RAFAEL SOARES POZZI MALHEIROS

PHYSIOLOGICAL, HORMONAL AND METABOLIC ALTERATIONS CAUSED BY SELENIUM IN RICE SEEDLINGS

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

Adviser: Dimas Mendes Ribeiro

Co-adviser: Agustin Zsögön

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APPROVED: July 30th, 2019.

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(Author)

Dimas Mendes Ribeiro
(Adviser)

To my mother Severina, my father Jailton, and my sisters

Jéssica and Anna Victória, for the support and love.

I dedicate.

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ABSTRACT

MALHEIROS, Rafael Soares Pozzi, D.Sc., Universidade Federal de Viçosa, July, 2019. **Physiological, hormonal and metabolic alterations caused by selenium in rice seedlings**. Adviser: Dimas Mendes Ribeiro. Co-adviser: Agustin Zsögön.

Selenium (Se) can alter the growth of some plant species and causes changes in levels of plant hormones, such as auxin and ethylene. However, the impact of Se supply on relationships between hormones biosynthesis and primary metabolism during growth of rice (*Oryza sativa* L.) seedlings is poorly understood. Thus, the hypothesis that Se induces changes in the interactions between auxin and ethylene to modulate primary metabolism and growth of rice seedlings was investigated. The application of selenite did not affect the of growth shoot of seedlings, but promoted the elongation of the primary root with reduction in the number and length of the lateral roots. There was a decrease in the concentrations of ethylene, 1-aminocyclopropane-1-carboxylic acid (ACC) and indole-3-acetic acid (IAA) in roots of rice seedlings treated with selenite as compared with the control. Selenite led to decreased expression of genes associated with the biosynthesis of auxin and ethylene, concomitantly with reduced production of these hormones by the roots. Moreover, selenite decreased the abundance of transcripts encoding auxin transport proteins. Treatment with IAA overrode the repressive effect of selenite on lateral root growth. The ethylene synthesis inhibitor L-α-(2-aminoethoxyvinyl)-glycine (AVG) increased elongation of primary root, whereas the ethylene precursor ACC resulted in the opposite effect. Moreover, soluble sugars accumulate in roots under selenite treatment. Together, these findings suggest that selenite affects primary and lateral root development by blocking ethylene and auxin biosynthesis, respectively. Experiments with organic Se showed that seleno-L-methionine (SeMet) at low concentration increased concentrations of hydrogen peroxide and superoxide anion production, inhibiting auxin biosynthesis and increasing ethylene production in both shoot and root. The effect of SeMet on seedlings was mediated by the inhibition of the abundance of transcripts encoding auxin transport and cell expansion proteins. Moreover, SeMet led to increased respiration, which was positively correlated with organic acids consumption, but negatively with sugars consumption, thereby decreasing seedling growth. In contrast with SeMet treatment, L-methionine did not affect reactive oxygen species production, hormone biosynthesis and growth of seedlings, indicating an exclusive Se effect. Treatment with 1,4-diazabicyclooctane (DABCO), a singlet oxygen scavenger, overrode the repressive effect of SeMet in seedling

growth. It is concluded that Se regulates physiological and metabolic processes in rice seedlings are dependent on the form of Se supplied.

Keywords: Cell expansion. Primary metabolism. Hormonal regulation. *Oryza sativa* L.

RESUMO

MALHEIROS, Rafael Soares Pozzi, D.Sc., Universidade Federal de Viçosa, julho de 2019. **Alterações fisiológicas, hormonais e metabólicas causadas pelo selênio em plântulas de arroz**. Orientador: Dimas Mendes Ribeiro. Coorientador: Agustin Zsögön.

O selênio (Se) pode alterar o crescimento de algumas espécies de plantas e causar modificações nos níveis de hormônios vegetais, tais como auxina e etileno. No entanto, o impacto do fornecimento de Se nas relações entre a biossíntese de hormônios e o metabolismo primário durante o crescimento de plântulas de arroz (*Oryza sativa* L.) é pouco compreendido. Assim, a hipótese de que o Se induz alterações nas interações entre auxina e etileno para modular o metabolismo primário e o crescimento de plântulas de arroz foi investigado. A aplicação de selenito não afetou o crescimento da parte aérea das plântulas, mas promoveu o alongamento da raiz primária com redução no número e comprimento das raízes laterais. Um decréscimo nas concentrações de etileno, ácido 1-carboxílico-1-aminociclopropano (ACC) e ácido indol-3-acético (AIA) nas raízes das tratadas com selenito foi observado em comparação ao controle. O selenito levou à diminuição da expressão de genes associados à biossíntese de auxina e etileno, concomitantemente à redução da produção desses hormônios pelas raízes. Além disso, o selenito diminuiu a abundância de transcritos que codificam proteínas transportadoras de auxina. O tratamento com AIA anulou o efeito repressivo do selenito no crescimento radicular lateral. O inibidor da síntese de etileno L-α-(2-aminoetoxivinil)-glicina (AVG) aumentou o alongamento da raiz primária, enquanto o precursor de etileno ACC resultou no efeito oposto. Além disso, os açúcares solúveis acumularam nas raízes submetidas ao tratamento com selenito. Juntos, esses resultados sugerem que o selenito afeta o desenvolvimento da raiz primária e lateral, bloqueando a biossíntese de etileno e auxina, respectivamente. Os experimentos com Se orgânico mostraram que a seleno-L-metionina (SeMet) em baixa concentração aumentou as concentrações de peróxido de hidrogênio e a produção de ânion superóxido, inibindo a biossíntese de auxinas e aumentando a produção de etileno na parte aérea e raiz. O efeito da SeMet nas plântulas foi mediado pela inibição da abundância de transcritos que codificam proteínas de transporte de auxinas e expansão celular. Além disso, a SeMet levou ao aumento da respiração, que foi positivamente correlacionada com o consumo de ácidos orgânicos, mas negativamente com o consumo de açúcares, diminuindo assim o crescimento das plântulas.

Em contraste com o tratamento com SeMet, a L-metionina não afetou a produção de espécies reativas de oxigênio, a biossíntese de hormônios e o crescimento de plântulas, indicando um efeito exclusivo de Se. O tratamento com 1,4-diazabiciclooctano (DABCO), um seqüestrador de oxigênio singleto, reverteu o efeito repressivo da SeMet no crescimento de plântulas. Portanto, a capacidade do Se em regular os processos fisiológicos e metabólicos em plântulas de arroz são dependentes da forma de Se fornecido.

Palavras-chave: Expansão celular. Metabolismo primário. Regulação Hormonal. *Oryza sativa* L.

SUMMARY

GENERAL INTRODUCTION

Selenium (Se) is a metalloid, belonging to the VIA group of the periodic table, essential for humans and animals, since it plays an important role in the antioxidant function, improved immune responses and protection against cancer (Combs, 2001; Rayman, 2012; Stranges et al., 2006; Zhong et al., 2016). In plants, Se is classified as a non-essential nutrient, but several studies report its beneficial effects on plant growth (Malik et al., 2011; Xue et al., 2001; Xue and Hartikainen, 2008). In addition, Se can act as an inducer of resistance against the effects of different abiotic stresses (Habibi, 2013; Hasanuzzaman et al., 2011; Saidi et al., 2014; Zhao et al., 2013). In this context, Se at low concentrations can eliminate excess free radicals, delay senescence, increase nitrogen assimilation and concentration of carbohydrate in plants (Hartikainen et al., 2000; Lin et al., 2012; Pilon-Smits and Quinn, 2010).

Concentrations of sodium selenite ≤ 20 μmol L^{-1} promote an increase in growth rate, yield, photosynthetic efficiency and starch content in *Landoltia punctata* L. (Zhong et al., 2016). In addition, selenite at concentrations between 5 and 10 μmol L^{-1} can provide a higher root volume, leaf numbers and increase in chlorophyll content in *Solanum lycopersicum* L. (Mozafariyan et al., 2017). On the other hand, Se at high concentrations can cause toxicity to plants by altering primary metabolism and concentrations of macro and micronutrients (Dimkovikj and Van Hoewyk, 2014; White, 2015; Ribeiro et al., 2016). Moreover, the toxic effect promoted by high concentrations of Se is also associated with the induction of oxidative stress (Van Hoewyk, 2013). Several studies have shown the occurrence of an increase in the production of reactive oxygen species in both shoot and roots of plants exposed to high concentrations of Se (Chen et al., 2014; Dimkovikj and Van Hoewyk, 2014; Lehotai et al., 2012).

The availability of Se for plants is dependent on soil geochemical parameters, for example, pH and redox condition (Winkel et al., 2012). Thus, Se can be found in the natural environment as elemental selenium (Se^0), selenide (Se_2), thioselenate ($HSeO_3^2$), selenate (SeO_4^{-2}) and selenite (SeO_3^{-2}; $HSeO_3^-$; H_2SeO_3) (Sors et al., 2005; White and Broadley, 2009). However, the forms that are predominantly available in soils for plants are selenate and selenite (Zhang et al., 2014a). Selenate is absorbed by the roots through the high-affinity sulphate transporters, while the selenite is absorbed by the roots through phosphate

transporters (Schiavon et al., 2016). Plants can also absorb organic compounds of Se, especially in the form of seleno-amino acids (Lima et al., 2018). Once absorbed, selenate and selenite are metabolized through the sulphate assimilation pathway to form seleno-amino acids (El Mehdawi et al., 2014). The seleno-amino acids (selenomethionine and selenocysteine) can be misincorporated into proteins (**Figure 1**), replacing methionine and cysteine, which leads to changes in the structure and function of these proteins (Freeman et al., 2010). In this context, high concentrations of seleno-amino acids can be toxic to cellular metabolism, since the alteration in the protein structure can inhibit the activity of these proteins (Stadtman, 1996).

Plants differ greatly in their ability to accumulate Se in their tissues (White, 2015). Plants capable of accumulating Se at high concentrations in the tissue (> 1000 mg Se kg^{1} dry mass), without damage to growth and metabolism, are known as hyperaccumulators and include species of the genus *Astragalus*, *Morinda*, *Neptunia* and *Xylorhiza* (Quinn et al., 2011; White, 2015). Non-accumulating plants, which are the majority of species, present Se concentrations below 25 mg Se kg^{-1} of dry mass in their tissues and do not tolerate high concentrations of Se in the growing environment (White et al., 2004).

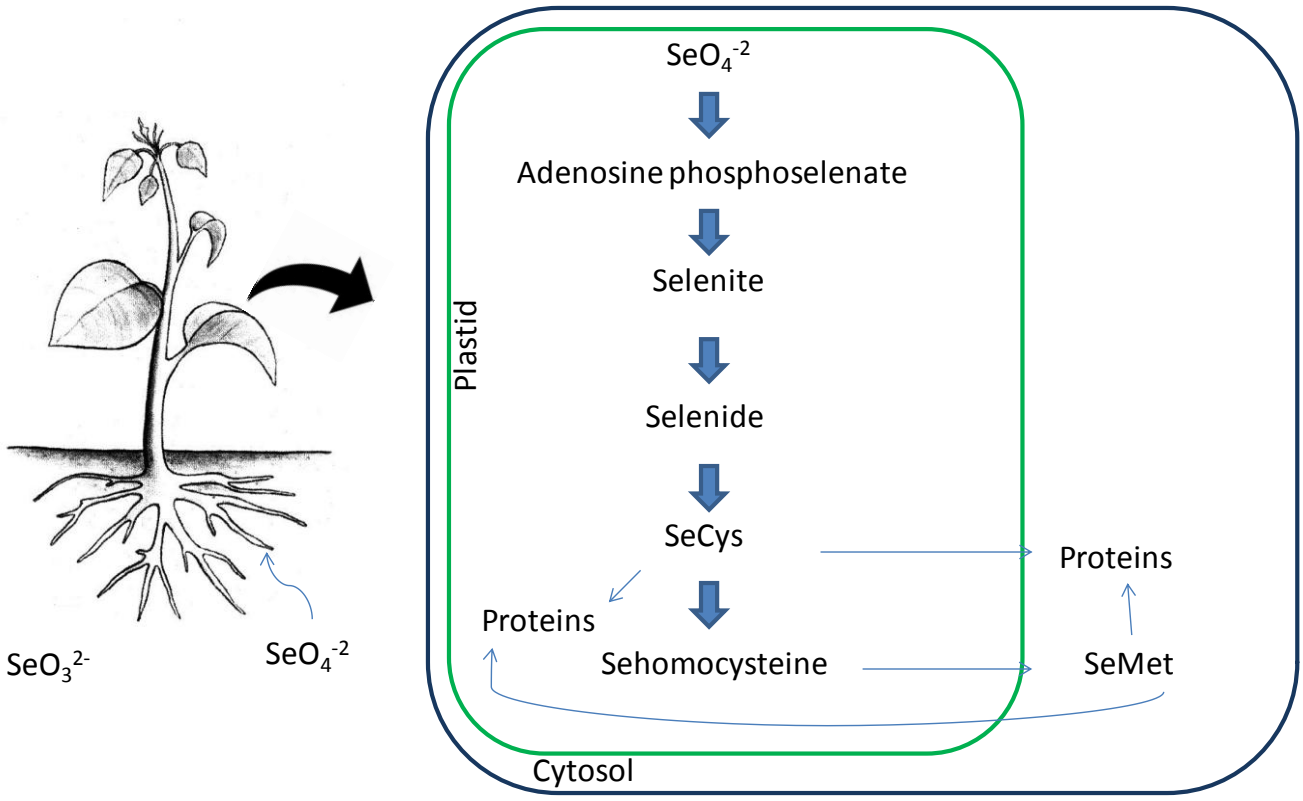

Figure 1. Schematic model of Se assimilation and metabolism in plant mesophyll cells.

Se can also alter the concentrations of hormones in plants (Tamaoki et al., 2008). Khan et al. (2015) demonstrated that the application of Se reduced the levels of ethylene in plants of *Triticum aestivum*. Similar to the effects on *Triticum aestivum*, Se inhibited the production of ethylene and the expression of genes from its biosynthesis pathway in fruits of *Solanum lycopersicum* (Zhu et al., 2017). On the other hand, selenomethionine is known to compete with methionine on the ethylene biosynthesis pathway, increasing ethylene production (Konze et al., 1978). Other plant hormones such as auxin, cytokinin, jasmonic acid, and salicylic acid also have their concentrations modified by the presence of Se in the culture medium (Jia et al., 2018; Lehotai et al., 2012; Tamaoki et al., 2008). Selenite concentrations (20 to 40 μM) caused a decrease in auxin biosynthesis as well as increased cytokinin and ethylene production in roots of *Arabidopsis thaliana* (Lehotai et al., 2012). The increase in auxin biosynthesis was also observed in roots of *Nicotiana tabacum* L. submitted to selenium treatment (Jia et al., 2018). These changes in hormone concentrations, especially with respect to ethylene and auxin, may promote modifications in the growth pattern of plants (Santisree et al., 2011).

Rice (*Oryza sativa* L.) mainly absorbs Se in the form of selenite, which is the predominant form found in soils for rice cultivation (Pilon-Smits et al., 2009; Zhang et al., 2006). Effects of low concentrations (2 and 6 mg L^{-1}) of selenite on the development of rice seedlings were described by Wang et al. (2012). These authors verified an increase in the carbohydrates metabolism and seedlings growth. On the other hand, relatively high concentrations of selenate (0.75 and 1.50 mM) promoted inhibition of growth and excessive accumulation of reactive oxygen species in rice plants (Mostofa et al., 2017). However, the pathways of hormone regulation induced by Se are still poorly understood in rice. In addition, in the literature, there are studies that demonstrate the effect of inorganic compounds of Se (selenate and selenite) on the growth and metabolism of rice (Kumar et al., 2016; Lin et al., 2012; Wang et al., 2012). However, little is known about the effects of direct application of organic compounds of Se, in particular, selenomethionine, on growth, hormone biosynthesis and primary metabolism of rice seedling. Thus, physiological, metabolic and molecular studies using organic and inorganic forms of Se become necessary for a better understanding of the effects of Se on the development of rice seedlings. Thus, the present study aimed to analyze the effect of the application of an inorganic form (selenite) and an organic form (selenomethionine) of Se on the growth of rice seedlings (japonica subspecies), examining the integrative actions between Se, ethylene and auxin in

the control of primary metabolism of rice seedlings. To achieve these objectives, this thesis was organized into two independent chapters. In Chapter 1 was investigated the effect of selenite on rice seedling growth, while in Chapter 2 was examined the action of SeMet on the growth of rice seedlings.

