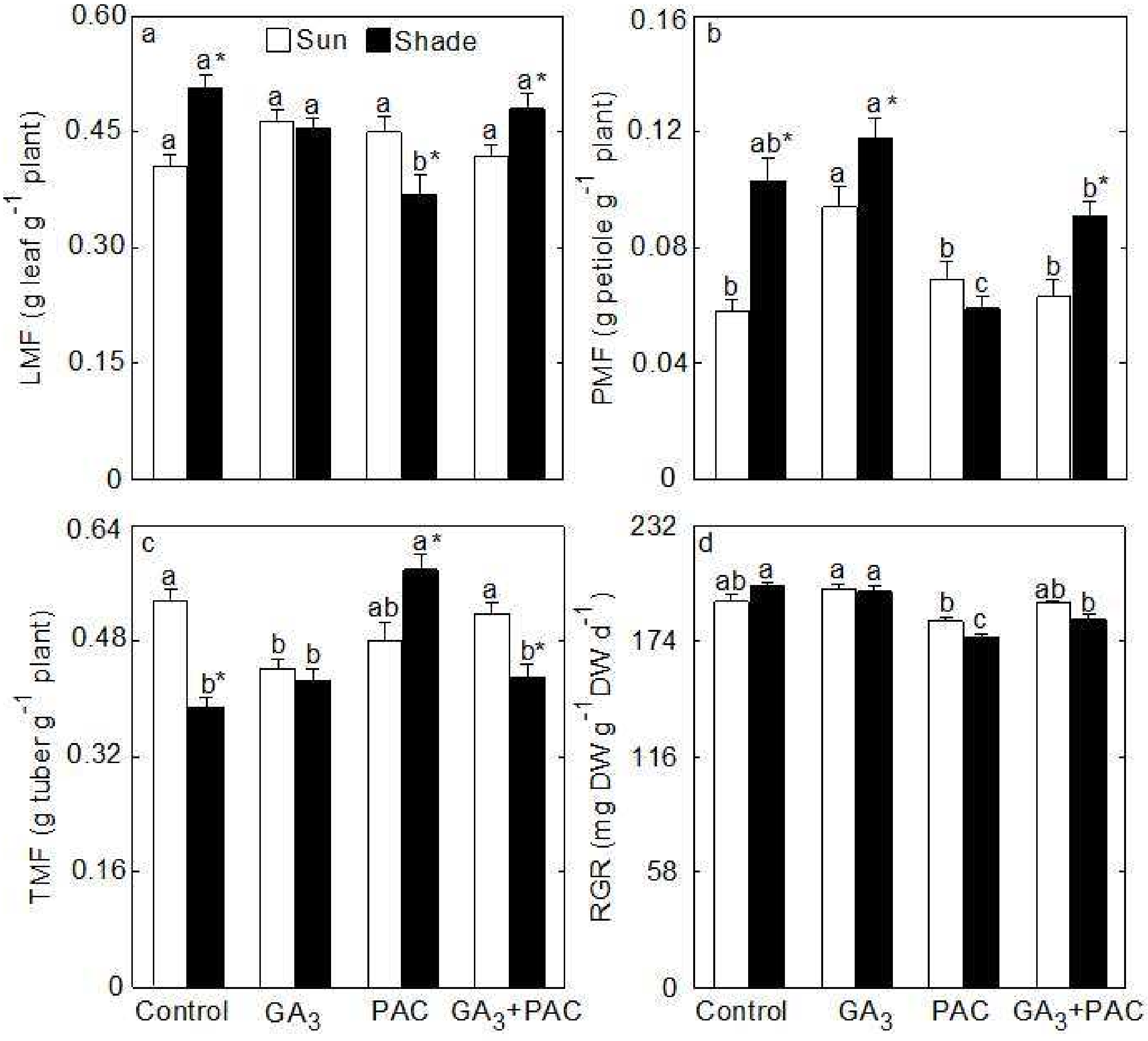

Figure S4. Phenotypic changes of radish plants caused by treatment with GA$_3$ and/or PAC grown under sun or shade conditions. (a) Leaf mass fraction. (b) Petiole mass fraction. (c) Tuber mass fraction. (d) Relative growth rate. The relative growth rate (RGR) was calculated between 5th and 30th day. The mass fraction for leaf (LMF), petiole (PMF) and tuber (TMF) was measured as the mass of leaf, petiole and tuber divided by the plant total mass. Bars labeled with the different letters indicate differences between treatments within the same irradiance condition by Tukey's test ($P \leq 0.05$). Asterisks indicate values determined by the Student's *t*-test to be significantly different in shade plants with respect to sun plants ($P \leq 0.05$). Data are mean + standard error of three independent experiments.

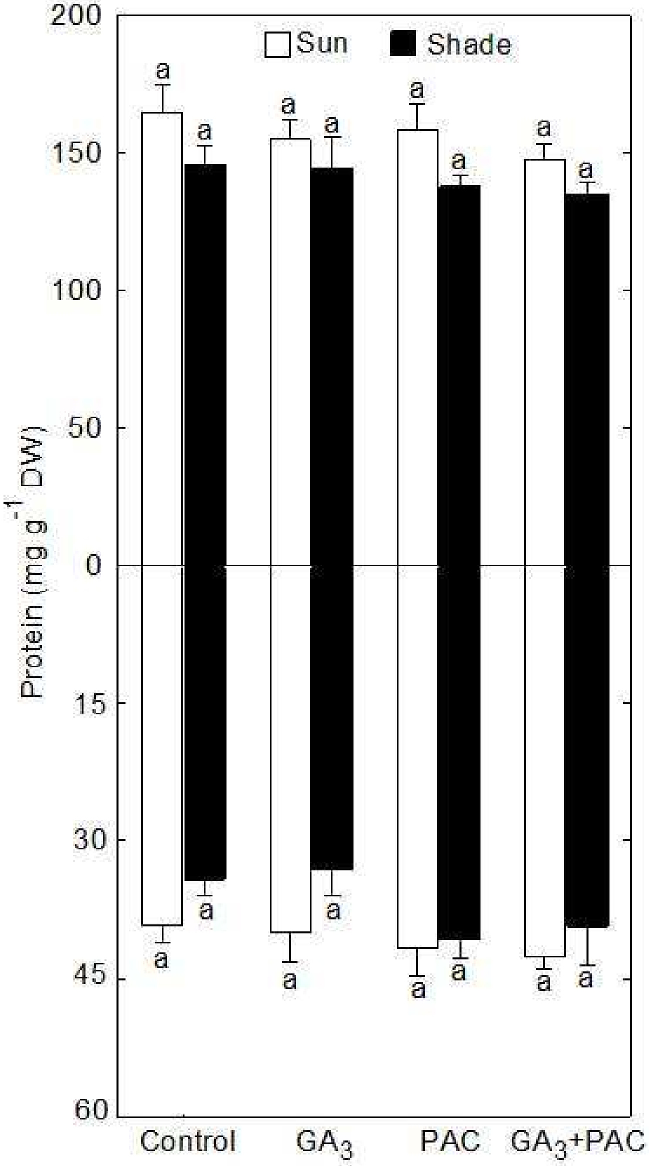

Figure S5. Protein concentration in shoot and tuber of radish plants treated with GA$_3$ and/or PAC grown under sun or shade conditions. Bars labeled with the different letters indicate differences between treatments within the same irradiance condition by Tukey's test ($P \leq 0.05$). Data are mean + standard error of three independent experiments.