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CHAPTER 1

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Selenium downregulates auxin and ethylene biosynthesis in rice seedlings to modify primary metabolism and root architecture

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Main conclusion **Selenium modulates the formation of primary and lateral roots through alterations in auxin and ethylene, leading to new patterns of root architecture in rice seedlings.**

Abstract

Selenium (Se) at low concentrations can control root growth through interaction with hormone biosynthesis. Auxin and ethylene have been shown to control the root architecture, with most of the information obtained from the eudicots such *Arabidopsis* and *Nicotiana tabacum*. Here, we presented the effects of Se on auxin and ethylene pathways and examined their impact on primary metabolism and root system architecture in rice (*Oryza sativa* L.) seedlings. Se treatment increased elongation of primary root, but decreased the number and length of lateral roots. Se led to decreased expression of genes associated with the

biosynthesis of auxin and ethylene, concomitantly with reduced production these hormones by the roots. Moreover, Se decreased the abundance of transcripts encoding auxin transport proteins. Indole-3-acetic acid (IAA) treatment overrode the repressive effect of Se on lateral root growth. The ethylene synthesis inhibitor L-α-(2-aminoethoxyvinyl)-glycine (AVG) increased elongation of primary root, whereas the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) resulted in the opposite effect. Moreover, soluble sugars accumulate in roots of rice seedlings under Se treatment. Thus, Se modulates the formation of primary and lateral roots through alterations in auxin and ethylene, leading to new patterns of root architecture in rice seedlings.

Keywords Cell expansion · *Oryza sativa* L. · Root development · Sodium selenite

Abbreviations

ACC 1-Aminocyclopropane-1-carboxylic acid
AVG L-α-(2-aminoethoxyvinyl)-glycine
IAA Indole-3-acetic acid
1-MCP 1-Methylcyclopropene
Se Selenium
TIBA 2,3,5-Triiodobenzoic acid

1 Introduction

Root development is modulated by the action of hormones, among which auxin and ethylene are central regulators (Muday et al. 2012; Hu et al. 2017). Biosynthesis of both auxin and ethylene is influenced by selenium (Se) (Tamaoki et al. 2008; Jia et al. 2018). Se at low concentration can stimulate primary root elongation and the number of lateral roots by triggering auxin biosynthesis and transport in tobacco (*Nicotiana tabacum*) (Jia et al. 2018). On the other hand, application of 10 μM Se reduces primary root growth, but increases the number of lateral roots in *Arabidopsis thaliana* (Lehotai et al. 2012). There is also conflicting evidence with respect to the effect of Se on expression of genes encoding auxin-responsive proteins. For example, Se increases the expression of genes associated with auxin biosynthesis and transport (Jia et al. 2018), but decreases the expression of several auxin-related genes in *Arabidopsis thaliana* (Van Hoewyk et al. 2008). Auxin might also control

growth of primary and lateral roots through interaction with the ethylene biosynthesis and signalling pathways in eudicots (Ruzicka et al. 2007; Van de Poel et al. 2015). The effects of the coaction between auxin and ethylene on development of primary root are fundamentally different from those of lateral root formation. In this regard, ethylene inhibits growth of the primary root by increasing auxin biosynthesis in the root apex, followed by subsequent auxin transport to the root elongation zone (Ruzicka et al. 2007). On the other hand, ethylene stimulates rootward auxin transport, which inhibits formation of auxin maxima that allow lateral root initiation (Lewis et al. 2011). The importance of auxin and ethylene for the control of root growth has been highlighted by the use of mutants altered in auxin transport or defective in auxin and ethylene biosynthesis and signaling, with most of the information gained from the model plant *Arabidopsis thaliana* (Negi et al. 2008; Lewis et al. 2011; Hu et al. 2017). Whether Se exerts its effects on root growth via alterations in the coordination between auxin and ethylene is not clear, particularly for monocots.

Rice (*Oryza sativa* L.) is a major food crop worldwide and is a model plant for monocot species (Li et al. 2018). The root system of rice consists of primary root, embryonic crown roots, postembryonic crown roots, small lateral roots and large lateral roots (Coudert et al. 2010). Convergence of auxin and ethylene in the control of root growth in rice occurs through members of the *YUCCA* gene family, which catalyze the conversion of indole-3-pyruvic acid to indole-3-acetic acid (IAA) (Zhao et al. 2018). Indeed, ethylene acts as a positive regulator of the expression of *YUCCA8/REIN7*, resulting in accumulation of free auxin and inhibition of primary root elongation of rice seedlings (Qin et al. 2017). Interestingly, Se at low concentration increases expression of *YUCCA* genes as well as the expression of the auxin efflux carrier *PIN FORMED* (*PIN*) gene family members in tobacco roots (Jia et al. 2018). In rice, overexpression of *PIN1* increases lateral root number (Luschnig et al. 1998; Xu et al. 2005). Moreover, genes involved in auxin signaling, such as those of the *auxin/indole-3-acetic acid* (*Aux/IAA*) and *auxin response factors* (*ARF*) gene families, impact morphological aspects of root formation (Inukai et al. 2005; Kitomi et al. 2012). For instance, gain-of-function mutation in *IAA11* has a regulatory effect in lateral root development in rice (Zhu et al. 2012), whereas the knockout of ARF12 inhibits primary root elongation in rice (Qi et al. 2012). In addition, auxin induces changes in expression of genes coding for expansin (*EXP*) and endoglucanases (*GLU*), which loosen cell walls to promote turgor-driven cell expansion (Cosgrove 2016). Consistent with this, auxins have been shown to control lateral root formation in rice through modulation of expression of *GLU5* and *GLU14* (Yoshida et al.

2006). Se can also upregulate *EXP* expression in *Arabidopsis* roots (Van Hoewyk et al. 2008). Taken together, these studies suggest that a balance between auxin and ethylene plays a significant role during root system growth in rice and that Se could in some way affect the auxin-ethylene balance.

In addition to auxin and ethylene, root growth is also sensitive to carbohydrates concentrations (Mudgil et al. 2016). Sugars serves as substrate for metabolism and as signaling molecules in multiple pathways, including those involved in root response to auxin (Mishra et al. 2009; Ruan 2014). In this context, it has been previously suggested that glucose affects the expression of genes related with auxin biosynthesis and transport (Sairanen et al. 2012; Yuan et al. 2013). Furthermore, Se has been proposed to increase concentration of glucose and amino acids in roots of *Brassica napus* (Dimkovikj and Van Hoewyk 2014). Thus, modulation of carbon metabolism is likely an important aspect of the root growth response to Se.

In this study, we investigated the hypothesis that Se induces changes in the interactions between auxin and ethylene to mediate control of primary and lateral root development in rice. In addition, we examined the ability of Se to modulate primary metabolism and root development.

2 Materials and methods

2.1 *Plant material and general conditions*

Seeds of rice (*O. sativa* L. ssp *japonica* cv Oochikara) were surface sterilized with 0.5% NaOCl for 10 min, washed with deionized water, and then germinated on filter paper in a growth chamber (Forma Scientific Inc, Ohio, USA), at 30 ^{o}C in the dark. Seeds with a radicle 2 cm long were transplanted to plastic pots (20 seedlings per pot) containing 2 L of half-strength Hoagland's solution (supplemented with 10 µM sodium selenite, 0.1 µM L-α-(2-aminoethoxyvinyl)-glycine (AVG), 5 µM 1-aminocyclopropane-1-carboxylic acid (ACC) and/or 10 µM IAA, depending on treatment). The solution was renewed every day. The pots were maintained in a growth chamber under a 16/8h day/night cycle (30/24 ^{o}C) with 60/75% relative humidity and 200 µmol m^{-2} s^{-1} light intensity. Rice seedlings were harvested after five days incubation in nutrient solution at the end of the photoperiod and thoroughly rinsed with deionized water.

2.2 *Analysis of root system*

At the end of the treatment period, the root system of rice seedlings was immediately stored in 30% (v/v) ethanol. At this stage, the root system was composed of a primary root with emerged lateral roots and two to three emerging crown roots that had no visible lateral roots. The crown roots were removed from the root system and the primary roots with emerged lateral roots were scanned using a flatbed scanner and analyzed using the image-processing software WinRhizo Pro (Regent Instruments Inc; Québec, Canada) as described by Zhu et al. (2005).

Image capture, documentation and analysis of cell size in the root elongation zone was performed as described by De Araújo et al. (2015).

2.3 *Measurements of IAA, ACC and ethylene*

Primary roots with emerged lateral roots were excised at the root-shoot junction, washed with deionized water, paper-dried and frozen in liquid nitrogen. Concentration of IAA and ACC in roots were quantified as described by Müller and Munné-Bosch (2011). For the ethylene assays, roots of rice seedlings were incubated in Erlenmeyer flasks capped with a rubber septum for 4 h. Afterwards, a gas sample 1 ml was taken from the headspace of the Erlenmeyer flask with a tight syringe and ethylene concentration was quantified by gas chromatography with a flame ionization detector as described by Ribeiro et al. (2010).

2.4 *Measurements of metabolites and selenium*

Shoots of rice seedlings were separated from primary roots with emerged lateral roots using a scalpel, frozen in liquid nitrogen and stored at -80^{o}C, until analysis. The analysis of metabolites was performed using 50 mg of freeze-dried samples of shoot and root. Sucrose, fructose, glucose, starch, total amino acids and total protein were extracted and quantified as described by Cross et al. (2006). Assays were performed in 96 well microplates and the absorbance was measured in a microplate reader (VersaMax, Molecular Devices, Sunny Valle, EUA).

To quantify the concentrations of Se, shoot and root tissues of rice seedlings were dried at 50 ^{o}C for 48 h and digested in nitric acid (El Mehdawi et al. 2018). The samples were

analyzed for Se concentrations using an inductively coupled plasma optical emission spectrometer (ICP-OES, Perkin-Elmer Optima 3000XL) as described by Cappa et al. (2014).

2.5 *Shoot and root respiration*

Oxygen consumption of shoot and root in the dark was measured using a Clark-type oxygen electrode as described by Schippers et al. (2008).

2.6 *Real-time PCR analysis*

Total RNA was extracted from primary root with emerged lateral roots using an RNeasy Plant Mini Kit (Qiagen) and cDNA was synthesized from 2 µg total RNAs using SuperscriptTM III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Real-time PCR reactions were performed as described by Schmidt et al. (2013a). Relative transcript abundance was calculated by the comparative cycle threshold (C_T) method (Livak and Schmittgen 2001). *ACTIN* (Os03g50885) was used as the reference gene according to Figueiredo et al. (2012). We selected candidate genes from the literature that have been shown to regulate auxin, ethylene and cell expansion in rice (Schmidt et al 2013a; Schmidt et al 2013b, Yu et al 2016; Xu et al 2017). PCR primers (Table S1) were designed using QuantPrime (Arvidsson et al. 2008).

2.7 *Statistical analysis*

The statistical design was completely randomized distribution. The experimental unit of the experiments consisted of 20 rice seedlings per plastic pot with five replicates per treatment. For real-time PCR analysis, three independent replicates containing 20 root systems per samples were assayed. Analysis of variance (ANOVA; $P < 0.05$) was performed to compare treatment effects. If ANOVA showed significant effects, a Tukey test or t-test ($P < 0.05$) was used to determine differences among treatments. The data were statistically evaluated with SPSS (Statistical Package for the Social Sciences) 11.0 version.

3 Results

3.1 *Regulation of primary and lateral root in response to selenium*

An increase in primary root length was observed for rice seedlings grown in the 10 µM Se treatment (Fig. 1a). Similarly, primary root length was increased by application of 0.1 µM AVG, an inhibitor of ethylene biosynthesis, and Se+AVG as compared with the control. Se, AVG and Se+AVG increased the primary root length by enhancing cell expansion (Fig. 1b). Primary root length increased by 26%, 31% and 47% in rice seedlings treated with Se, AVG and Se+AVG, respectively, whereas the same treatments resulted in an increase in root cell length of 20%, 26% and 30%, respectively (Fig. 1c, d). Moreover, relative root growth rate was increased in rice seedlings grown in Se, AVG and Se+AVG solution compared with the control (Fig. 1e).

Rice seedlings grown in 10 µM Se solution showed a >2-fold decrease in elongation and number of lateral roots compared with the control (Fig. 1a, f, g). However, number and length of lateral roots were increased in seedlings treated with 0.1 µM AVG. In addition, 0.1 µM AVG effects on lateral root development were inhibited in rice seedlings grown in Se+AVG solution (Fig. 1a, f, g).

It is important to emphasize that Se alone affect primary root elongation as well as number and length of lateral roots in a concentration-dependent manner, suggesting a quantitative relationship between the concentration of this compound and root growth of rice seedlings (Suppl. Fig. S1). The maximum significant effect induced by Se occurred at 10 µM. Additionally, inhibition induced by 10 µM Se on length and number of lateral roots was not suppressed by AVG in the range of 0.05-20 µM. However, AVG increased primary root length of rice seedlings treated with 10 µM Se in a dose-dependent manner (Suppl. Fig. S1).

3.2 *Selenium modifies primary and lateral root growth in response to ACC and IAA*

To investigate how Se affects primary and lateral root development, seedlings were grown on IAA and ACC together with Se. ACC inhibited primary root length in a dose-dependent manner (Suppl. Fig. S1). The maximum significant effect induced by ACC occurred at 5 µM when primary root length was reduced by 31% compared with control (Fig. 2a, b and Suppl. Fig. S1). No further significant reduction in primary root length occurred in the range 7.5-10 µM (Suppl. Fig. S1). Moreover, treatment of rice seedlings with 5 µM ACC had no significant effect on the number of lateral roots, but decreased length of lateral roots by 22% compared to control (Fig. 2a, c, d and Suppl. Fig. S1). The inhibitory effect of 5 µM ACC on primary root length was overcome when rice seedlings were treated with 10 µM Se

(Fig. 2b). In addition, 10 µM Se treatment combined with 5 µM ACC treatment promoted identical inhibition in length and number of lateral roots to 10 µM Se added alone (Fig 2c, d and Suppl. Fig. S1).

The relationship between auxin and lateral root development under Se treatment was investigated by treating rice seedlings with IAA in the range of 2-14 µM plus 10 µM Se. Length and number of lateral roots increased with increasing exogenous IAA concentration in rice seedlings treated with 10 µM Se (Suppl. Fig. S1). The maximum effect in lateral root development induced by applied IAA occurred at 10 µM (Suppl. Fig. S1). In this context, 10 µM IAA combined with 10 µM Se treatment increased the length (50%) and number (49%) of lateral roots of the rice seedlings, when compared to the pure Se solution (Fig. 2a, c, d). On the other hand, treatment with 10 µM Se+10 µM IAA produced the same elongation of primary root as Se alone (Fig. 2a, b and Suppl. Fig. S1). Interestingly, IAA alone in the range of 2-14 µM had no effect on primary and lateral root development, indicating that without Se the growth-promoting response to IAA is saturated (Suppl. Fig. S1).

We also examined the effect of 10 µM Se treatment on primary and lateral root development in the presence and absence of polar auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA). Treatment with 10 µM TIBA decreased the number and length of lateral roots, without changes in elongation of primary root compared to control (Fig. 3a-d). However, treatment of rice seedlings with Se+TIBA produced the same elongation of primary root as well as the same number and length of lateral roots as Se alone (Fig. 3a-d).

3.3 *Selenium modulates changes in ethylene and auxin biosynthesis in whole roots*

To investigate whether Se modifies primary and lateral root development altering ethylene and auxin biosynthesis, we quantified ethylene, ACC and IAA, as well as the expression of ethylene- and auxin-related genes in roots of seedlings treated with Se, AVG and Se+AVG. There was a decrease in the concentrations of ethylene, ACC and IAA in roots of rice seedlings treated with Se as compared with the control (Fig. 4a-c). It is interesting to note that the concentrations of ethylene and ACC in roots of seedlings treated with Se were similar to those observed in the AVG treatment. AVG at concentration of 0.1 µM did not influence the concentration of IAA in roots. On the other hand, roots of seedlings grown in Se+AVG solution showed a reduction in the concentrations of ethylene (63%), ACC (75%) and IAA (50%) in relation to control (Fig. 4a-c).

We also observed strong effects of Se on the expression of genes encoding proteins involved in ethylene and auxin biosynthesis (Fig. 4d). The expression of ethylene biosynthesis genes *ACS2*, *ACS6*, *ACO1* and *ACO7* was down-regulated in plants treated with Se and Se+AVG. Interestingly, changes in the expression of genes involved in ethylene biosynthesis induced by Se were comparable to those of plants treated with AVG alone. Moreover, *YUCCA1* and *YUCCA3* showed a significant down-regulation upon Se and Se+AVG treatment, whereas AVG alone did not affected these genes (Fig. 4d).

3.4 *Changes in the expression of auxin- and cell-wall related genes are interlinked with selenium treatment*

Treatments with Se or Se+AVG resulted in a decrease in the expression of genes associated with auxin signaling including *auxin response factor* (*ARF*) *4*, *ARF10*, *ARF19*, *auxin-responsive Gretchen Hagen* (*GH*) *3.3* and *GH3.4*, but no effect of AVG on the expression of these genes was observed in roots of rice seedlings (Fig. 5). Moreover, *Auxin/Indole-3-Acetic Acid* genes (i.e. *IAA3, IAA11* and *IAA20*), also involved in auxin signaling, showed upregulation upon Se and Se+AVG treatment, but AVG itself no longer induced expression of these genes in roots. Genes related to auxin transport such as *PIN1A*, *PIN1B* and *PIN3* were repressed upon Se and Se+AVG treatment, but induced by AVG treatment (Fig. 5). On the other hand, *PIN2* was upregulated in roots of seedlings grown in Se, AVG and Se+AVG. Two α-class *expansin* (*EXP*) genes, *EXPA8* and *EXPA14*, and two β-class *EXP* genes, *EXPB2* and *EXPB3*, were found to be positively regulated in roots of seedlings treated with Se, AVG and Se+AVG (Fig. 5). Additionally, treatment with Se and Se+AVG decreased the expression of *endoglucanase* (*GLU*) *5* and *GLU14* genes in roots, whereas AVG increased these genes.

3.5 *Influence of selenium on primary metabolism*

In an attempt to clarify the effect of Se on growth of primary and lateral roots, primary metabolism was analyzed in seedlings treated with Se in presence and absence of AVG, IAA and ACC. Concentrations of sucrose, glucose and fructose increased in both shoot and roots of rice seedlings treated with Se, Se+AVG and Se+ACC as compared with control (Fig. 6a-c). However, Se+IAA did not lead to significant changes in the concentrations of sucrose,

glucose and fructose in either shoot or roots of rice seedlings. There were no differences in concentrations of starch in both shoot and roots across treatments (Fig. 6d). The concentrations of amino acids were unaltered in shoot of seedlings treated with Se, AVG and Se+IAA, but Se+AVG and Se+ACC led to an increase of amino acids concentrations in shoot (Fig. 6e). Moreover, amino acids were significantly increased in roots of seedlings treated with Se, Se+AVG and Se+ACC, but not in roots of seedlings grown upon AVG and Se+IAA treatment compared with the control (Fig. 6e). Protein concentrations decreased in shoot of plants treated with AVG alone, while protein concentration remained stable in shoots of rice seedlings treated with Se, Se+AVG, Se+IAA and Se+ACC (Fig. 6f). However, Se, Se+AVG and Se+ACC treatment increased protein in roots, while AVG and Se+IAA did not change the concentrations of protein in root of rice seedlings (Fig. 6f). Our results also revealed that shoot and root respiration remained at the same level in all treatments (Fig. 6g). There was an increased Se concentration in both shoot and root of seedlings treated with sodium selenite and sodium selenite together with AVG, IAA and ACC, indicating that these treatments had no effect on Se uptake (Fig. 6h).

4 Discussion

Plant growth and development are controlled by the action of hormones, among which auxin and ethylene interact to control processes such as primary root elongation and lateral root development (Muday et al. 2012; Hu et al. 2017). In tobacco, Se stimulates primary root elongation and lateral root growth by triggering auxin biosynthesis and transport (Jia et al. 2018). Moreover, Se has been proposed to decrease total number of lateral roots in Arabidopsis, which was associated with the increased ethylene production (Feigl et al. 2019). However, the coordination of auxin and ethylene action by Se remains poorly defined, particularly during root development in rice. The results of the present study have revealed that Se decreased ethylene and auxin biosynthesis and suppressed number and elongation of lateral roots, while increasing primary root length.

In rice, ethylene stimulates auxin biosynthesis by effecting *YUCCA8/REIN7* transcription, allowing inhibition of primary root elongation (Qin et al. 2017). It has also been documented that enhanced ethylene production stimulates auxin transport and inhibits lateral root formation and elongation (Lewis et al. 2011). Our analyses revealed that the ethylene biosynthesis inhibitor AVG increased both primary and lateral root length. However, AVG

may also negatively regulate auxin biosynthesis (Le Deunff and Lecourt 2015). Free IAA concentration was reduced by ~20% and ~68% in seedlings of *Arabidopsis* treated with 4 µM and 40 µM AVG, respectively (Soeno et al. 2010). In our experimental setup, the concentration of the AVG was at least 40-fold less than employed in the earlier investigation. In this context, 0.1 µM AVG did not have any significant effect on expression of auxin biosynthesis genes and IAA concentration in root of rice seedlings compared with control (Fig. 4). Interestingly, Se application to AVG-treated seedlings reduced elongation and number of lateral root as well as IAA concentration whereas length of primary root was increased compared with the control (Figs 1, 4). Together, these findings suggest that Se affects primary and lateral root development by blocking ethylene and auxin biosynthesis, respectively. Consistent with this finding, inhibition of primary root elongation induced by ACC was reversed by application of Se (Fig. 2). The observation that seedlings treated with Se, 1-methylcyclopropene (1-MCP) and Se+1-MCP exhibit a primary root phenotype similar to AVG- and Se+AVG-treated seedlings supports the importance of ethylene in this process (Suppl. Fig. S2). Moreover, auxin application to Se-treated seedlings did not influence the growth of primary root, but increased number and length of lateral root compared with Se alone (Fig. 2). This observation is somewhat at odds with a previous study showing that lateral root development of *Arabidopsis* involves changes in ethylene and auxin (Lewis et al. 2011). Several factors could explain this discrepancy, including the fact that we are studying different species subject to different types of nutrition. Irrespective of the reason underlying the different conclusion of these studies, our work suggested that Se modulates root architecture of rice seedlings through alteration in ethylene and auxin.

Se at low concentration increased primary root length and the number of lateral roots of tobacco plants by increased expression of auxin synthesis genes *YUCCAs* and auxin efflux carriers *PINs* (Jia et al. 2018). In our experiments, *YUCCA1* and *YUCCA3* transcript abundance decreased after Se and Se+AVG treatment, but AVG alone did not affect expression of these genes (Fig. 4). Previously, it was demonstrated that overexpression of *PIN1* causes increased lateral root density in rice (Xu et al. 2005). Our study revealed that expression of *PIN1A*, *PIN1B* and *PIN3* decreased with Se and Se+AVG treatment and increased with AVG application (Fig. 5). Similar to the effects of the AVG solution, Se and Se+AVG resulted in a decrease in the expression genes associated with ethylene biosynthesis (Fig. 4). Se-dependent decreased in IAA concentration and number and length of lateral roots were not observed in seedling treated with AVG alone (Figs 1, 4). In addition, seedlings

treated with Se+1-MCP developed fewer and shorter lateral roots as compared to 1-MCP alone (Suppl. Fig. S2). Thus, the initiation and elongation of lateral roots of Se treated rice seedlings might be driven by a direct effect on the biosynthesis and transport of auxin rather than ethylene production. In agreement with this observation, TIBA inhibited the number and length of lateral roots, without changes in elongation of primary root compared to control (Fig. 3). On the other hand, primary and lateral root development of seedlings treated with Se+TIBA was similar to that observed in seedlings treated with Se alone (Fig. 3). Moreover, Se-induced inhibition on length and number of lateral roots was not suppressed by ACC (range 2.5-10 µM), but IAA increased lateral root development of rice seedlings treated with Se in a dose-dependent manner (Suppl. Fig. S1). These findings further support the idea that ethylene and auxin may act independently to control lateral root formation in rice seedlings treated with Se. In fact, ACC did not have any significant effect on lateral root formation of *Arabidopsis* ethylene-insensitive mutant (*etr1*) (Negi et al. 2008), whereas auxin treatment increased lateral root development of *etr1* mutant (Ivanchenko et al. 2010). In this context, the expression of *GLU5* and *GLU14* was decreased in root of rice seedlings treated with Se and Se+AVG, but AVG alone increased the expression of these genes (Fig. 5). This finding is consistent with previous reports that auxin positively regulates the expression of *GLU5* and *GLU14* during lateral root formation in rice (Yoshida et al. 2006). On the other hand, our study suggests that the effect of Se on primary root growth is dependent of changes in the transcription of ethylene biosynthesis genes, which in turn decreased ethylene production (Figs 1, 4). This finding is supported by the fact that the effects of the Se on length of primary root, expression of genes involved in ethylene biosynthesis and on ethylene production mimicked those of AVG treatment (Figs 1, 4). Similar to the effects of AVG, Se increased the expression of *EXPA8*, *EXPA14*, *EXPB2* and *EXPB3* compared to control (Fig. 5). The increase of ethylene concentration causes inhibition of cell division and cell expansion by decreasing the expression of *EXP* genes (Dubois et al. 2018). It seems therefore possible that Se mediates upregulation of *EXP* genes through inhibition of ethylene production. Collectively, these findings indicate that the differential effect of Se on primary and lateral root of rice seedlings could be associated to their distinct ontogeny and the balance between auxin and ethylene.

Growth of primary and lateral root is known to be under the control of carbon availability (Freixes et al. 2002; Willaume and Pages 2006). Sucrose and hexoses increased in both shoot and root of seedling grown under Se and Se+AVG treatment (Fig. 6). Se and

Se+AVG had no significant effect on shoot growth of rice seedlings as compared with control (Suppl. Fig. S3). On the other hand, treatment of seedlings with Se and Se+AVG increased primary root growth, but decreased number and length of lateral roots compared with control (Fig 1). It seems possible, therefore, that Se altered the utilization of carbon in the lateral roots but not in the primary root. In this context, soluble sugars accumulation in the shoot of seedlings grown under Se can be caused by impaired sugars usage in the lateral roots, which is followed by a buildup of sugars concentrations in the shoot. Consistent with this view, AVG treatment did not preserve the sucrose and hexoses pools and increased length and number of lateral roots compared with Se+AVG (Figs 1, 6). These observations were also supported by the demonstration that sucrose and hexoses increased in root and shoot of rice seedlings treated with Se+ACC, but Se+IAA did not affect the concentrations of sugars in shoot and root as compared with the control (Fig. 6). Treatment of rice seedlings with Se+ACC promoted identical inhibition in number and length of lateral roots in relation to the Se alone, whereas Se+IAA increased number and length of lateral roots compared with Se (Fig. 2). These results imply that Se modulates the effect of auxin on lateral root development and leads to changes in primary metabolism. Plant biomass enhancement is dependent of protein synthesis (Sulpice et al. 2013). The fact that protein and amino acids concentrations increased in the root when Se inhibited lateral root growth (Fig. 6) strengthens the idea that Se differentially tunes the relationship between lateral root growth and primary carbohydrate metabolism. However, it has been pointed out that Se can alter respiration in *Brassica napus* roots (Dimkovikj and Van Hoewyk 2014). In our experiments, Se did not influence respiration rates in both shoot and root of rice seedlings (Fig. 6). Moreover, shoot of rice seedlings treated with Se contained similar concentrations of starch and proteins as well as higher concentration of sucrose compared with control (Fig. 6), suggesting that there are changes in the regulation of sugars metabolism. The alteration of sugars metabolism under Se treatment has also been reported in *Arabidopsis* plants (Ribeiro et al. 2016). Thus, it seems that Se decreases the total sink strength of the root system of rice seedlings by decreasing the number and elongation of lateral roots. Consistent with this observation, the development of greater number and length of lateral roots increases the sink strength of the root system in maize (Postma et al. 2014).

Glucose is also known to be involved in control of root growth through auxin signal transduction (Mishra et al. 2009). The fact that glucose accumulated considerably in roots of rice seedlings under Se treatment is interesting because it suggests that control of root growth

in response to Se could be mediated by the combined effect of glucose and auxin. Treatments with Se or Se+AVG influenced the expression of major classes of auxin-responsive genes, including the *Auxin/Indole-3-Acetic Acid* (*Aux/IAA*) family, *auxin response factor* (*ARF*) family and *auxin-responsive Gretchen Hagen 3* (*GH3*) family, but no effect of AVG on the expression of these genes was observed in rice root (Fig. 5). Given that Se reduced number and length of lateral roots, it is feasible that increased glucose concentration upon Se treatment affects the responsiveness of lateral root towards auxin. In fact, addition of external glucose decreased the number of lateral root and root hair length in auxin signaling mutants (Mishra et al. 2009).

Our results are integrated in a model presented in Fig. 7 in which Se causes a decrease in concentrations of auxin and ethylene in rice roots. Thus, the inhibition of ethylene biosynthesis and signaling by Se increases primary root length. On the other hand, Se treatment inhibits auxin synthesis, transport and signaling, leading to decrease in number and length of lateral roots. The consequence of inhibition of lateral root growth is that sugars, amino acids and proteins are being utilized more slowly in roots of rice seedlings treated with Se. Moreover, the accumulation of metabolites in roots of seedlings treated with Se was accompanied by an increase in sugars in the shoot of rice seedlings.

5 Conclusions

In summary, Se increases primary root length, but decreases the number and length of lateral roots through alteration of the auxin and ethylene balance. Se downregulates genes associated with the biosynthesis of auxin and ethylene, which in turn decreases auxin and ethylene concentration. IAA treatment overrides the inhibitory effect of Se in the lateral roots. In addition, Se negatively regulates the expression of genes associated with auxin transport. Hence, these results are consistent with Se altering auxin biosynthesis and transport, and thereby inhibiting lateral root formation. The ethylene synthesis inhibitor AVG increases elongation of the primary root, whereas ACC results in the opposite effects. Therefore, Se-induced elongation of primary roots probably occurs through its inhibition of ethylene biosynthesis. Carbon accumulation in shoot and roots under Se treatment are likely to be related to reduction of lateral root growth under influence of auxin. Thus, our study highlights the potential role of Se in modulating the formation of primary and lateral roots through alterations in auxin and ethylene, leading to new patterns of root architecture in rice seedlings.

Further investigation is required to evaluate the importance of phenotypic changes of root system caused by treatment with Se in rice seedlings. We suggest that an increase in primary root elongation associated with a reduction in lateral roots development and carbon demand by the roots deserves consideration as a root phenotype to utilize soil water resources during the drying season without as much penalty for growth of rice seedlings.