CHAPTER 3

Effects of nitrogen supply and its relation to auxin on root architecture of *Arabidopsis thaliana*

Abstract

Nitrogen is essential for plant growth and development. Therefore, nitrogen imbalance strongly alters plant growth. In addition to nitrogen, polar transport of auxin is required for both primary root and lateral root growth. Among the auxin transporters, ATP-binding cassette class B (ABCB), are known to act in auxin homeostasis due to their efflux and/or conditional efflux/influx of auxin under high and low auxin levels, respectively. It is known that nitrogen supply affects auxin levels by altering gene expression and auxin influx in a pH-dependent manner. However, the mechanisms underlying auxin homeostasis through ABCB transport in roots under altered nitrogen status remain unclear. In this sense, it was hypothesized that the changes in root architecture due to nitrogen status are related to auxin transport by ABCBs. Here, mutants of *Arabidopsis thaliana* defectives in *abcb1*, *abcb19*, *abcb4, abcb21*, *abcb6* and *abcb20* grown under different nitrogen supplies (nitrate, ammonium and glutamine) were used to investigate if auxin homeostasis is altered in these conditions and how it affects root architecture. The results showed that ammonium strongly inhibits primary root growth and lateral root emergence, nitrate induces primary root growth and glutamine increases lateral root density. Interestingly, the inhibitory effect of NH_4^+ was reversed in the doubles *abcb1 abcb19* and *abcb6 abcb20* mutants. Besides that, ammonium and glutamine affected root bending in all genotypes, which was even more evident in the doubles *abcb1 abcb19* and *abcb6 abcb20* mutants.

Keywords: Auxin transporters · Nitrogen · Plant nutrition · Root bending · Root growth

1 Introduction

Plant growth is determined by the interaction of internal signals and environmental factors. Among the environmental factors, nutrient availability is critical for plant development.

Consequently, plants developed strategies to overcome the heterogeneity of nutrients on soil by modulating their root morphology and physiology (Robinson, 1994; Dong et al., 2018). Root adjustments are also known as root foraging plasticity, and they include changes on root architecture and nutrient uptake rates (Malamy, 2005; Dong et al., 2018). Nitrogen (N) is a major nutrient determining plant growth and productivity and, due to its scarcity on soils, N availability strongly affects root architecture (Kiba and Krapp, 2016). Under agronomic and ecological conditions, N is mostly acquired by plants as the combination of nitrate (NO_3^-) and ammonium (NH_4^+), the N inorganic forms (Marschner, 2011). Besides the inorganic forms, N can also be assimilated as the organic forms urea, amino acids and peptides (Tegeder and Rentsch, 2010; Kiba and Krapp, 2016).

Studies have shown that severe N depletion impairs primary root (PR) and lateral root (LR) growth, whereas mild N limitation induces PR and LR elongation (Gruber et al., 2013; Giehl et al., 2014; Araya et al., 2015). Besides the availability, the N source also affects the root architecture. While local nitrate supply in the normal concentration range (around 5 mM) can stimulate PR growth, increased NO_3^- can inhibit PR elongation and stimulate LR elongation (Walch-Liu and Forde, 2008; Vidal et al., 2013; Forde, 2014). High local supply of ammonium, in turn, results in shorter but highly branched lateral roots (Lima et al., 2010; Giehl et al., 2014; Meier et al., 2020). This LR branching is thought to occur due to root apoplast acidification caused by ammonium uptake through ammonium transporters (AMT), which induces the influx of protonated auxin through AUX1 LAX3 transporters into overlaying cells of lateral root primordia (Meier et al., 2020). Considering that imbalances on N nutrition can cause growth limitation and toxicity in plants, it is important to understand how N sources and levels can affect root architecture and, consequently, crop production (Li et al., 2014; Esteban et al., 2016).

Root growth responses to nitrogen are dependent of a complex signaling cascade, which also involves hormonal regulation (Gojon et al., 2011; Giehl et al., 2014; Ma et al., 2014). Auxin, for example, is a plant hormone known by its role in root growth and by its interaction with N (Krouk et al., 2010; Ma et al., 2014). Growth of primary and lateral roots is determined by local auxin maxima and minima generated over tissues by polar and lateral auxin transport (Adamowski and Friml, 2015). Local auxin levels are modulated by three main families of auxin transporters: PIN (PIN-FORMED) proteins, that are efflux carriers basally localized on cells; AUX1/LAX (AUXIN1/LIKE AUXIN1) influx permeases, that are H^+ symporters asymmetrically cell-localized; and ABCB (ATP Binding Cassette subfamily B) transporters, that can either act as influx and efflux carriers depending on auxin level and their localization (Yang and Murphy, 2009; Geisler et al., 2017; Swarup and Bhosale, 2019).

The *Arabidopsis* ABCB subfamily comprises around 20 members and presents nonpolar localization on the plasma membrane (Geisler and Murphy, 2006). Among them, some isoforms are thought to have overlapping activities due to its sequence similarity (Noh et al., 2001; Zhang et al., 2018; Jenness et al., 2019). This is the case of ABCB1 and ABCB19, that function as auxin exporters and integrate the rootward auxin transport and the shootward auxin stream from the root apex (Kerr and Bennett, 2007; Knöller et al., 2010). *ABCB1/B19* loss of function results in reduced adventitious roots formation and waiving phenotype (Noh et al., 2001). ABCB4 and ABCB21, in turn, are facultative auxin influx/efflux transporters which function under low and high intracellular auxin concentrations, respectively (Kamimoto et al., 2012). The conditional influx/efflux activity of ABCB4/21 provides a fine regulation of auxin levels, and their loss of function causes reduced rootward auxin transport and defective lateral root outgrowth (Jenness et al., 2019). Another pair of isomers, ABCB6 and ABCB20, is involved in basipetal auxin movement in the shoot, and their loss of function results in reduced root length (Zhang et al., 2018). In addition to these auxin transporters, a NO_3^- influx carrier NITRATE TRANSPORTER1.1 (NRT1.1, also called CHL1), is a nonspecific transporter that also transports auxin and, thus, modulates local auxin level (Krouk et al., 2010; Mounier et al., 2014; Maghiaoui et al., 2020). The CHL1 affinity for auxin is inversely proportional to its affinity for NO_3^- in a concentration-dependent manner (Krouk et al., 2010). Thus, at low NO_3^- conditions, CHL1 affinity for auxin is increased, lowering the auxin levels at lateral root primordia and reducing the LR emergence. On the other hand, when NO_3^- is high, CHL1 reduces auxin transport, accumulating the hormone in LR primordia and inducing LR growth (Krouk et al., 2010; Maghiaoui et al., 2020).

Considering the importance of root system architecture (RSA) to provide mechanic support and water and nutrient acquisition, it is important to understand the physiologic mechanisms underlying the control of RSA and how it works under different N sources and levels. Although the root responses to auxin and N are relatively well understood, the interaction between those factors and the role of ABCB transporters still unclear. In this sense, it was hypothesized that the changes in root architecture in response to nitrogen status are related to local levels of active auxin modulated through ABCB transporters.

2 Materials and methods

2.1 *Plant material and growth conditions*

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type for all experiments. The single mutant *A. thaliana* lines *abcb1-100*, *abcb19-101*, *abcb4-1*, *abcb21-2*, *abcb6*-1, and the double mutant lines, *abcb1-100 abcb19-101* (*b1b19*)*, abcb4-1 abcb21-2* (*b4b21*) and *abcb6-1 abcb20-2* (*b6b20*) were used for nitrogen experiments (Table 1).