Author contribution statement Rafael S.P. Malheiros, Lucas C. Costa, Rodrigo T. Ávila, Thaline M. Pimenta, Lubia S. Teixeira and Fred A.L. Brito conducted experiments and statistical analysis. Rafael S.P. Malheiros, Lucas C. Costa, Lubia S. Teixeira, Agustín Zsögön and Dimas M. Ribeiro performed literature survey. Rafael S.P. Malheiros, Wagner L. Araújo, Agustín Zsögön and Dimas M. Ribeiro designed the research and interpreted the results. All authors the authors contributed to the writing of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Figures

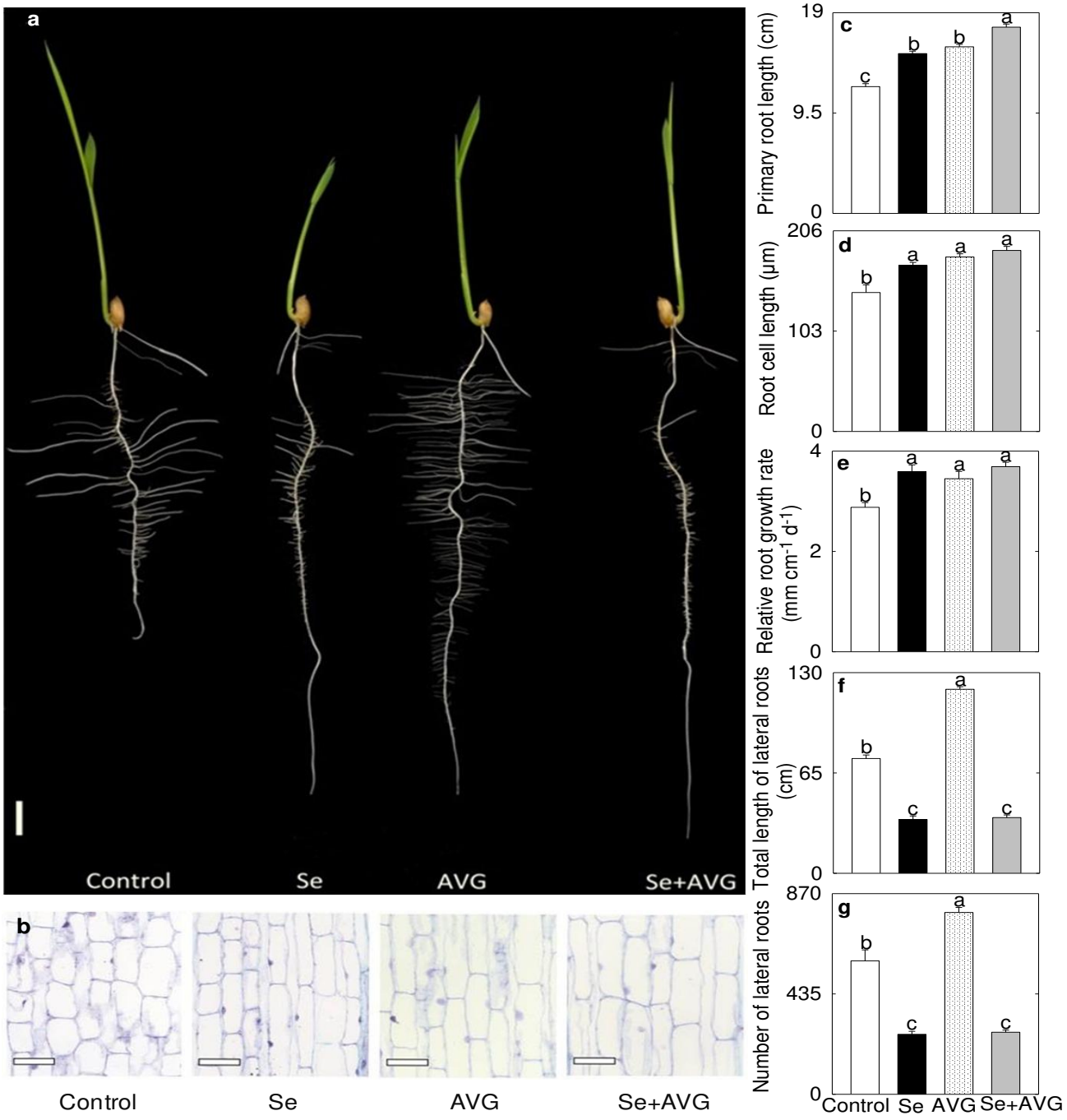


Fig. 1 Phenotypic changes of rice seedlings caused by treatment with Se, AVG and Se+AVG. **a** Photography of seedlings incubated for 5 days in half-strength Hoagland's solution (control) alone or also containing 10 μM sodium selenite (Se), 0.1 μM AVG and Se+AVG. The scale bars represent 10 mm. **b** Photomicrographs of elongation zone of rice primary root. The scale bars represent 50 μm. **c** Primary root elongation. **d** Root cell length. **e** Relative root growth rate. **f** Total length of lateral roots. **g** Number of lateral roots. Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Data are means ± standard error of three separate experiments, with five replicates each.

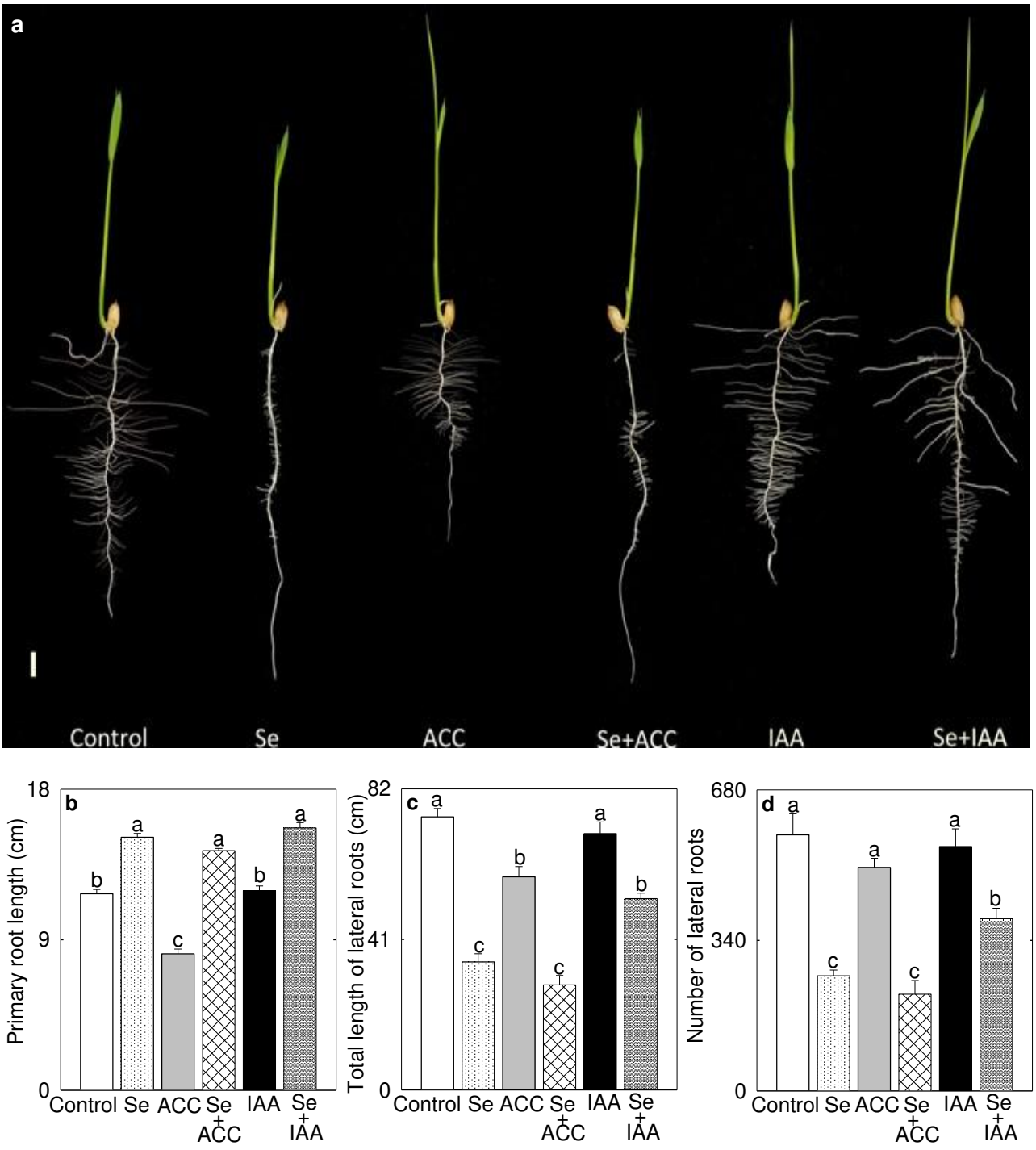


Fig. 2 Response to ACC and IAA of rice seedlings treated with Se. **a** Photography of seedlings incubated for 5 days in half-strength Hoagland's solution (control) alone or also containing 10 µM sodium selenite (Se), 5 µM ACC, Se+ACC, 10 µM IAA and Se+IAA. The scale bars represent 10 mm. **b** Primary root length. **c** Total length of lateral roots. **d** Number of lateral roots. Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Data are means ± standard error of three separate experiments, with five replicates each.

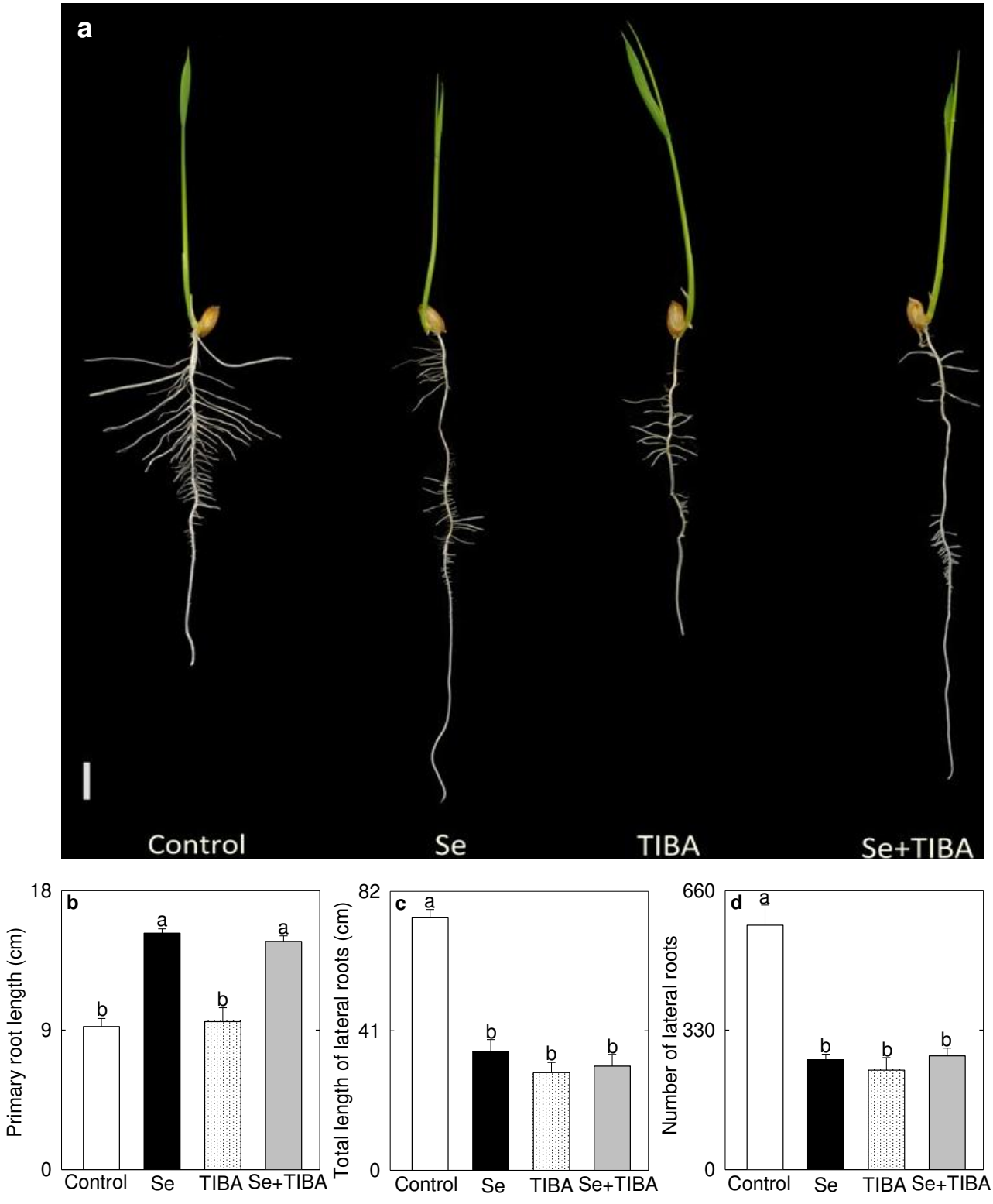


Fig. 3 Effect of Se and TIBA on roots of rice seedlings. **a** Photography of seedlings incubated for 5 days in half-strength Hoagland's solution (control) alone or also containing 10 µM Se or 10 µM TIBA. The scale bars represent 10 mm. **b** Primary root length. **c** Total length of lateral roots. **d** Number of lateral roots. Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Data are means ± standard error of three separate experiments, with five replicates each

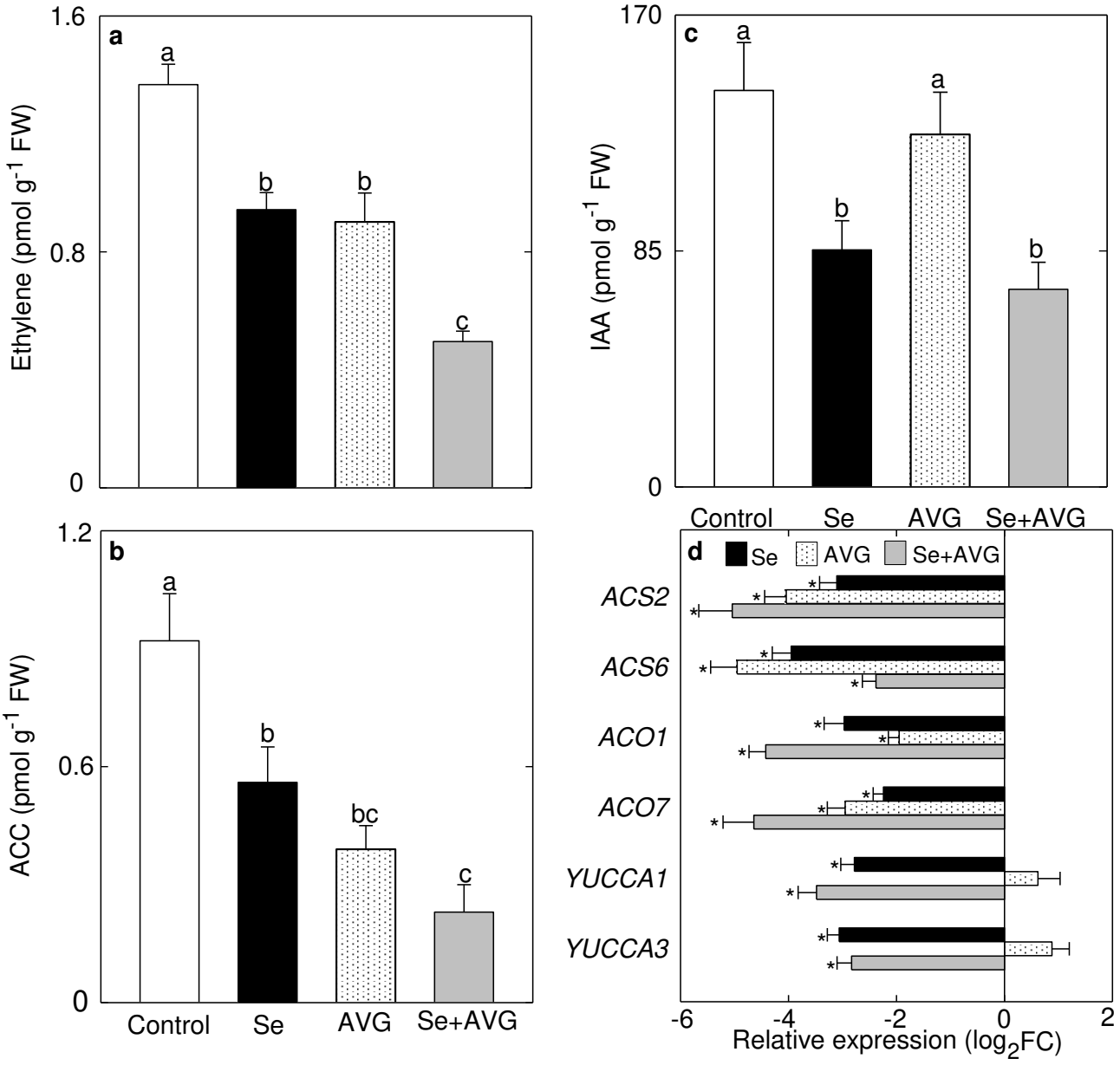


Fig. 4 Concentration of ethylene, ACC and IAA as well as the expression of genes related with ethylene and IAA biosynthesis in roots of rice seedlings treated with 10 µM sodium selenite (Se), 0.1 µM AVG and Se+AVG. **a** Ethylene. **b** ACC. **c** IAA. **d** Gene expression. Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Asterisks indicate values determined by the Student's *t*-test to be significantly different from the control ($P < 0.05$). Data are means ± standard error of three separate experiments, with five replicates, for concentrations of ethylene, ACC and IAA, and three replicates for gene expression.

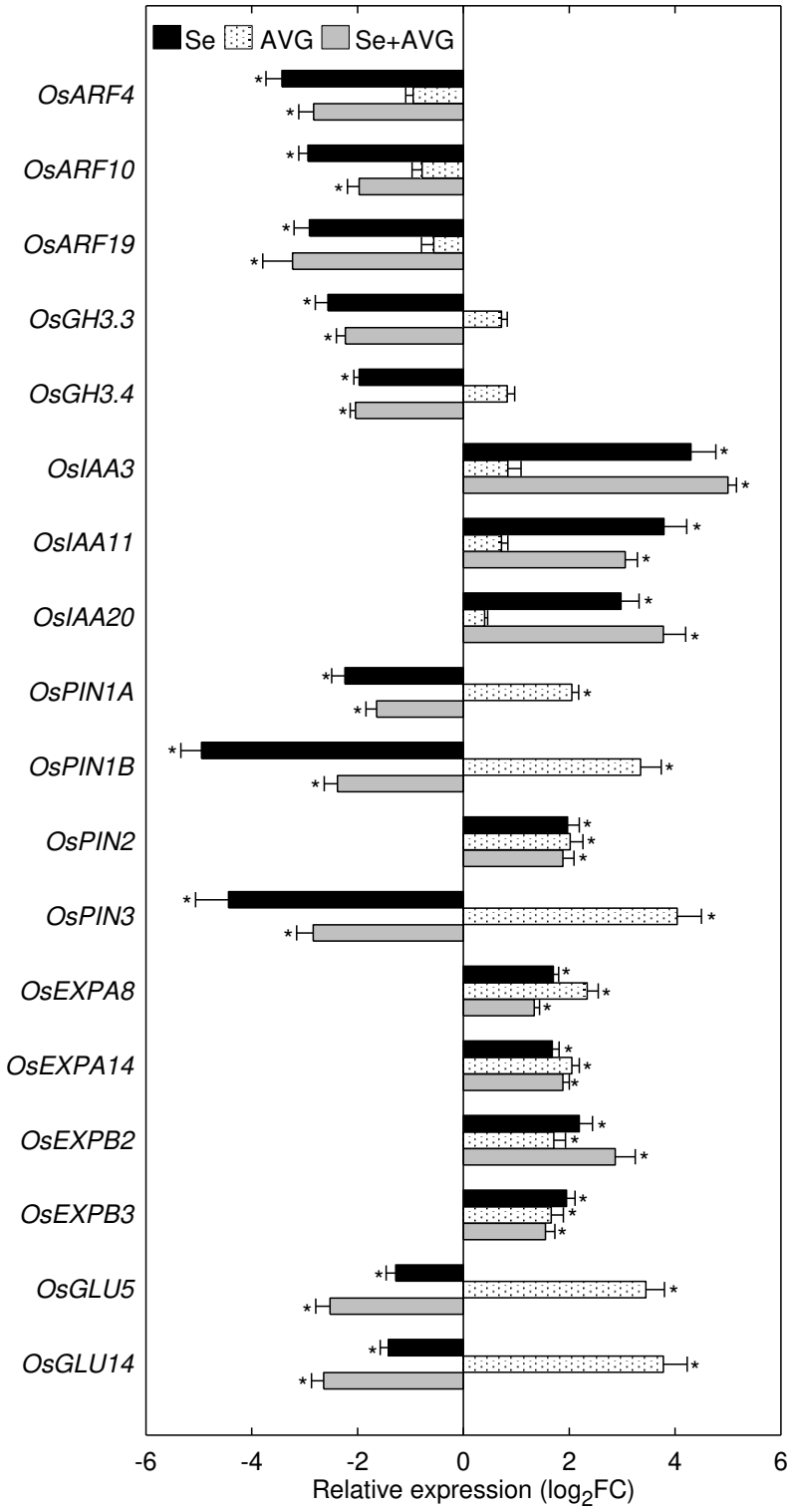


Fig. 5 Changes in gene expression in root of rice seedlings treated with 10 μM sodium selenite (Se), 0.1 μM AVG and Se+AVG. Primers sequences are listed in Supplementary Table S1. Asterisks indicate values determined by the Student's *t*-test to be significantly different from the control ($P < 0.05$). Data are means ± standard error of three separate experiments, with three replicates each.

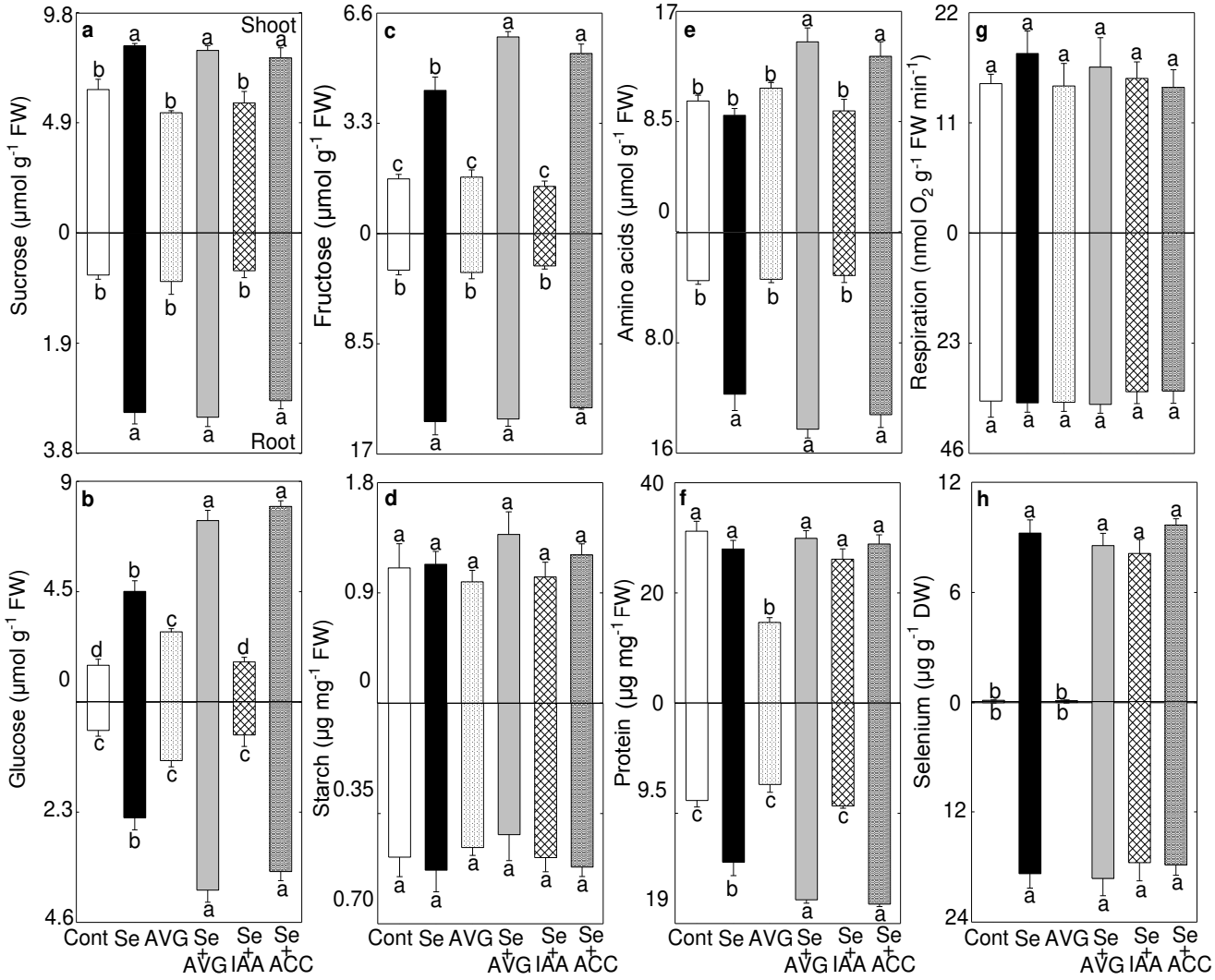


Fig. 6 Concentrations of major metabolites and selenium as well as respiration rate in shoot and root of rice seedlings treated with 10 μM sodium selenite (Se), 0.1 μM AVG, Se+AVG, Se+10 μM IAA and Se+5 μM ACC. **a** Sucrose. **b** Glucose. **c** Fructose. **d** Starch. **e** Total amino acids. **f** Total protein. **g** Respiration rate in the dark. **h** Selenium. Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Data are means ± standard error of three separate experiments, with five replicates each.

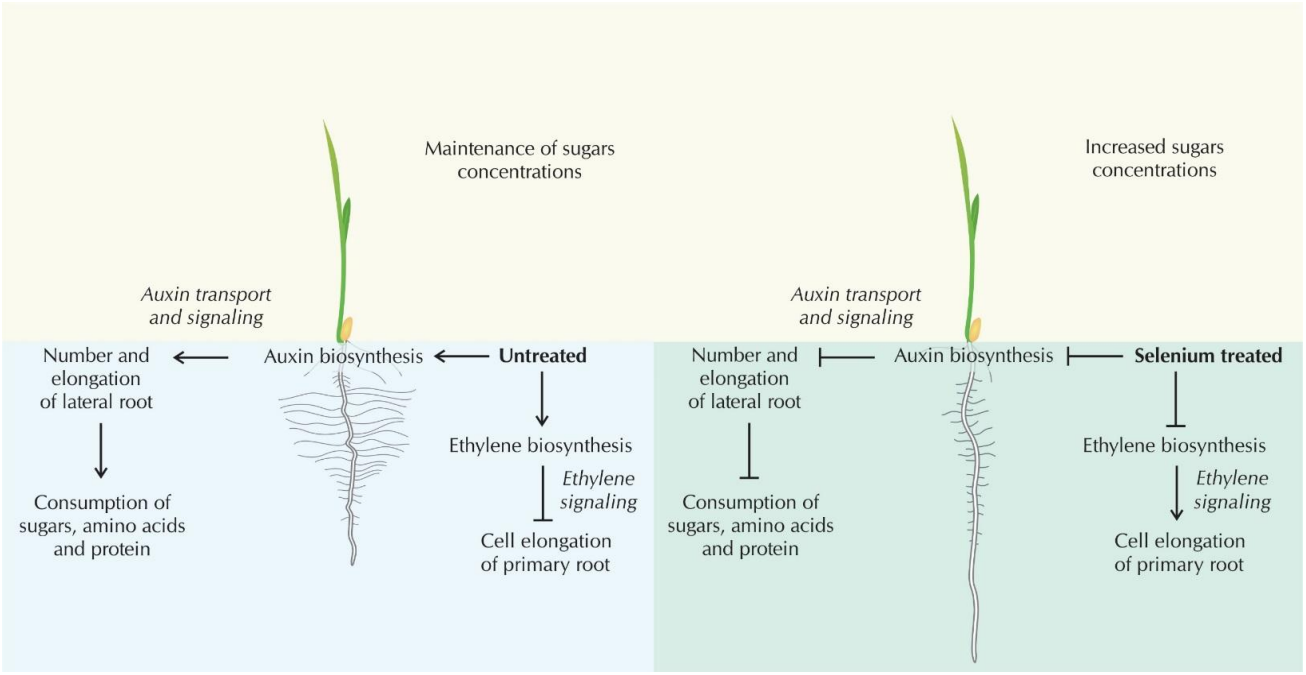
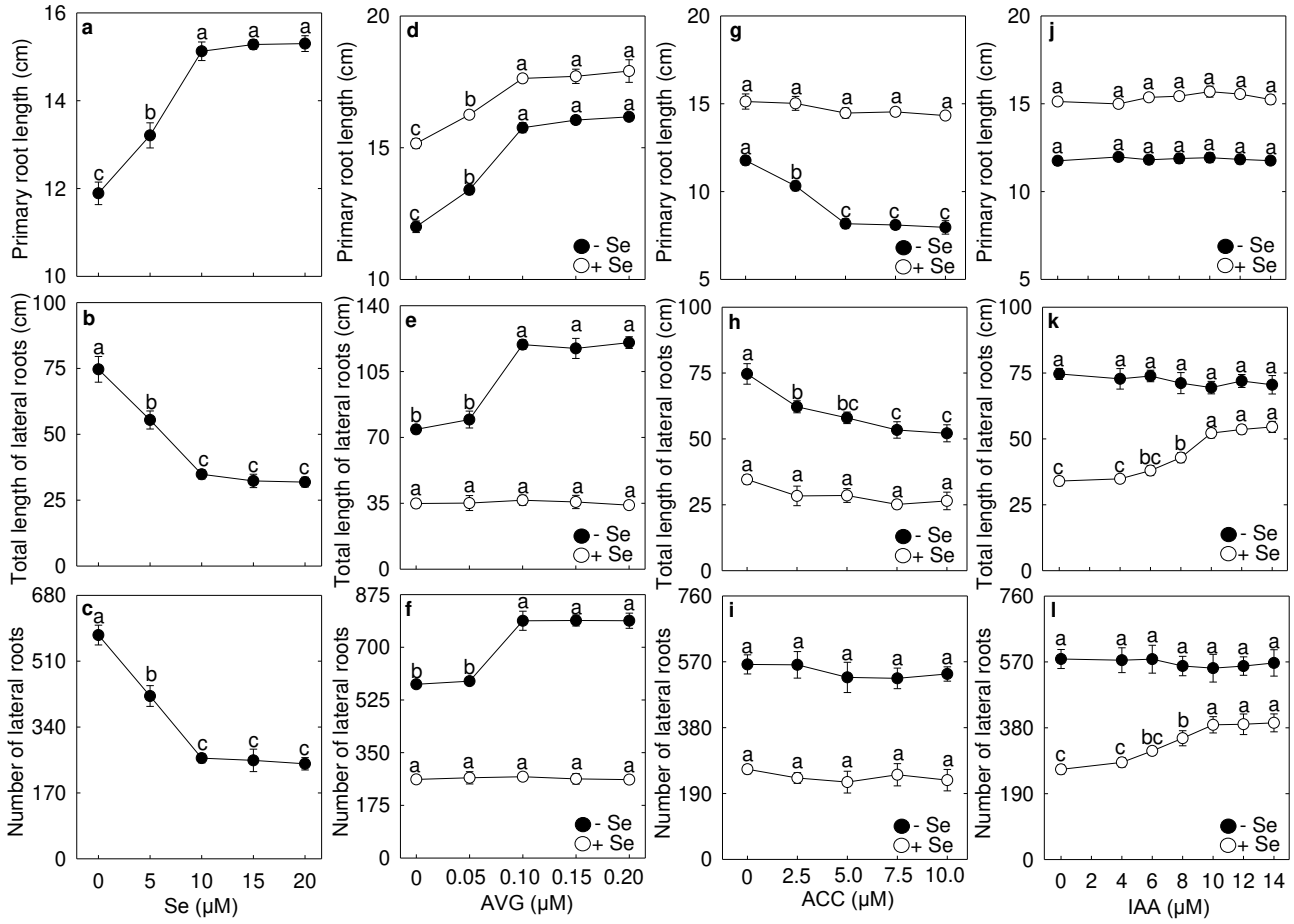
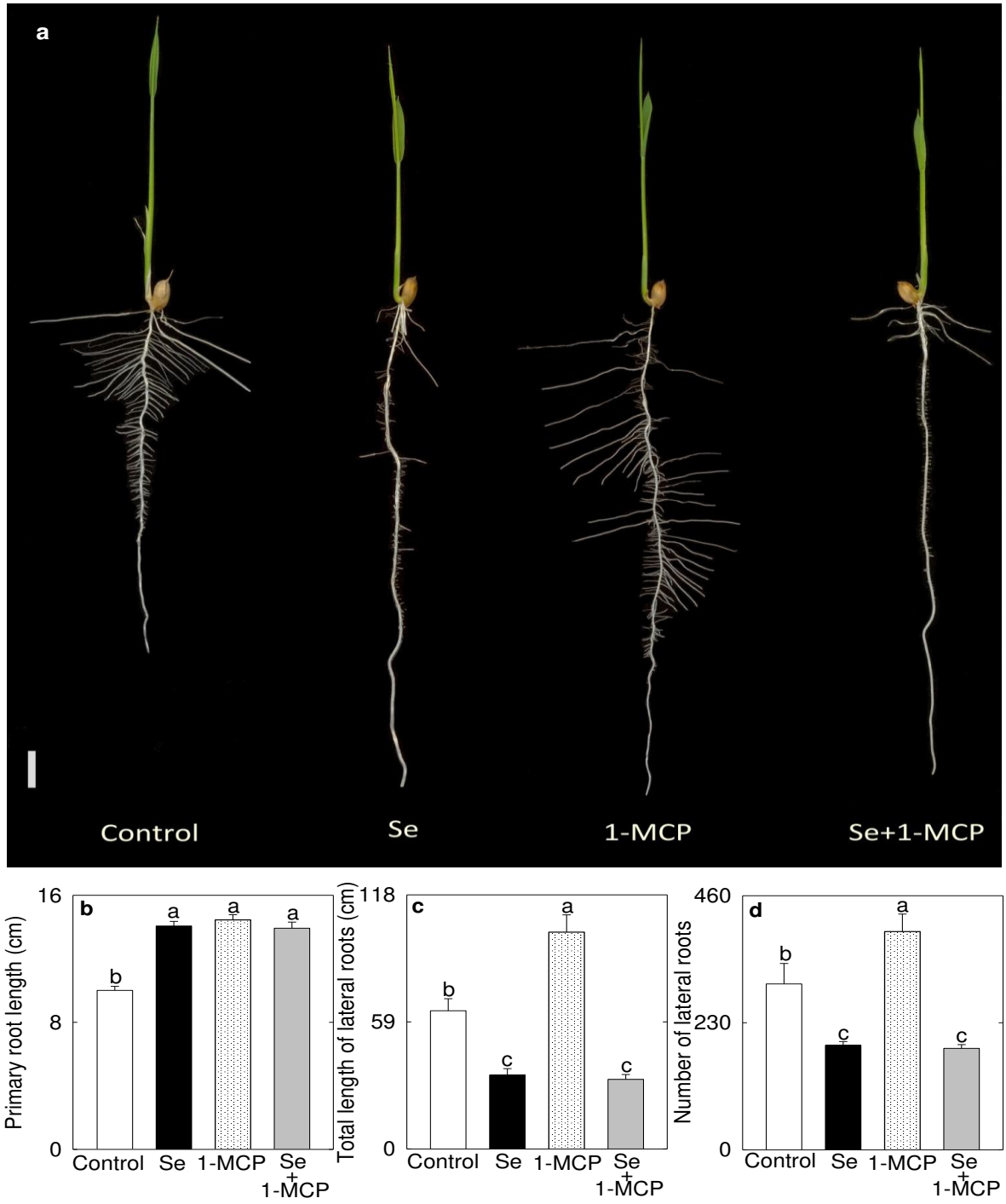


Fig. 7 Scheme summarizing the effect of Se on roots of rice seedlings. Diagrams of rice roots untreated (left) and treated (right) with Se. Treatment of rice seedlings with Se decreases auxin biosynthesis, transport and signaling, which may then inhibit the lateral root development while decrease consumption of sugars, amino acids and protein. This effect may be responsible for the accumulation of sugars in shoot of rice seedlings. On the other hand, Se decreases ethylene biosynthesis and signaling, thereby reducing primary root elongation.