Table 1. Mutant lines used in this study.

Gene Locus	Protein	Mutant	T-DNA Insertion	Reference
AT2G36910	ABCB1	*abcb1-100*	Salk_083649	Lin and Wang, 2005
AT3G28860	ABCB19	*abcb19-101*	Salk_033455	Lin and Wang, 2005
AT2G47000	ABCB4	*abcb4-1*	Salk_063720	Terasaka et al., 2005
AT3G62150	ABCB21	*abcb21-2*	Gabi_954H06	Jenness et al., 2019
AT2G39480	ABCB6	*abcb6-1*	GABI_401D12	Zhang et al., 2018
AT3G55320	ABCB20	*abcb20-2*	GABI_387F09	Zhang et al., 2018

Seeds were surface sterilized with 70% ethanol and sown in plates containing one-quarter-strength Murashige and Skoog medium (1962) (¼ MS – pH 5.6; Caisson Labs, Smithfield, UT, USA) with 1 g L^{-1} MES, 0.5% sucrose, and 0.8% agar. After sowing, seeds were stratified at 4 °C for 2 days, then grown vertically in square plates (130 x 130 x 17 mm) under continuous light (100 µmol m^{-2} s^{-1}) at 22 °C. Treatments consisted of one-quarter-strength MS medium with (control) or without nitrogen (other treatments). Nitrogen source and concentration were altered by adding the following nitrogen forms – 5 mM Glutamine, 5 mM KNO_3, 10 µM KNO_3 and 2.5 mM $(NH_4)_2SO_4$. Considering that K_2SO_4 was used to balance the supplemented medium, a sulfate control composed by complete ¼ MS medium plus K_2SO_4 was included. Detailed information about media composition is presented in Table 2.

Primarily tests were performed with seeds sown either directly on the modified media (non-precultured, NPC) or sown initially on ¼ MS complete medium for 5 days (pre-cultured, PC). After pre-culture, uniformly grown seedlings were transferred to new plates containing the nitrogen treatments. All experiments with mutants were pre-cultured on complete ¼ MS media and then transferred to nitrogen treatments. Plants were collected for root measurements and IAA quantitation analysis 10 days after sowing.

Table 2. Nitrogen treatments used in this study.

Treatment	**Media**	**Added nitrogen form**	**K_2SO_4 added to balance**
Control	¼ MS, complete medium	Nothing*	Nothing
K_2SO_4 control	¼ MS, complete medium	Nothing*	4.9 mM of K_2SO_4
Glutamine	¼ MS without nitrogen	5 mM Glutamine	6.2 mM of K_2SO_4
Nitrate	¼ MS without nitrogen	5 mM KNO_3	3.7 mM of K_2SO_4
Low nitrate	¼ MS without nitrogen	10 µM KNO_3	6.2 mM of K_2SO_4
Ammonium	¼ MS without nitrogen	2.5 mM $(NH_4)_2SO_4$	6.2 mM of K_2SO_4

*Complete solution containing 5.15 mM of NH_4NO_3 and 4.69 mM KNO_3

2.2 *Root Measurements*

Seedlings grown on the plates with medium were scanned (Epson Stylus Photo RX700), and the primary root length (PR) and lateral root density (LR) of at least 25 plants per treatment were determined. Measurements of non pre-cultured roots were obtained from the entire root, while on pre-cultured roots only the root grew after transfer to treatments was considered. Images obtained were analyzed using the ImageJ software (http://rsbweb.nih.gov/ij/). For GUS staining, a *DR5::GUS* fusion was used to visualize auxin localization on tissues. For this, roots were incubated in 90% cold acetone for 20 min on ice, then immersed in staining solution (50 mM sodium phosphate buffer (pH 7.0), 0.1% Triton X-100, 0.5 mM potassium ferrocyanide, 0.5mM potassium ferricyanide, and 1 mM X-gluc) and incubated in the dark at 37◦C for 5 h. Stained samples were cleared with 70% ethanol before imaging. Density of emerged, stage I-IV, stage V-VII and total lateral roots were determined.

2.3 *IAA quantitation*

Levels of free IAA, oxIAA, IAA-Asp, IAA-Glc, IAA-Glu, IAA-Ala and Trp were determined by liquid chromatography coupled to a mass spectrometer (LC-MS/MS) as described by Novák et al. (2012), with modifications. Briefly, 10 – 20 mg of root tissue were extracted at 4 °C by adding sodium phosphate buffer (50 mM, pH 7) containing 1% diethyldithiocarbamic acid (DETC) and indolproprionic acid (internal standard). After extraction, samples were filtered with HLB purification columns (Oasis®), conditioned with 1 mL of methanol, followed by 1 mL of water and 0.5 mL of acidic sodium phosphate buffer (pH 2.7). Then, the pH of the supernatant was adjusted to 3 using 1 N HCl. After loading the column with samples, the column was washed with 2 mL of 5% (v/v) methanol. Then the compounds were eluted in 2 mL of 80% methanol (v/v) and dried with nitrogen flow. Dry samples were resuspended in 0.5 mL of methanol and filtered through PTFE filters (0.2 µm, Fisher Scientific, 03–391-4E). For LC-MS/MS quantification, 1 µL of each sample was injected to a chromatograph Agilent 6460 triple quadrupole LC-MS/MS.

2.4 *RNA isolation and quantitative real-time PCR (qRT-PCR)*

Total RNA was extracted from approximately 50 mg of root tissue, using the TRIzol® reagent method (Invitrogen, Carlsbad, CA, USA) and treated with DNAse I (Invitrogen). Total RNA (1.5 µg) was used for first-strand synthesis using SuperScript III reverse transcriptase (Thermo Fisher Scientific). qRT-PCR was performed on a CFX Connect (Bio-Rad Laboratories) using EvaGreen qPCR master mix (Biotium) according to manufacturer's instructions. For *ABCB19* gene expression the following primers were used: forward 5'-AGGATTGACCCGGATGATGCTGAT-3' and reverse 5'-TCGGGTCTTGAAGGGTAAGCGAAA-3'. Transcript levels were normalized against *PP2A* (AT1G69960).

3 Results

Treatments containing 10 µM NO_3^-, 5 mM NH_4^+ and 5 mM glutamine (Gln) as the only N source resulted in shorter PR compared to control and 5 mM NO_3^- in both, pre-cultured (PC) and non pre-cultured (NPC) plants (Fig. 1A). Pre-cultured roots treated with 5 mM NO_3^- were longer than control roots. LR density was reduced (NPC) or not affected (PC) by 5 mM NO_3^-. Lateral root density of PC and NPC plants were strongly reduced by low NO_3^- (10 µM) and NH_4^+ (5 mM) supply, with almost absent LR emergence. Glutamine, in turn, reduced LR density on NPC plants but increased on PC plants compared to control (Fig. 1B). Treatment with 4.9 mM of K_2SO_4 only slightly reduced PR length of PC plants compared to control, not affecting LR density. Considering that root growth limitation caused by low NO_3^- and NH_4^+ was stronger in plants sown directly on plates containing nitrogen treatments (NPC), the next experiments were all performed with pre-culture on basal MS medium (PC). To determine the effects of N treatments on LR development, *DR5::GUS* roots were stained and classified according to their developmental stage: I-IV (LR primordia); stage V-VIII (late LR primordia); and emerged LR (Fig. 2).