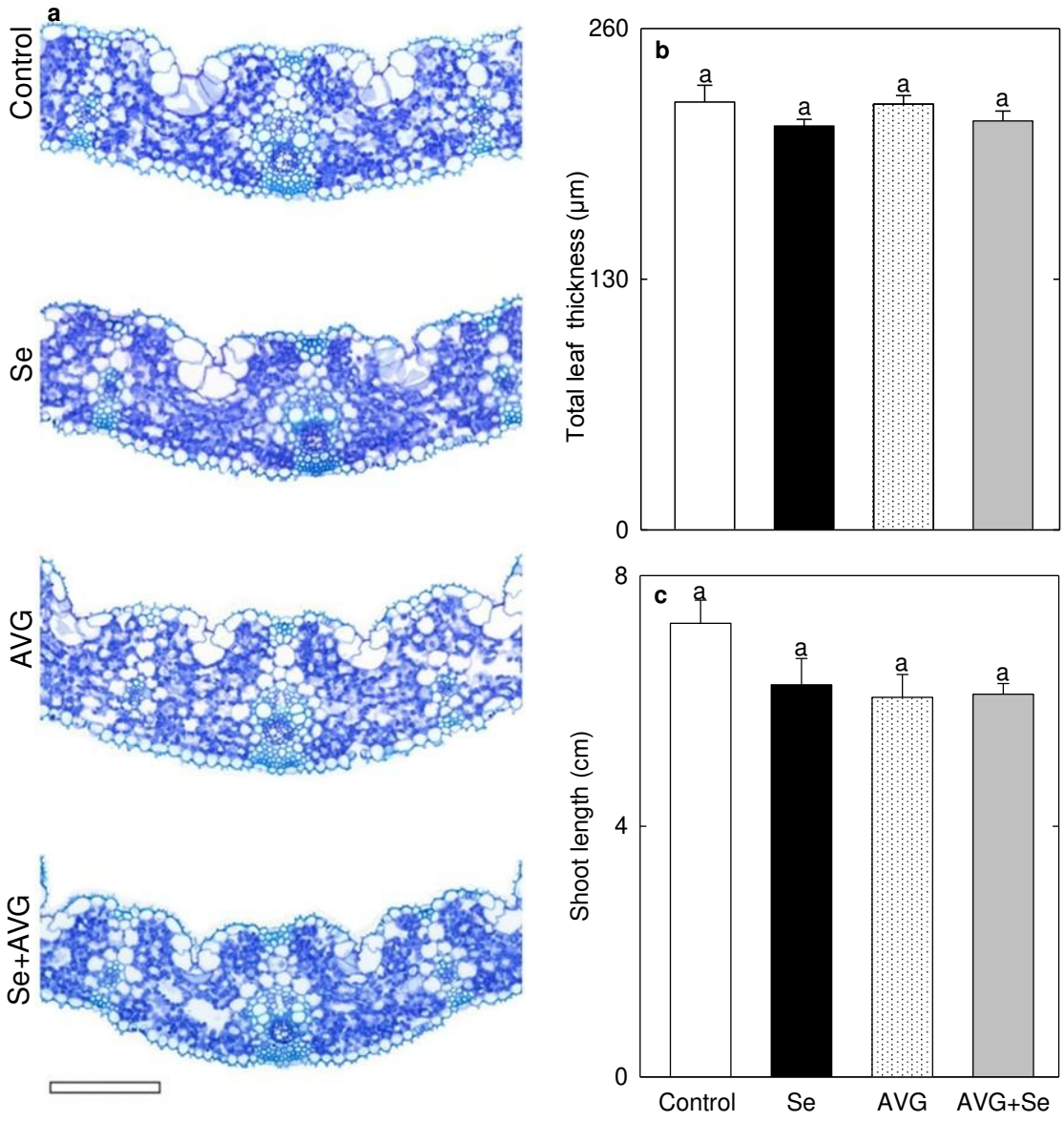
Supplementary material



Suppl. Fig. S1 Effect of Se alone or Se supplied in AVG, ACC and IAA solution on roots of rice seedlings. Length of primary and lateral roots as well as number of lateral roots were assayed 5 days from the start of seedlings incubation in half-strength Hoagland's solution (control) alone or also containing Se, AVG, ACC and IAA. **a** Se dose-response curves for primary root length, **b** total length of lateral roots and **c** number of lateral roots. **d** AVG dose-response curves for primary root length, **e** total length of lateral roots and **f** number of lateral roots with or without 10 µM Se. **g** ACC dose-response curves for primary root length, **h** total length of lateral roots and **i** number of lateral roots with or without 10 µM Se. **j** IAA dose-response curves for primary root length, **k** total length of lateral roots and **l** number of lateral roots with or without 10 µM Se. Values with the same letter do not differ statistically at the 5% level by Tukey test. Data are means ± standard error of three separate experiments, with five replicates each.



Suppl. Fig. S2 Effects of Se, 1-MCP and Se+1-MCP on roots of rice seedlings. **a** Phenotypes of rice seedlings incubated for 5 days in half-strength Hoagland's solution (control) alone or also containing 10 µM sodium selenite (Se) and exposed either to air or 1-MCP (2 µL L^{-1}) gas in a sealed environment. The scale bars represent 10 mm. **b** Primary root length. **c** Total length of lateral roots. **d** Number of lateral roots. 1-MCP, an inhibitor of ethylene perception, was applied as described by Tucker et al. (2017). Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Data are means ± standard error of three separate experiments, with five replicates each.



Suppl. Fig. S3 Leaf size parameters. **a** Representative images of leaf lamina cross-sections of rice seedlings incubated for 5 days in half-strength Hoagland's solution (control) alone or also containing Se, AVG and Se+AVG. The scale bars represent 50 µm. **b** Leaf width. **c** Shoot elongation. Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Data are means ± standard error of three separate experiments, with five replicates each.

Suppl. Table S1 Primers sequences used for real-time PCR

Name	Primer sequences	
	Forward	Reverse
IAA3	ACAAGGATGGTGACTGGATGCTG	GCAAGAGTCGGTGAACATCTCC
IAA11	AGCAGCTGAAGGAGAGCAATAAGC	TGCACGACTCGACGAACATCTC
IAA20	GCGAAATGGGCAACAAGAGGAG	AGGATGGACAAGTCCAGCTTCC
ARF4	TCGACGAGGAGTTGTACAAAGGTC	ATCCACAGATCTGCCAAGTGCAAC
ARF10	CAGCATTGGCAGCGATGAACTG	ATTTCACGAACGCGCAGAATGG
ARF19	AAGCGGCCCAGGCAATTAGATG	AGCCCAGGCATTATGGTGTTCTG
GH3.3	TTCAACGAGGAGCTCGTCAAGTC	TTCTCCACCGGAAACTCCCTGTTC
GH3.4	GCAACAAGCAGTGGATCAGCAG	AGCCAAGCTATCACAGGTCGTC
EXPA8	TACACCTCCTCGGCTCAGTTCTAC	TGCCACGTCGATCAAAGCATAC
EXPA14	CCCGGTTATCTACCAAAGGGTTCC	AGTCGTGCCCGTTAATGGTAAAC
EXPB2	CAACCAGTACCCGTTCATGTCC	GTTGTTGGTGCACCGTATCTGG
EXPB3	TGCGGGTTCAAGAACACCAACC	GGGTGGTTGACGCATCTTATCTGG
ACO1	TGGAGCAGCTGGATGATGCTTG	AGATGCCGTGGTTCAGGATCTC
ACO7	ATCGTCGTGTAGTACGCAGGGTTC	AGTTACCGTGATAACCACCCAACC
ACS2	TTTGGCGCCTTGACGGCCTC	AAAGGGAGCGCACCATGGCC
ACS6	CCGGGCGACACGTTCAGCTT	ACAGCGCGAACGGGTTCCAG
PIN1A	TCATCTGGTCGCTCGTCTGC	CGAACGTCGCCACCTTGTTC
PIN1B	TGCACCCTAGCATTCTCAGCA	CCCTCCTCCCAAATTCTACTTC
PIN2	CAGGGCTAGGAATGGCTATGT	GCAAACACAAACGGGACAA
PIN3	ATCCTGAGCACAGCGGTAAT	CAATGTCCGACAACAGGCTA
YUCCA1	TCATCGGACGCCCTCAACGTCGC	GGCAGAGCAAGATTATCAGTC
YUCCA3	GTGAGAACGGGCTCTACTCGGTCG	GCTTATGCATGACCGATGAACACG
GLU5	AACTCCTGCAACACTCTAAACAGC	ACGGTGATGCTGTTGTCGTACC
GLU14	AGCAGGACGACTTCACCTTCAG	TGCTCTGCAAGAATCCTTTGGTG
ACTIN	TGGATTGGAGGATCCATCTTGGC	CCTTGGCAATCCACATCTGCTG

CHAPTER 2

Selenomethionine induces oxidative stress and modifies growth in rice (*Oryza sativa* L.) seedlings through effects on hormone biosynthesis and primary metabolism

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Abbreviations: ACC 1-aminocyclopropane-1-carboxylic acid; APX ascorbate peroxidase; CAT catalase; DABCO 1,4-diazabicyclooctane; GPX glutathione peroxidase; IAA Indole-3-acetic acid; MetL L-methionine; MDA malondialdehyde; ROS reactive oxygen species; SeMet seleno-L-methionine; SOD superoxide dismutase

Abstract

Inorganic selenium inhibits auxin and ethylene biosynthesis in roots of rice (*Oryza sativa* L.) seedlings, resulting in the reduction of number and elongation of lateral roots together with increased of primary root length. Experiments with organic selenium showed that seleno-L-methionine (SeMet) at low concentration increased concentrations of hydrogen peroxide and superoxide anion production, inhibiting auxin biosynthesis and increasing ethylene production in both shoot and root of rice seedlings. We also show that the effect of SeMet on rice seedlings was mediated by the inhibition of the abundance of transcripts encoding auxin transport and cell expansion proteins. Moreover, SeMet led to increased seedling respiration, which was positively correlated with organic acids consumption, but negatively with sugars consumption, thereby decreasing seedling growth. In contrast with SeMet treatment, L-methionine did not affect reactive oxygen species production, hormone biosynthesis and growth of rice seedlings, indicating an exclusive selenium effect. Treatment with 1,4-

diazabicyclooctane (DABCO), a singlet oxygen scavenger, overrode the repressive effect of SeMet in seedling growth. Our results demonstrate a phytotoxic effect of SeMet at low concentration for rice seedlings and reveal a relationship between reactive oxygen species, hormone homeostasis and carbon availability, which regulates growth responses.

Keywords: Reactive oxygen species; growth inhibition; primary metabolism, hormonal regulation.

1 Introduction

The selenate and selenite are the major inorganic forms of selenium present in most soils (El-Ramady et al., 2015). These inorganic forms absorbed by plant roots can be converted to organic form of selenium such as selenocysteine and selenomethionine (SeMet) (Wang et al., 2012). SeMet might control ethylene production in seed tissues of *Stylosanthes humilis* through alteration in the 1-aminocyclopropane-1-carboxilic acid (ACC) biosynthesis pathway (Pinheiro et al., 2008). In this regards, SeMet has been proposed to compete with methionine (Met) in the ACC biosynthesis pathway, which was associated with increased of ethylene production in mature flower of *Ipomoea tricolor* (Konze et al., 1978). On the other hand, selenite decreased ethylene biosynthesis, which was associated with the increased primary root elongation of rice seedlings (Malheiros et al., 2019). Additionally, selenite decreased lateral root formation by reducing auxin biosynthesis and transport in rice (Malheiros et al., 2019). These results suggest that although inorganic form of Se inhibits ethylene and auxin biosynthesis in rice, the effects of SeMet are unclear. It may be that the ability of selenium regulated hormone biosynthesis and hormone-dependent physiological processes in rice are dependent on the form of Se supplied.

The toxicity of inorganic forms of selenium such as selenite and selenite is known to induce reactive oxygen species (ROS) production in both shoot and roots of plants, which

contributed to a reduced biomass (Chen et al., 2014; Dimkovikj and Van Hoewyk, 2014). In this regards, the stress induced by inorganic selenium could affect the activity of antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT) and ascorbate peroxidase (APX) (Gupta and Gupta, 2016). Inorganic selenium assimilated into selenoamino acids can be misincorporated into selenoproteins (Terry et al., 2000). Proteomic analysis in rice has demonstrated that the treatment with selenium increases the abundance of proteasome subunits, which seems to be associated with the need to remove selenoproteins (Wang et al., 2012). In this context, plants need to maintain a high production of ATP, since the substitution of malformed selenoproteins is an energy-expensive process (Ribeiro et al., 2016). Therefore, the accumulation of selenoproteins in plants can result in the generation of ROS (Van Hoewyk, 2013). ROS might control plant growth through interaction with hormones such as salicylic acid, jasmonic acid, gibberellin, abscisic acid, ethylene and auxin (Mhamdi and Van Breusegem, 2018). The balance between auxin and ethylene plays an important role in modulating root architecture in rice seedlings treated with inorganic selenium (Malheiros et al., 2019). In rice, selenite modulates root growth by regulating expression of *expansin* (*EXP*), *pin formed* (*PIN*) and *endoglucanases* (*GLU*) genes (Malheiros et al., 2019). Given that inorganic and organic forms of selenium often occur together in plants (Jiang et al., 2018), we asked whether SeMet exerts its effects on growth of rice seedlings via alterations in the coordination between ROS and hormones.

It has been described that selenium coordinates the regulation of primary metabolism in eudicots and monocots (Dimkovikj and Van Hoewyk, 2014; Malheiros et al., 2019; Ribeiro et al., 2016). Selenite reduces tricarboxylic acid (TCA) cycle metabolites, while enhancing concentrations of glucose and amino acids, with both events occurring to overcome the effects of oxidative stress in roots of *Brassica napus* (Dimkovikj and Van Hoewyk, 2014). On the other hand, selenite induces changes in the concentrations of sugars,

amino acids and protein in rice seedlings, which defines root system architecture (Malheiros et al., 2019). Moreover, selenate altered the concentrations of TCA intermediates in shoot of *Arabidopsis thaliana*, but no effect of selenate on the concentration of these metabolites was observed in roots (Ribeiro et al., 2016). These results highlight the flexibility of the inorganic form of selenium in the modulation of primary metabolism in plants. However, relatively little is known about the role of organic form of selenium in the coordination of primary metabolism during seedling development in rice.