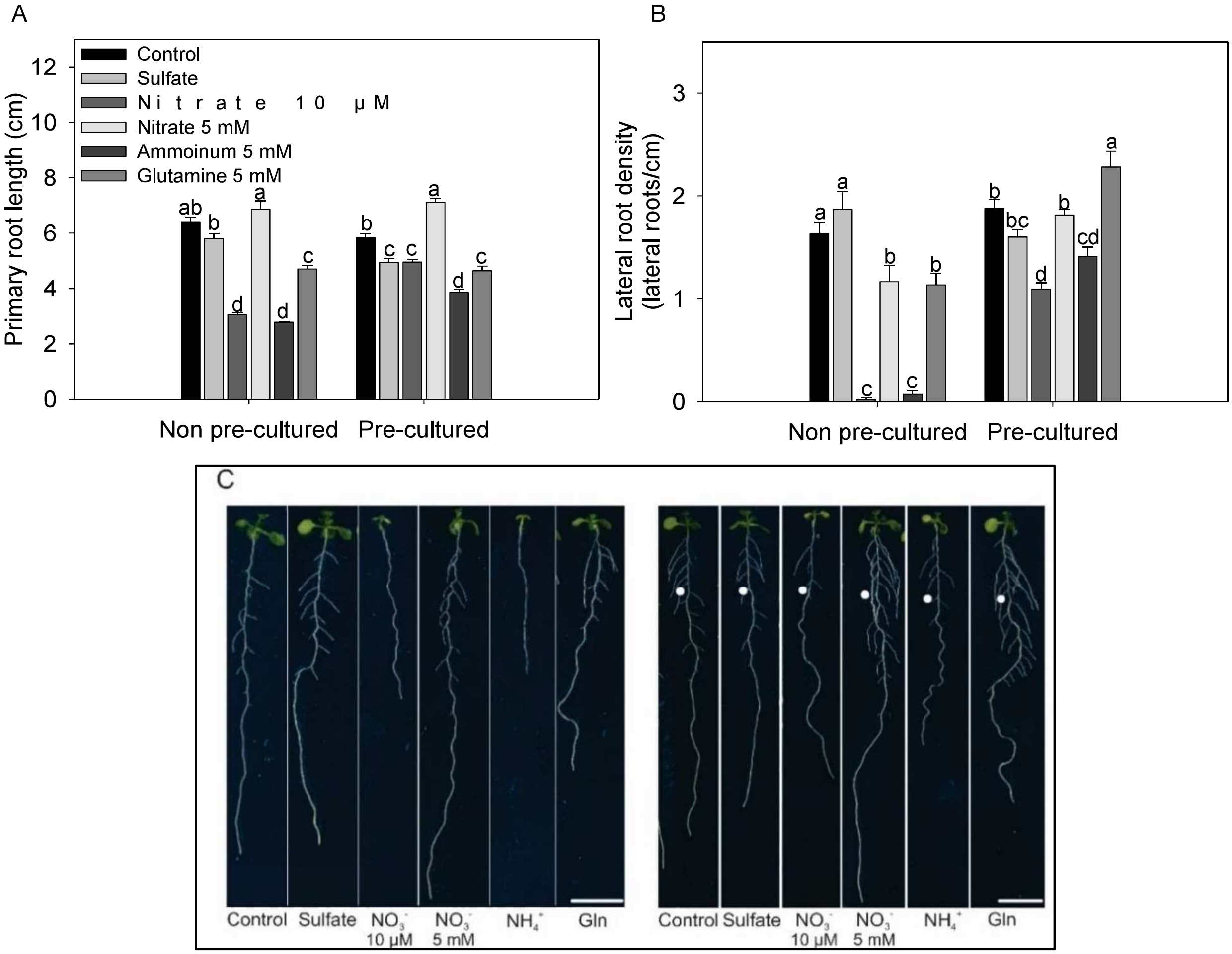

Figure 1. Root parameters of non pre-cultured and pre-cultured of 10-day-old Col-0 plants grown under different nitrogen sources. (A) Primary root length. (B) Lateral root density. (C) Pictures of NPC (left) and PC (right) plants. White bars on the bottom of (C) correspond to 1 cm and white circles indicate root length in the moment of transfer to treatments. Bars represent mean values + SE; n = at least 20 individual biological replicates per treatment. Different letters indicate significant differences by Tukey test (*P* ≤ 0.05).

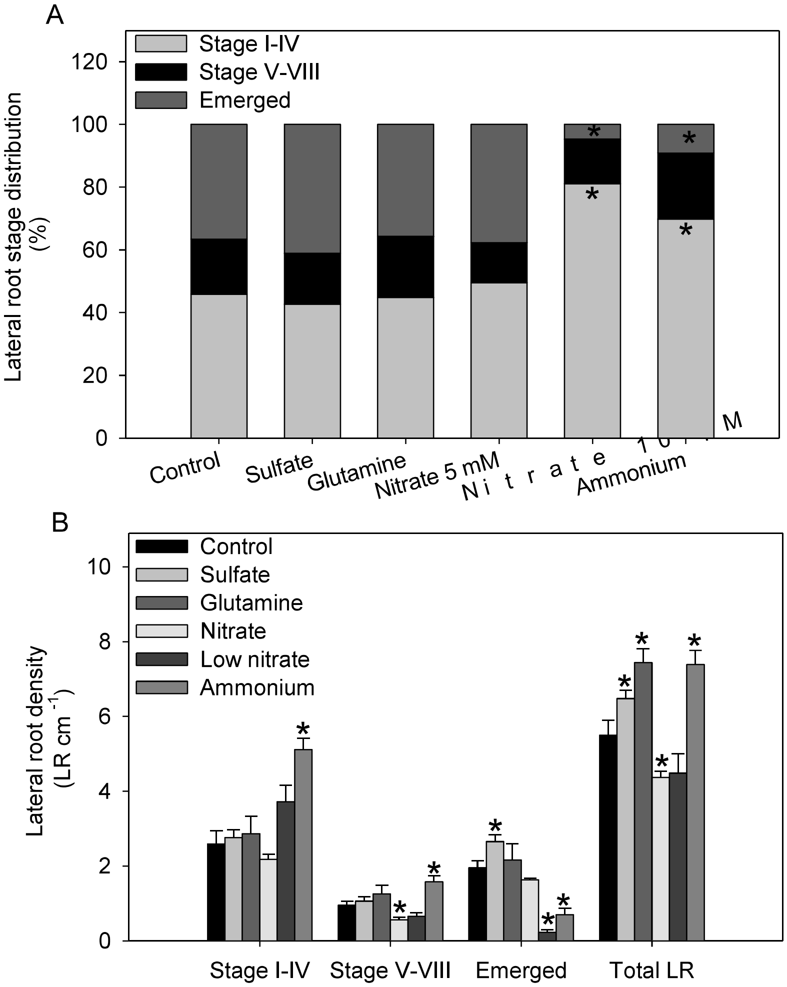

Figure 2. Lateral root distribution, lateral root density and total lateral root density of *DR5::GUS* plants grown under different nitrogen sources. Bars represent mean values + SE; n = at least 10 individual biological replicates per treatment. Asterisks indicate differences between control (¼ MS) and nitrogen treatments by Student's *t*-test ($P \leq 0.05$).

Low NO_3^- and NH_4^+ treatments increased the percentage of LR on stage I-IV and reduced the percentage of emerged LR, suggesting that LR primordia initiation occurred, but LRs failed to emerge (Fig. 2A). Moreover, the increase in LR primordia caused by NH_4^+ resulted in higher total LR (Fig. 2B). Treatment with 5 mM NO_3^- did not affect emerged LR density, but reduced LR primordia, resulting in reduced total LR density (Fig. 2B). Glutamine and sulfate treatments did not alter LR primordia but increased total LR density. In general, PR length of Col-0 plants was increased under NO_3^- in a normal concentration range (5 mM), and reduced under other N treatments, with stronger reduction under NH_4^+ treatment (Fig. 1A).

The ABCB mutant assays showed the same response pattern to N treatments as Col-0, with increased PR length under 5 mM NO_3^-, and reduced PR length under the other treatments (Fig. 3A-H). Double *abcb4 abcb21* (*b4b21*) mutant presented a slight increase in PR length on control plants compared to Col-0 control plants, but no difference was found for the other treatments (Fig. 3A). The single *abcb21* mutant, in turn, presented higher PR length under all treatments, except under low NO_3^-, which was not different from Col-0. PR length of double *b1b19* was reduced under all treatments, except under 5 mM NO_3^-. On the other hand, the PR length of single mutants *abcb1* and *abcb19* was increased by 5 mM NO_3^- compared to Col-0, but not by other N treatments (Fig. 3E). The PR length of double *b6b20* was slightly reduced in control, sulfate and 5 mM NO_3^- plants, while no differences were found in the single *abcb6* mutant compared to Col-0 (Fig. 3G).

The emergence of LRs in Col-0 plants was induced under glutamine supply, but strongly impaired by low NO_3^- (10 µM) and NH_4^+ (Figs. 1 and 2). This response pattern to N was not observed for all ABCB mutants studied here. LR density of *b4b21* plants, for example, was not induced under glutamine treatment, but it was increased in control plants and under sulfate supply. Besides that, LR emergence under NH_4^+ supply was even lower in *b4b21* than in Col-0 (Fig. 3B). In the single *abcb21*, in turn, LR emergence was induced by glutamine, following the Col-0 response pattern. Moreover, LR emergence under 10 µM NO_3^- was lower in *abcb21* than in Col-0 plants (Fig. 3F). LR density in the double *b1b19* was also increased by glutamine, being even higher than in Col-0 plants. In contrast to the results observed in Col-0 plants, LR emergence in the doubles *b1b19* and *b6b20* and in the single *abcb19* mutants was increased under NH_4^+. However, the LR density of single *abcb1* and *abcb6* mutants was not different from Col-0 plants.

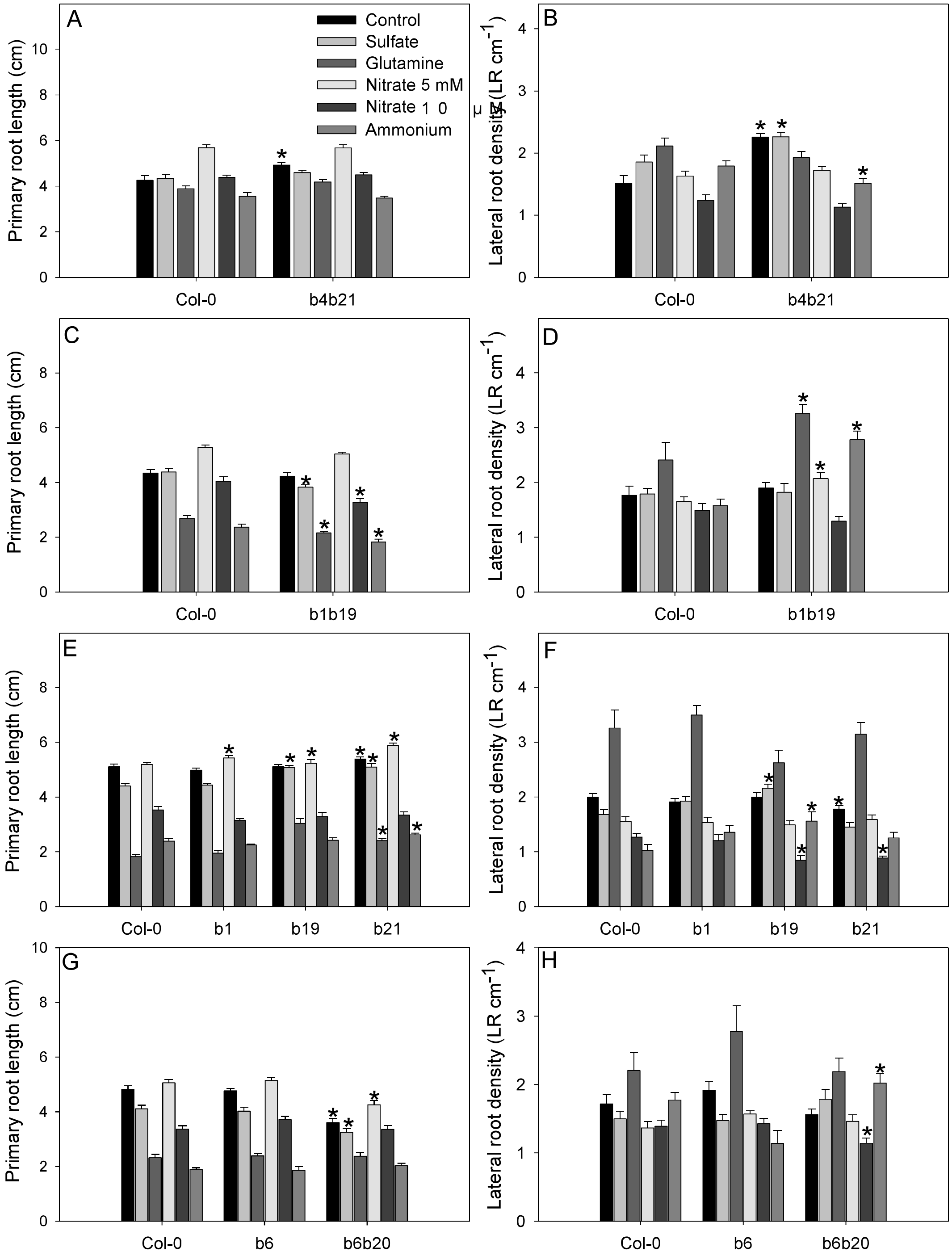

Figure 3. Primary root length and lateral root density of 10-day-old Col-0, *b1b19*, *b4b21*, *b1*, *b19*, *b21, b6*, and *b6b20* plants grown under different nitrogen sources. Bars represent mean values + SE; n = at least 12 individual biological replicates per treatment. Asterisks indicate differences between treated Col-0 and mutants by Student's *t*-test ($P \leq 0.05$).