In this study, we tested the hypothesis that SeMet at low concentration alters auxin and ethylene biosynthesis by triggering ROS production and thus regulates the development of rice seedlings. Finally, we examined whether the SeMet-associated processes affect primary metabolism of rice seedlings.

2 Materials and methods

2.1 *Plant material and general conditions*

Seeds were collected from plants of rice (*O. sativa* L. ssp *japonica* cv Oochikara) growing in a greenhouse in Viçosa (20^{o} 45'S, 42^{o} 15'W), Minas Gerais, Brazil. Growth in hydroponic culture was performed as described by Malheiros et al. (2019). Rice seedlings were exposed to half-strength Hoagland's solution supplemented with 5µM Met, 5µM SeMet and 5µM SeMet+1mM 1,4-diazabicyclooctane (DABCO). The pots containing the seedlings were placed at 30/24 ^{o}C and 60/75% relative humidity (day/night) with a day length of 16 h and a light intensity of 200 µmol m^{-2} s^{-1} for 5 days.

2.2 *Phenotypical analysis*

Measurements of root lengths and number of lateral roots were performed as described by Malheiros et al. (2019) using the image-processing software WinRhizo Pro (Regent Instruments Inc; Quebec, Canada). For measuring dry-weight biomass, shoot and root of rice

seedlings were separated using a scalpel and placed in a drying oven at 70 ^{o}C and measured for dry mass after 72 h.

2.3 *Measurement of metabolite, IAA, ACC and ethylene*

The rice seedlings were harvested after 5 days on nutrient solution at the end of the photoperiod and through washed with deionised water. Afterwards, seedlings were blotted between two sheets of filter paper to remove excess water and the shoot was separated from root, frozen in liquid nitrogen and stored at –80 ^{o}C until analysis. The analysis of metabolites by spectrophotometer was performed using 50 mg of frozen shoot and root powder. Sucrose, fructose, glucose, total amino acids and total protein were extracted and quantified following the protocol described by Cross et al. (2006). The concentrations of malate and fumarate in shoot and root of rice seedlings were determined as described by Nunes-Nesi et al. (2007). IAA and ACC in rice seedlings were determined following the technique described by Müller and Munné-Bosch (2011). Ethylene production by the shoot and root of rice seedlings was measured using a gas chromatography with a flame ionization detector as described by Pelacani et al. (2005).

2.4 *In situ localization and quantification of hydrogen peroxide and superoxide anion*

Hydrogen peroxide (H_2O_2) accumulation in shoot and root of rice seedlings was analyzed using 3,3'-diaminobenzidine (DAB) according to Daudi and O'Brien (2012). Concentrations of H_2O_2 in rice tissues were measured as described by Okuda et al. (1991). Superoxide anion ($O_2^{\bullet -}$) was detected in rice seedlings by nitroblue tetrazolium (NBT) staining according to Dunand et al. (2007). Concentrations of $O_2^{\bullet -}$ in shoot and root of rice seedlings were determined according to Achary et al. (2012).

2.5 *Enzyme assays*

A total of 200 mg of frozen shoot and root powder was extracted by vigorous shaking with 4 ml of extraction buffer containing 50 mM potassium phosphate buffer (pH 6.8), 0.1 mM EDTA, 1 mM phenylmethyl-sulphonyl fluoride (PMSF), and 2%(w/v) polyvinylpyrrolidone (PVP) and centrifuged at 12,000 *g* at 4 ^{o}C for 15 min. The supernatant

was used to determine the activity of antioxidant enzymes. The activity of SOD (EC 1.15.11) was determined according to Kono (1978). The activity of APX (EC 1.11.1.11) was assayed as described by Nakano and Asada (1981). The activity of CAT (EC 1.11.1.6) was determined following the protocol described by (Aebi, 1974). The extraction and assay of GPX (EC 1.11.1.9) were performed according to Hasanuzzaman et al. (2011).

2.6 *Quantification of lipid peroxidation and respiration*

Oxygen consumption of shoot and root was quantified in rice seedlings kept in the dark with a Clark-type oxygen electrode as described by Schippers et al. (2008). Lipid peroxidation in shoot and root of rice seedlings were determined by estimating of the thiobarbituric acid reactive substances which was expressed as the malondialdehyde (MDA) concentration as described by Dhindsa et al. (1981).

2.7 *Real-time quantitative RT-PCR*

Total RNA was extracted from shoot and root of rice seedlings using the RNeasy kit (Qiagen) according to the manufacturer's instructions. The isolated RNA was treated with DNase I (Invitrogen) and cDNA was synthesized from 2 μg of total RNAs using SuperScript^{TM} III reverse transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative real-time PCR was performed with Power SYBR Green PCR Master Mix using gene-specific primers (Supplemental Table 1) and the rice *ACTIN* (Os03g50885) gene as an internal reference for normalization (Malheiros et al., 2019).

2.8 *Statistical analysis*

The statistical design was completely randomized distribution. The experimental unit of the experiments consisted of 20 rice seedlings per plastic pot with five replicates per treatment. Analysis of variance ($P < 0.05$) was carried out to determine effects of the treatments, and then mean values were compared through Tukey test or t-test at the 5% level of significance. All comparisons were performed with SPSS (Statistical Package for the Social Sciences) 11.0 version.

3 Results

3.1 *Regulation of growth process and ROS production in response to SeMet*

Growth of rice seedlings was not affected by 5µM Met compared to control (Fig. 1A). However, 5µM SeMet led to an inhibition of seedlings growth, while no growth repression was observed in rice seedlings treated with SeMet together with DABCO, a singlet oxygen scavenger (Fig. 1A). The ability of SeMet to inhibit length of shoot and root in a concentration-dependent manner indicates a quantitative relationship between the concentration of SeMet and growth of rice seedlings (Fig. S1A, B). In this context, treatment of rice seedlings with SeMet decreased shoot and root dry weight by 45% and 32%, respectively (Fig. 1B, C). Consequently, the total biomass was reduced in SeMet-treated seedlings compared to control (Fig. 1D). The inhibitory effect of SeMet on shoot and root biomass was overcome when rice seedlings were treated with DABCO (Fig. 1B-D). It is interesting to note that the DABCO alone had no effect on growth of rice seedlings (Fig. S1C, D). On the other hand, DABCO increased length of shoot and root seedlings treated with 5µM SeMet in a dose-dependent manner (Fig. S1E, F). There was also a change in the overall root architecture, seedlings treated with SeMet showed a 41% reduction in primary root length and developed fewer and shorter lateral roots compared to control (Fig. 1E-G). DABCO reversed the inhibition on primary and lateral root development promoted by SeMet. In contrast with SeMet treatment, Met did not induce architectural alterations in the root system of rice seedlings compared to control (Fig. 1E-G).

To investigate if SeMet triggers an increased ROS concentration modifying the activities of antioxidant enzymes by the seedlings, we analyzed the concentrations of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$) as well as the activity of SOD, APX, GPX and CAT in both shoot and root of rice seedlings. The H_2O_2 and $O_2^{\bullet-}$ accumulation visualized by DAB and NBT staining, respectively, were increased in both shoot and root of rice seedlings treated with SeMet, but not in seedlings grown upon Met and SeMet+DABCO compared to control (Fig. 2A). To confirm that SeMet-induced ROS production in rice seedlings, we determined the concentrations of H_2O_2 and $O_2^{\bullet-}$ in both shoot and root. Concentrations of H_2O_2 and $O_2^{\bullet-}$ increased in both shoot and root of rice seedlings treated with SeMet compared to control, a result that was associated with increase in MDA concentration (Fig. 2B-D). However, Met and SeMet+DABCO did not lead to significant

changes in concentrations of ROS and MDA in either shoot or root. Moreover, seedlings incubated in SeMet solution showed a significant increase in the activities of SOD, CAT, APX and GPX in both shoot and root, while Met and SeMet+DABCO did not affect the activities of these enzymes (Fig. 3A-D).

3.2 *SeMet modifies ethylene and auxin biosynthesis in rice seedlings*

Given that ethylene and auxin affect rice seedlings development (Malheiros et al., 2019), we asked whether SeMet modifies ROS production altering biosynthesis of ethylene and auxin. There was a significant increase in the concentrations of ACC and ethylene in both shoot and roots of seedlings treated with SeMet, but not in seedlings treated with Met and SeMet+DABCO compared to control (Fig. 4A, B). On the other hand, SeMet supply decreased auxin concentration by 52% and 50% in shoot and root, respectively (Fig. 4C). Met and SeMet+DABCO did not influence the concentrations of auxin in either shoot or root compared to control. Additionally, we observed that the expression of genes associated with ethylene biosynthesis including *ACS2*, *ACS6*, *ACO1* and *ACO7* was increased in shoot and roots of seedlings treated with SeMet, while Met and SeMet+DABCO did not affect these genes (Fig. 4D, E). Treatment with SeMet repressed the expression of genes involved in auxin biosynthesis (i.e. *YUCCA1* and *YUCCA3*) in both shoot and root, but Met and SeMet+DABCO no longer affected expression of these genes (Fig. 4D, E).

3.3 *Changes in primary metabolism in rice seedlings in response to SeMet*

In order to determine the effect of SeMet on primary metabolism, analyzes of sugars, amino acids, protein, organic acids and respiration rate were performed in shoot and root of rice seedlings. Treatment with Met did not change the concentrations of glucose, fructose, sucrose, amino acids, protein, malate and fumarate in both shoot and root of rice seedlings as compared with control (Fig. 5A-G). There were no differences in shoot and root respiration of seedlings treated with Met compared to control (Fig. 5H). A significant increase in glucose, fructose and sucrose was observed in both shoot and root of seedlings treated with SeMet and SeMet+DABCO compared to control (Fig. 5A-C). Amino acids were increased in both shoot and root by SeMet treatment, while SeMet+DABCO increased the amino acids concentration in shoot, with no change in root compared to control (Fig. 5D). SeMet and SeMet+DABCO

increased protein concentration in roots, but not in shoot compared to control (Fig. 5E). On the other hand, SeMet treatment decreased concentrations of malate and fumarate in both shoot and roots, while SeMet+DABCO did not change the concentrations of organic acids in rice seedlings (Fig. 5F, G). Our results also revealed that SeMet increased shoot (91%) and root (55%) respiration, while respirations of rice seedlings was unaltered by SeMet+DABCO treatment (Fig. 5H). It is important to emphasize that DABCO alone did not lead to significant changes in the respiration rate as well as in the concentrations of sucrose, glucose, fructose, amino acids, protein, malate and fumarate in both shoot and roots of rice seedlings (Fig. S2).

3.4 *Effect of SeMet on the expression of genes associated with auxin transport and cell wall biosynthesis/modification*

As expression of genes involved in auxin transport and cell wall modification are modulated by inorganic selenium during rice seedling development (Malheiros et al., 2019), the response of those genes towards SeMet was tested (Fig. 6A, B). The expression of *PIN1A*, *PIN1B*, *PIN2* and *PIN3*, encoding polar-auxin-transport efflux carriers, was downregulated in both shoot and roots of seedlings treated with SeMet, but Met and SeMet+DABCO no longer repressed the expression of these genes (Fig. 6A, B). *EXPA4* was downregulated only in roots by SeMet application. On the other hand, SeMet treatment repressed the expression of *EXPA8* and *EXPA14* in both shoot and root, while Met and SeMet+DABCO did not show an effect (Fig. 6A, B). *EXPB2* was found to be repressed upon SeMet treatment in both shoot and root, but induced by SeMet+DABCO. Next to that, Met upregulated *EXPB2* expression in root. Treatment with SeMet downregulated *EXPB3* expression in shoot, whereas SeMet+DABCO upregulated *EXPB3* expression compared to control (Fig. 6A, B). Moreover, Met, SeMet and SeMet+DABCO did not affect *EXPB3* expression in root of rice seedlings. We did observed any significant changes in expression of *GLU5* and *GLU14* in shoot of rice seedlings treated with Met, SeMet and SeMet+DABCO compared to control (Fig. 6A). However, SeMet repressed *GLU5* and *GLU14* in roots of rice seedlings, whereas Met and SeMet+DABCO induced the expression these genes (Fig. 6B).

4 Discussion

The initial reason for testing the effect of SeMet on growth of rice seedlings was the observation that selenite increases primary root elongation, but decreases elongation of lateral roots of rice seedlings through inhibition of auxin and ethylene biosynthesis (Malheiros et al., 2019). Moreover, treatment of rice seedlings with 10 µM selenite had no effect on shoot growth (Malheiros et al., 2019). Our results demonstrated that SeMet decreases both shoot and root growth of rice seedling and inhibited auxin biosynthesis, while increasing ethylene and ROS production. The inhibition of rice seedling growth by 5 µM SeMet is surprising, as studies have shown that selenite is efficiently converted to SeMet in rice (Hu et al., 2018; Wang et al., 2015). Concentrations of ACC and ethylene were increased in shoot and roots of seedlings treated with SeMet, but not in seedlings grown upon Met treatment compared to control (Fig. 4). This observation is somewhat at odds with a previous study showing that the affinity of methionine adenosyltransferase is higher for Met than for SeMet (Konze and Kende, 1979). Irrespective of Met and SeMet working as an ethylene precursor, our study suggests that SeMet leads to a stress condition that triggers ethylene biosynthesis in rice seedlings, by directly inducing *ACS2*, *ACS6*, *ACO1* and *ACO7* transcription (Fig. 4). In rice, ethylene increases auxin accumulation by triggering *YUC8/RIN7* transcription, resulting in a decrease in primary root growth (Qin et al. 2017). Although SeMet have been increased ethylene production in rice seedlings, the expression of auxin biosynthesis genes *YUCs* was repressed, which was followed by a decrease at IAA concentration in the shoot and roots (Fig. 4). Together, these findings suggest that SeMet induces stress that affects ethylene and auxin balance, which in turn reduces shoot and root development of rice seedlings.

In rice, inorganic forms of selenium at concentration as low as 25 µM has been shown to increase plant stress tolerance by decreasing H_2O_2 and $O_2^{\bullet-}$ production (Kumar et al., 2014; Pandey and Gupta, 2018). However, it has been pointed out that inorganic forms of selenium at concentration above 250 µM can trigger ROS formation and membrane damage in rice (Mostofa et al., 2017). In our experimental setup, treatment of rice seedlings with 5 µM SeMet increased concentrations of ROS and MDA in both shoot and root, which was accompanied by increased activity of ROS-scavenging enzymes, including SOD, APX, GPX and CAT (Figs 2 and 3). These results imply that organic form of Se such as SeMet at low concentration stimulates ROS production and affects membrane integrity, thereby inhibiting shoot and root growth of rice seedlings. Consistent with this view, inhibition of rice seedlings

growth induced by SeMet was reversed by application of DABCO, a singlet oxygen scavenger (Fig. 1). However, the increase in activities of antioxidant enzymes was not efficient to protect the rice tissues from the SeMet-induced cellular damages. The replacement of Met in proteins by SeMet may result in the generation of ROS in plant tissues (Terry et al., 2000; Van Hoewyk, 2013). Rice proteomic analysis has shown that selenium increases the abundance of proteasome subunits, which seems to be associated with the need to remove malformed selenoproteins (Wang et al., 2012). In this sense, plants need to maintain a high production of ATP to adapt to selenium, since the substitution of malformed selenoproteins is an energy-intensive process (Ribeiro et al., 2016). Therefore, the accumulation of selenoproteins in plants can result in the generation of ROS (Van Hoewyk, 2013). Thus, the application of SeMet in rice seedlings could be leading to oxidative stress by the accumulation of malformed selenoproteins, which in turn triggers to an inadequate response in the antioxidant system. The observation that seedlings treated with Met exhibit concentrations of ROS and activities of antioxidant enzymes similar to control supports the importance of SeMet in this process (Figs 2 and 3).