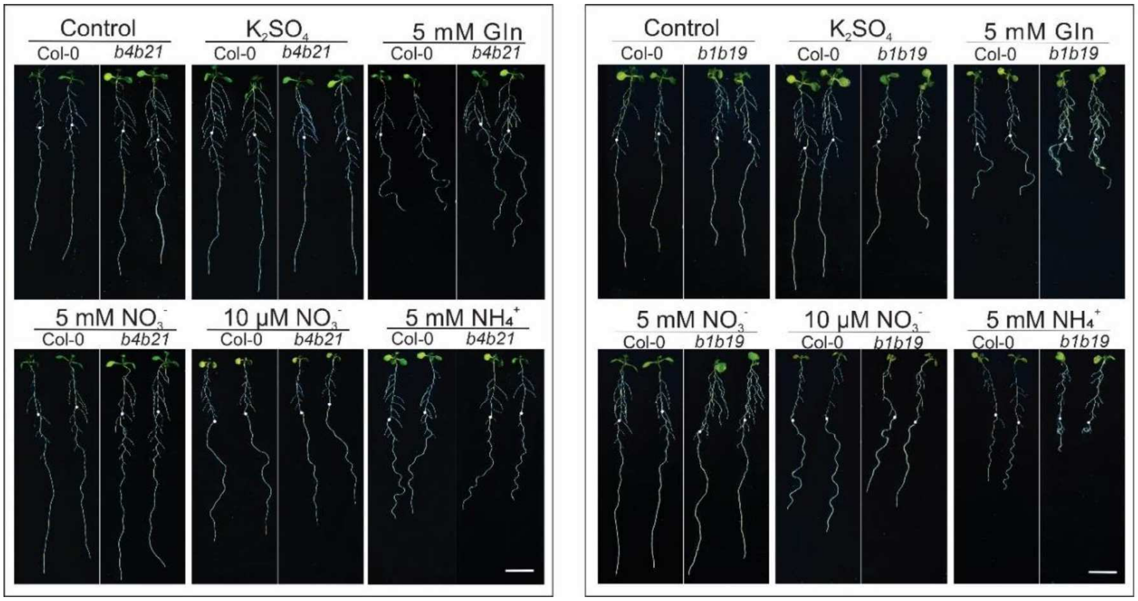

Figure 4. Pictures of 10-day-old Col-0, *b4b21* and *b1b19* plants grown under different nitrogen sources. White bars on the bottom correspond to 1 cm and white circles indicate root length in the moment of transfer to treatments.

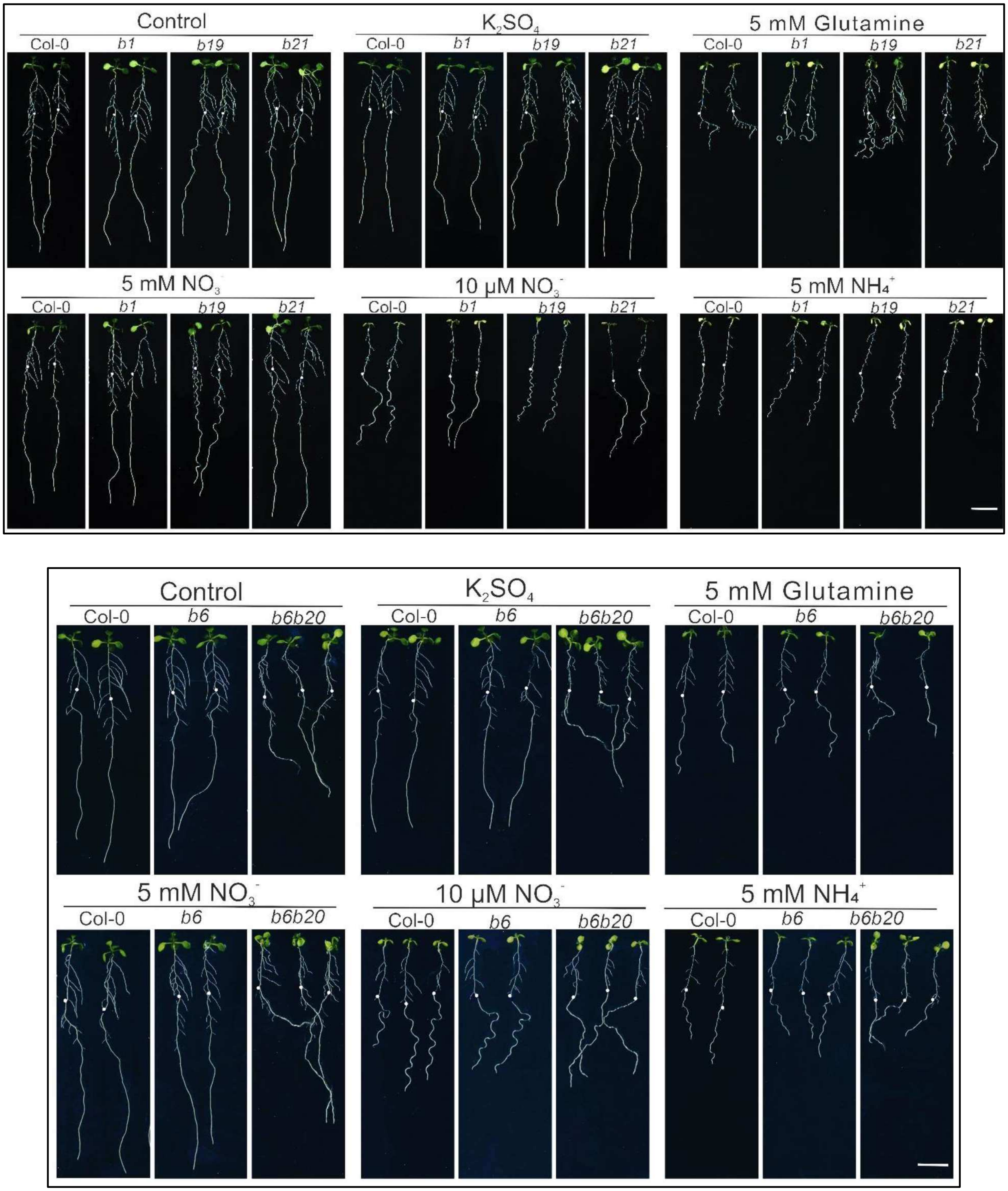

Figure 5. Pictures of 10-day-old Col-0, *abcb1*, *abcb19*, *abcb21*, *abcb6* and *b6b20* plants grown under different nitrogen sources. White bars on the bottom correspond to 1 cm and white circles indicate root length in the moment of transfer to treatments.

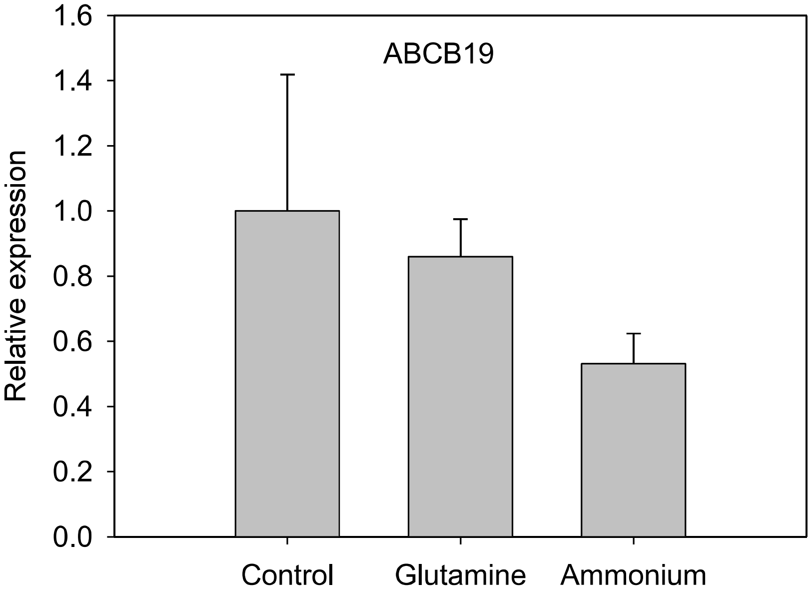

Figure 6. Relative expression of *ABCB19* on Col-0 plants grown under different nitrogen supplies. Bars represent mean values + SE; n = at least 20 individual biological replicates per treatment. Different letters indicate differences among treatments by Tukey test ($P \leq 0.05$).

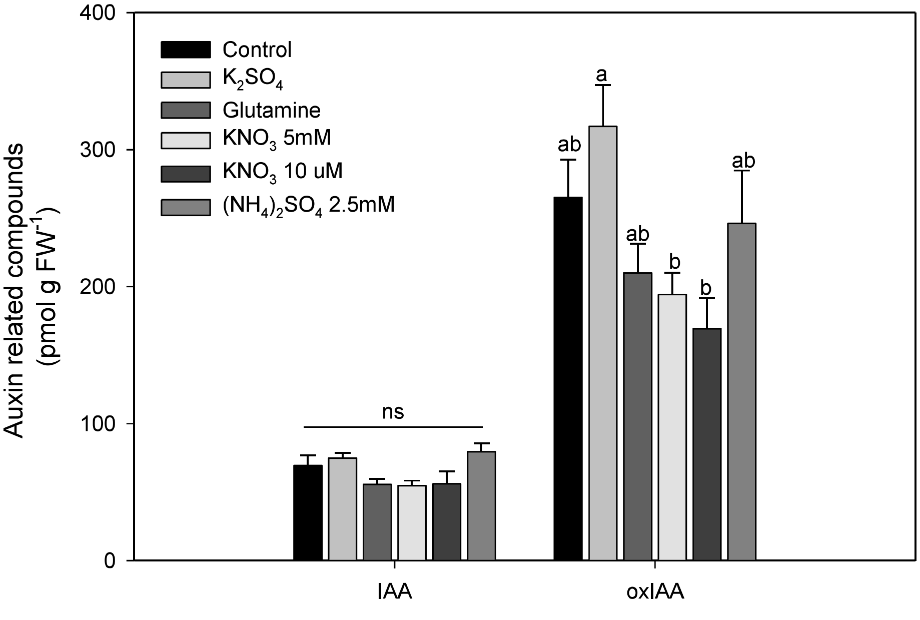

Figure 7. Free IAA and oxIAA levels of Col-0 plants grown under different nitrogen supplies. Bars represent mean values + SE; n = at least 6 individual biological replicates per treatment. Different letters indicate differences among treatments by Tukey test ($P \leq 0.05$).

Another aspect observed was the root bending of plants grown with glutamine as the only N source (Figs. 4 and 5). Although both, Col-0 and ABCB mutants, showed altered root bending, this response was stronger in *b1b19*, *b1*, *b19* and *b6b20*. Moreover, NH_4^+ and NO_3^- treatments resulted in a waiving root phenotype for all genotypes tested, being the waiving phenotype even more evident in *b1b19* plants, which presented root coiling. In contrast to the other mutants, the double *b6b20* showed altered root bending under all treatments (Fig. 5). Considering that the waiving phenotype under glutamine and NH_4^+ treatments were evident for all genotypes and even more evident in the *abcb19* and *abcb1 abcb19* mutants, the relative expression of *ABCB19* was determined in Col-0 plants under these treatments. However, no difference was observed (Fig. 6). During auxin quantitation in Col-0 plants grown under different N sources, free IAA and oxIAA were detected, but no differences were observed for IAA (Fig. 7). Treatments with 10 µM and 5 mM NO_3^- reduced oxIAA level compared to K_2SO_4 treatment, but no difference was found compared to control.

4 Discussion

Auxin is a main factor controlling the primary root growth and the lateral root initiation and elongation (Péret et al., 2009; Olatunji et al., 2017). It was observed here that ammonium as the only N source strongly reduces PR length and LR density, although does not reduce LR primordia initiation. The root growth inhibition caused by NH_4^+ is a result of several events, such as extra- and intra-cellular pH changes, overproduction of ROS, NH_4^+ efflux in the elongation zone and decreased protein glycosylation (Liu and Von Wirén, 2017). Accordingly, Yang et al. (2015) found that ammonium inhibits primary and lateral root growth, which is followed by drastic reduction in auxin response on primary root tips and lateral root primordia and tips. Moreover, they observed that root growth inhibition caused by NH_4^+ was partially rescued by exogenous auxin (Yang et al., 2015). Likewise, wheat plants supplied exclusively with NH_4^+ presented inhibition of root growth together with lower auxin contents (Kudoyarova et al., 1997). On the other hand, Liu et al. (2013) found that ammonium inhibits primary root growth in *Arabidopsis*, which is followed by impaired lateral distribution of auxin in the root apex but not in auxin maximum in the quiescent center and stem cell niche. In addition, Meier et al. (2020) have shown that root responses to ammonium are also regulated by pH changes. Briefly, they found that NH_4^+ uptake by ammonium transporters acidifies the apoplast, accumulating auxin in the vasculature and allowing the protonated IAA to bypass the auxin importers AUX1 and LAX3 towards cortex and epidermic cells. This increase in auxin promotes lateral root primordia initiation, mainly in high-order LRs (Meier et al., 2020). This mechanism might explain the increased LR initiation found here, but not the reduced LR emergence (Fig. 2B), which probably occurred because here only first-order LRs were evaluated and NH_4^+ was applied into the medium instead of locally.

After the uptake of inorganic nitrogen (NO_3^- and NH_4^+), it is converted into glutamine and glutamate through the action of the enzymes glutamine synthetase (GS) and glutamine-oxoglutarate aminotransferase (GOGAT) (Xu et al., 2012). Glutamine can also be absorbed directly from the soil, being used by plants as an effective N source (Forsum et al., 2008). However, studies have shown that Gln impairs LR growth by preventing auxin to accumulate in LR tips in a NRT1.1-dependet manner (Forsum et al., 2008; Krouk et al., 2010; Kan et al., 2015; Hassan et al., 2020). Moreover, in rice seedlings low concentration of Gln (< 1 mM) increases root length, while high concentration (> 1 mM) inhibits root elongation (Kan et al., 2015). Here, the results showed that plants growing with glutamine (Gln) as the sole N source presented shorter PRs but slightly increased LR density (Figs. 1 and 2).

The low nitrate supply (10 µM NO_3^-) used in this study strongly inhibited growth of PR and LR emergence, not affecting LR initiation (Figs. 1 and 2). In contrast, 5 mM nitrate increased PR length and slightly reduced LRs in stage V-VII, not affecting LR emergence. These results might be related to the dual-affinity of the nitrate transporter NRT1.1, which directs root growth towards soil regions with high nitrogen and inhibits root growth into low N regions (Ho et al., 2009; Mounier et al., 2014; Maghiaoui et al., 2020). Moreover, NRT1.1 also controls root growth by directly transports auxin in a concentration-dependent manner, and by regulates auxin biosynthesis (Krouk et al., 2010; Maghiaoui et al., 2020). In other words, NRT1.1 transports auxin under low NO_3^-, preventing auxin accumulation in the LR tip and inhibiting root growth, whereas under high NO_3^-, NRT1.1 transports nitrate, accumulating auxin in the LR tip and inducing root growth (Krouk et a., 2010). In addition to that, Meier at al. (2020) suggest that inhibition of LR formation in N-deficient roots is caused by a pH-dependent suppression of lateral auxin movement rather than by a lack of N for biomass formation. This could explain the fact that low NO_3^- reduced LR emergence but not LR initiation. The fact that IAA levels were not altered here suggests that the growth responses to the nitrogen sources resulted from local auxin redistribution instead of changes in total auxin level (Fig. 7).

Auxin is transported from shoots to roots through the vasculature in a rootward movement to the meristem. After reaching the root meristem, auxin is laterally redirected in a shootward direction, either controlling lateral root initiation or being reintroduced into the vasculature (Grieneisen et al., 2007). ABCBs are thought to act, along with PINs, in both long-distance (rootward) and lateral auxin movement (shootward), also preventing apoplastic auxin reflux (Blakeslee et al., 2007; Geisler et al., 2017). ABCB1 and ABCB19, for example, are efflux carriers with overlapping functions that control the shootward and rootward auxin transport (Geisler et al., 2017). ABCB4 and ABCB21, in turn, are facultative efflux/influx carriers localized respectively in the root epidermis and pericycle, being involved in the shootward and outward auxin transport in roots (Kamimoto et al., 2012). On the other hand, ABCB6 and ABCB20 are auxin efflux carriers involved in rootward auxin movement (Zhang et al., 2018). The results found here indicate that, overall, *abcb* mutants followed the same response pattern to the different nitrogen sources as Col-0. The single *abcb21* and the double *abcb4 abcb21* mutants presented longer primary roots than Col-0, which is in accordance with Jenness et al. (2019), who showed that loss of ABCB21 increases primary root length. On the other hand, here, the double *abcb4 abcb21* presented increased LR density, whereas the single *abcb21* presented reduced or unchanged LR density. Moreover, the doubles *abcb1 abcb19* and *abcb6 abcb20* presented slightly shorter primary roots. Zhang et al. (2018) also observed a slight reduction of PR length in *abcb6 abcb20* plants, but the reduced PR length is not a characteristic well documented in *abcb1 abcb19* (Lewis et al., 2007). Although loss-of-function of ABCB19 has been shown to reduce or even arrests LR elongation (Wu et al., 2007), it was not observed here. Interestingly, the doubles, but not the singles, *abcb1 abcb19* and *abcb6 abcb20* mutants presented increased LR density under NH_4^+ supply (Fig. 3), indicating that the lateral root inhibition caused by ammonium might be dependent on auxin transport by these transporters.

Considering that lateral auxin redistribution in the root apex is essential for gravitropic responses (Geisler et al., 2014; Singh et al., 2017), the waiving phenotype observed under NH_4^+, low NO_3^- and glutamine supply can be related to disorders in lateral auxin transport. This is in accordance with studies showing that ammonium impairs root bending and delays lateral auxin redistribution in roots (Zou et al., 2012; Liu et al., 2013). Additionally, studies have shown that nitrate deficiency impairs root bending and causes root coiling through a mechanism involving nitrate sensing by NRT1.1 and auxin transport by PIN2 and AUX1 (Chai et al., 2020). The fact that ABCB1, ABCB4 and ABCB19 are not found in the columella cells suggests that auxin transport through ABCBs is unlikely to control gravitropic responses (Geisler et al., 2017). In fact, waiving phenotype of single mutants *abcb1, abcb4, abcb19* and double mutants *abcb4 abcb21* and *abcb1 abcb19* was only found under NH_4^+, low NO_3^- and Gln, but not under control conditions (Figs. 4 and 5). These results suggest that the waiving phenotype occurs, at least in part, independently of these auxin transporters. However, the fact that root waiving was stronger in the double *abcb1 abcb19* under NH_4^+ indicates that the loss-of-function of both ABCB1 and ABCB19 aggravates the root waiving caused by ammonium. Besides that, the fact that *ABCB19* expression was not altered under ammonium and glutamine treatments suggests that the waiving phenotype might be related to post-transcriptional events. Although the role of ABCB6 and ABCB20 auxin transporters has been recently characterized during shoot growth and development (Zhang et al., 2018), their action during root growth and root bending, as well as their relation to nutrient availability remains unclear. The results found here showed that the double mutant *abcb6 abcb20*, but not the single *abcb6*, presented altered root bending under all treatments, indicating that the auxin stream dependent of ABCB6 and ABCB20 is required for gravitropic responses.

Besides that, root responses to ammonium supply seem to be dependent of auxin transporters PIN2, AUX1 and LAX3 (Liu et al., 2013; Meier et al., 2020). ABCB1/19 are known for their role in polar auxin transport and are also thought to prevent auxin reflux in root apoplast (Geisler et al., 2017). In its turn, ABCB6/20 loss of function results in slightly reduced root length, but their effect on LR emergence remains unclear (Zhang et al., 2018). Our results for the double *b1b19* and *b6b20* mutants indicate that the lateral root emergence impairment caused by ammonium might be, at least partially, related to these ABCB transporters. In addition, further analysis of root bending responses, gene expression and auxin quantitation are required to better understand the relation of ABCB transporter and root responses to nitrogen.

5 Conclusions

In summary, the results found here confirm that NO_3^- functions as a signal directing root growth to nitrate-rich regions. Besides that, LR emergence inhibition caused by ammonium is, at least partially, dependent of auxin transport by ABCB1/ABCB19 and ABCB6/ABCB20. Moreover, the results indicate a probable role for ABCB1/ABCB19 in root bending disturbance caused by glutamine and ammonium and for ABCB6/ABCB20, irrespective to nitrogen sources.

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GENERAL CONCLUSIONS

In summary, this study shows that the reduction of GAs levels by PAC inhibits shoot and root growth of radish plants, but the inhibition is stronger in shoots. Besides that, radish plants respond distinctly to shade caused by neighbors canopy (high-density - HD) and to low irradiance caused by black nylon nettings (~400 µmol m^{-2} s^{-1}). HD of plants only affected leaf area and specific leaf area, probably increasing leaf expansion independently of GAs. Moreover, PAC treatment does not prevent shade avoidance responses under the HD of neighbors investigated here. On the other hand, radish responses to low irradiance are regulated by gibberellins. Being that low irradiance modulates gibberellin and auxin metabolism, which in turn increases the expression of the cell-wall remodeling genes *EXP* and *XTH* in petiole and leaf lamina. This upregulation of *EXP* and *XTH* results in increased petiole length and lamina area, reflecting in reduced root growth. These responses to low irradiance were also observed upon GA_3 treatment, with GA_3 regulating mainly petiole growth. PAC treatment, in turn, inhibited expression of *EXP* and *XTH*, resulting in reduced growth of shoots relative to roots. These results indicate that GAs regulate biomass allocation in radish in response to irradiance and that inhibition of GAs biosynthesis under low light intensity leads to changes in biomass allocation that contribute to the nutritional quality of radish tuber with no yield reduction. Concerning the role of auxin transport during root growth responses of *Arabidopsis thaliana* seedlings to nitrogen availability, this study shows that these responses are a result of auxin redistribution rather than global changes in auxin levels in roots. Accordingly, inhibition of lateral roots emergence by ammonium supply is, at least partially, dependent of auxin transport by ABCB1/ABCB19 and ABCB6/ABCB20. Additionally, ABCB1/ABCB19 are involved in root bending observed under glutamine and ammonium supply, whereas ABCB6/ABCB20 modulate root bending irrespective to nitrogen sources.