ROS production is known to influence the ethylene and auxin homeostasis (Xia et al., 2015). Our findings reveal that ROS acts as a link between ethylene and auxin biosynthesis by coordinating the modulation of SeMet-inhibited rice seedling growth (Figs 1, 2 and 3). In rice seedlings, primary and lateral root development regulated by selenite are ethylene and auxin dependent through alterations on the expression of genes related with cell wall and auxin transport (Malheiros et al., 2019). PIN proteins regulates the polar auxin transport (Adamowski and Friml, 2015) and the regulation of cell expansion is driven by proteins such as expansins (EXP) and endoglucanases (GLU) that affect cell wall loosening (Cosgrove, 2016; Wang et al., 2014). Our study indicated that SeMet negatively regulated the expression of several *PIN* and *EXP* genes in both shoot and roots of rice seedlings (Fig. 6). Interestingly, SeMet repressed the expression of genes involved with cell wall remodeling (i.e. *GLU5* and *GLU14*), whereas Met and SeMet+DABCO induced these genes in roots of rice seedlings. These results support the notion that SeMet functions as a negative regulator of genes related with cell wall and auxin transport. The ability of SeMet to inhibit growth of rice seedlings is related to the inhibition of auxin biosynthesis and enhanced ethylene production (Fig. 4). It seems possible, therefore, that auxin and ethylene act in a dependent manner of ROS to control shoot and root development in rice seedlings treated with SeMet. This is supported by the demonstration that DABCO inhibited the physiological alterations caused by SeMet in

rice seedlings. Thus, the level of ROS production by seedlings could be considered as biomarker to indicate degree of SeMet-induce phytotoxicity in rice.

The primary metabolism of plants is known to be affected by selenium (Ribeiro et al., 2016; Wang et al., 2012). The concentrations of sugars such as sucrose, fructose and glucose increased in both shoot and root of rice seedlings treated with SeMet and SeMet+DABCO compared to control (Fig. 5). Moreover, malate and fumarate decreased in both shoot and root of seedlings grown under SeMet treatment, but SeMet+DABCO did not affect the concentrations of organic acids compared to control. These data suggest that low concentrations of organic acids in SeMet-treated seedlings might serve to decrease carbon utilization for respiration, since seedlings grown under SeMet treatment showed an increase in both shoot and root respiration compared to seedlings treated with SeMet+DABCO (Fig. 5). In fact, overexpression of NADP-malic enzyme in *Arabidopsis* decreased biosynthesis of malate and fumarate and increased accumulation of sugars (Fahnenstich et al., 2007). Oxidative stress can impose higher energy demand to repair damaged organelles (Dimkovikj and Van Hoewyk, 2014). To meet these higher energy demand, metabolic adjustments are required to regulate respiratory potential (Jacoby et al., 2011). In this context, plants allocate the carbon that would be used in growth for respiration to maintain cell homeostasis (Møller et al., 2007). In this sense, it is possible that the high respiratory rate found in both shoot and root of seedlings treated with SeMet has affected the growth of rice seedlings, since the organic acids may have been redirected to maintain high cellular respiration. Consistent with this view, SeMet inhibited rice seedling growth, but SeMet together with DABCO completely rescued their growth. Plant growth consumes sugars and amino acids (Griffiths et al., 2016). Despite seedlings treated with SeMet and SeMet+DABCO displaying similar concentration of protein, amino acids increased in shoot and root of seedlings treated with SeMet whereas SeMet+DABCO increased amino acids in shoot with no changes in root compared to control. Thus, these data suggest that amino acids is also a part of metabolic effect responsible for the distinctive phenotype of seedlings treated with SeMet.

5 Conclusions

In summary, SeMet at low concentration inhibits growth of rice seedlings by triggering ROS production, which leads to an alteration of the auxin and ethylene balance

(Fig. 7). The consequence of alteration of ROS production and hormonal homeostasis is increased respiration rates in both shoot and root of rice seedlings (Fig. 7). This was associated with faster utilization of malate and fumarate and increased accumulation of sugars when seedlings were treated with SeMet, supporting the idea that low concentration of organic acids might serve to decrease carbon utilization for respiration of rice seedlings. Taken together, our results support a model in which ROS integrates the modulation of hormones biosynthesis and primary metabolism to restrict rice growth in response to SeMet. Therefore, it could be hypothesized that the level of ROS production may be an important biochemical trait for SeMet tolerance in rice seedlings.

Contribution Rafael S.P. Malheiros, Fabrício C.M. Gonçalves, Fred A.L. Brito and Dimas M. Ribeiro conducted experiments and statistical analysis. Rafael S.P. Malheiros and Fabrício C.M. Gonçalves performed literature survey. Rafael S.P. Malheiros, Agustín Zsögön and Dimas M. Ribeiro designed the research, interpreted the results. All authors the authors contributed to the writing of the manuscript.

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Figures

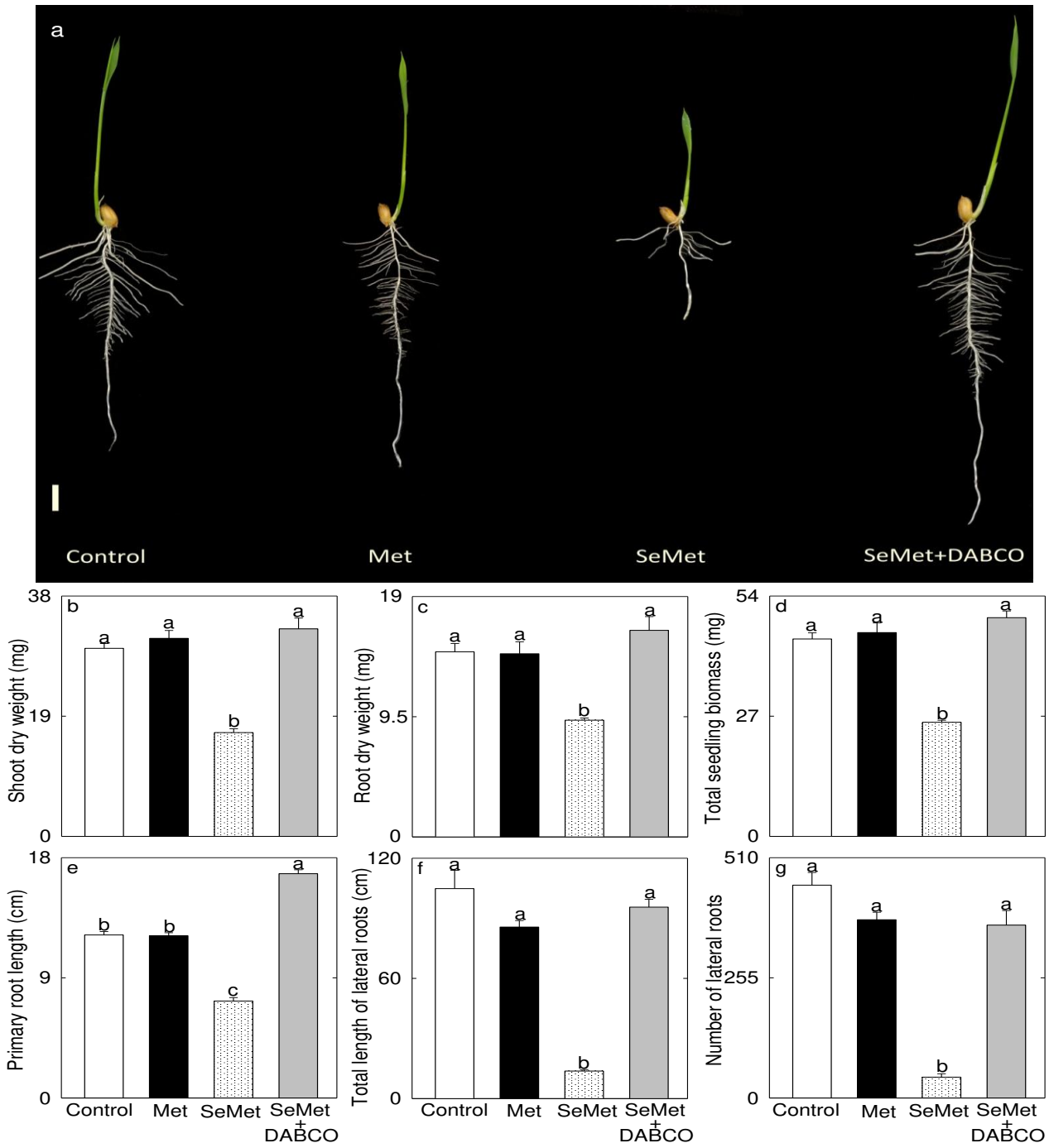

Fig. 1. Phenotypic changes of rice seedlings caused by treatment with Met, SeMet and SeMet+DABCO. **a** Photography of seedlings incubated for 5 days in half-strength Hoagland's solution (control) alone or also containing 5μM Methionine (Met), 5μM Seleno-L-methionine (SeMet) and SeMet+1mM 1,4-diazabicyclooctane (DABCO) (SeMet+DABCO). The scale bars represent 10 mm. **b** Shoot dry weight. **c** Root dry weight. **d** Total seedling biomass. **e** Primary root length. **f** Total length of lateral roots. **g** Number of lateral roots. Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Data are mean ± standard error of three separate experiments, with five replicates each.

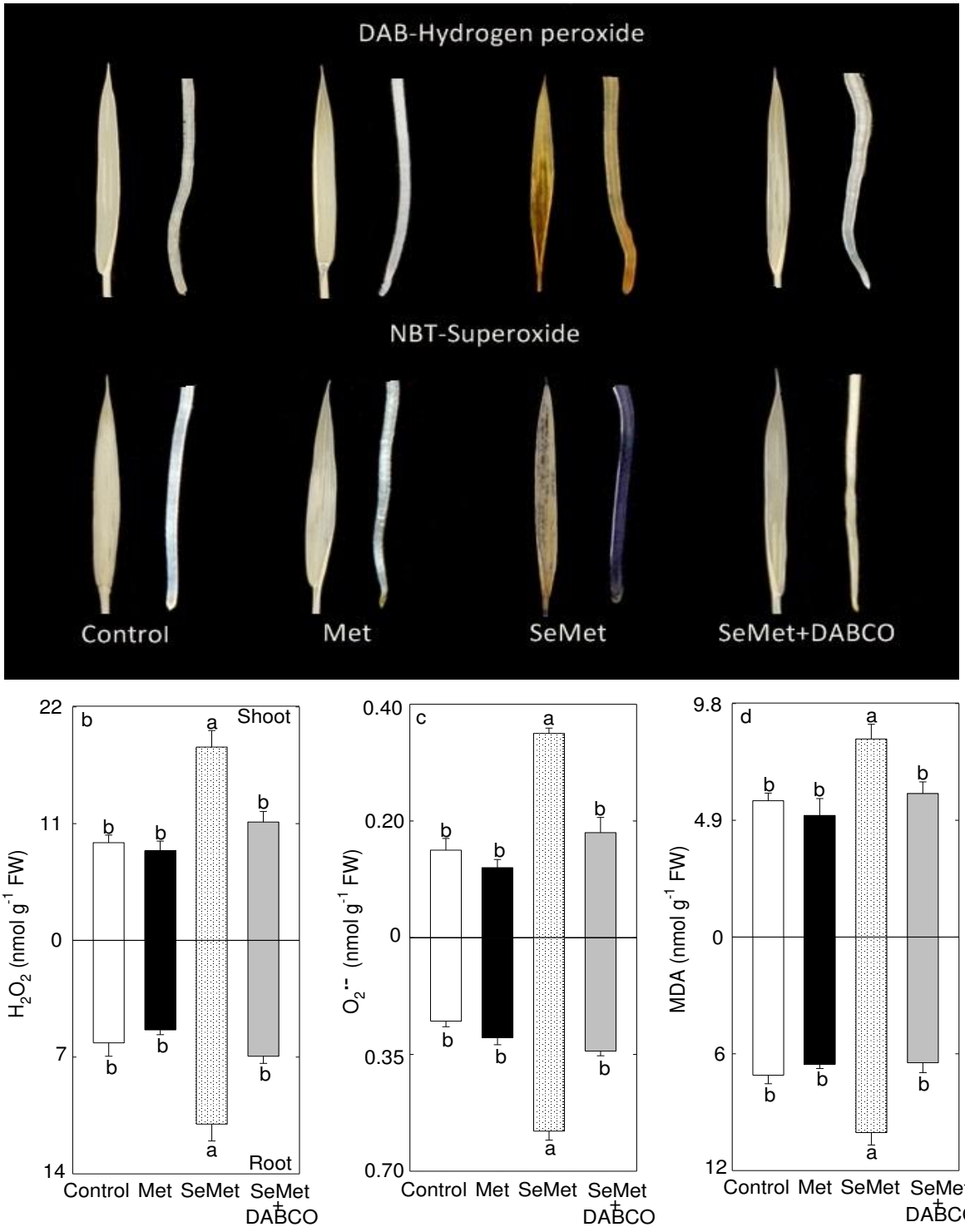

Fig. 2. Effects of Met, SeMet and SeMet+DABCO on hydrogen peroxide and superoxide anion production and on malondialdehyde concentration in rice seedlings. **a** Hydrogen peroxide and superoxide anion accumulation in leaves and roots visualized by the presence of brown DAB or blue formazan precipitates, respectively. **b** Hydrogen peroxide concentration. **c** superoxide anion concentration. **d** malondialdehyde concentration. Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Data are mean ± standard error of three separate experiments, with five replicates each.

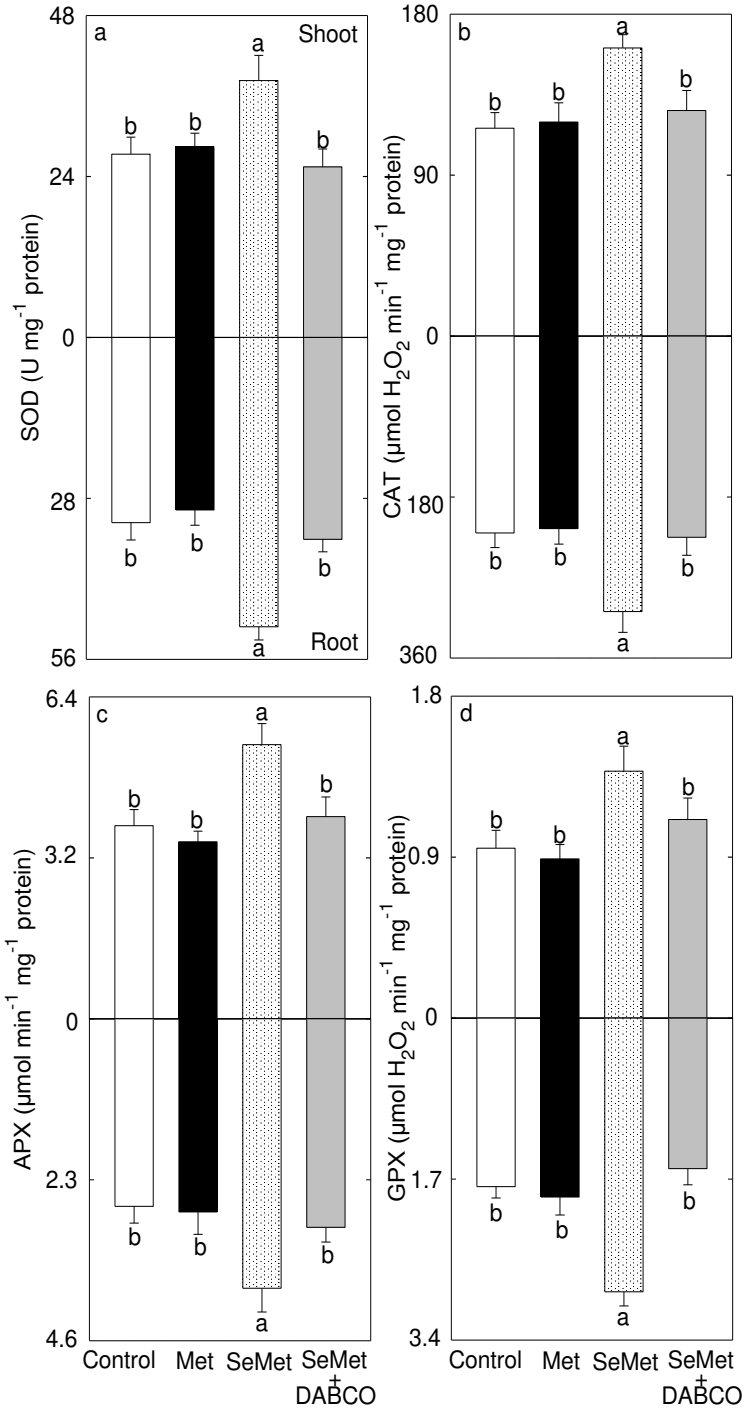

Fig. 3. Activities of superoxide dismutase, catalase, ascorbate peroxidase and glutathione peroxidase in shoot and root of rice seedlings treated with Met, SeMet and SeMet+DABCO. **a** Superoxide dismutase (SOD). **b** Catalase (CAT). **c** Ascorbate peroxidase (APX). **d** Glutathione peroxidase (GPX). Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Data are mean ± standard error of three separate experiments, with five replicates each.

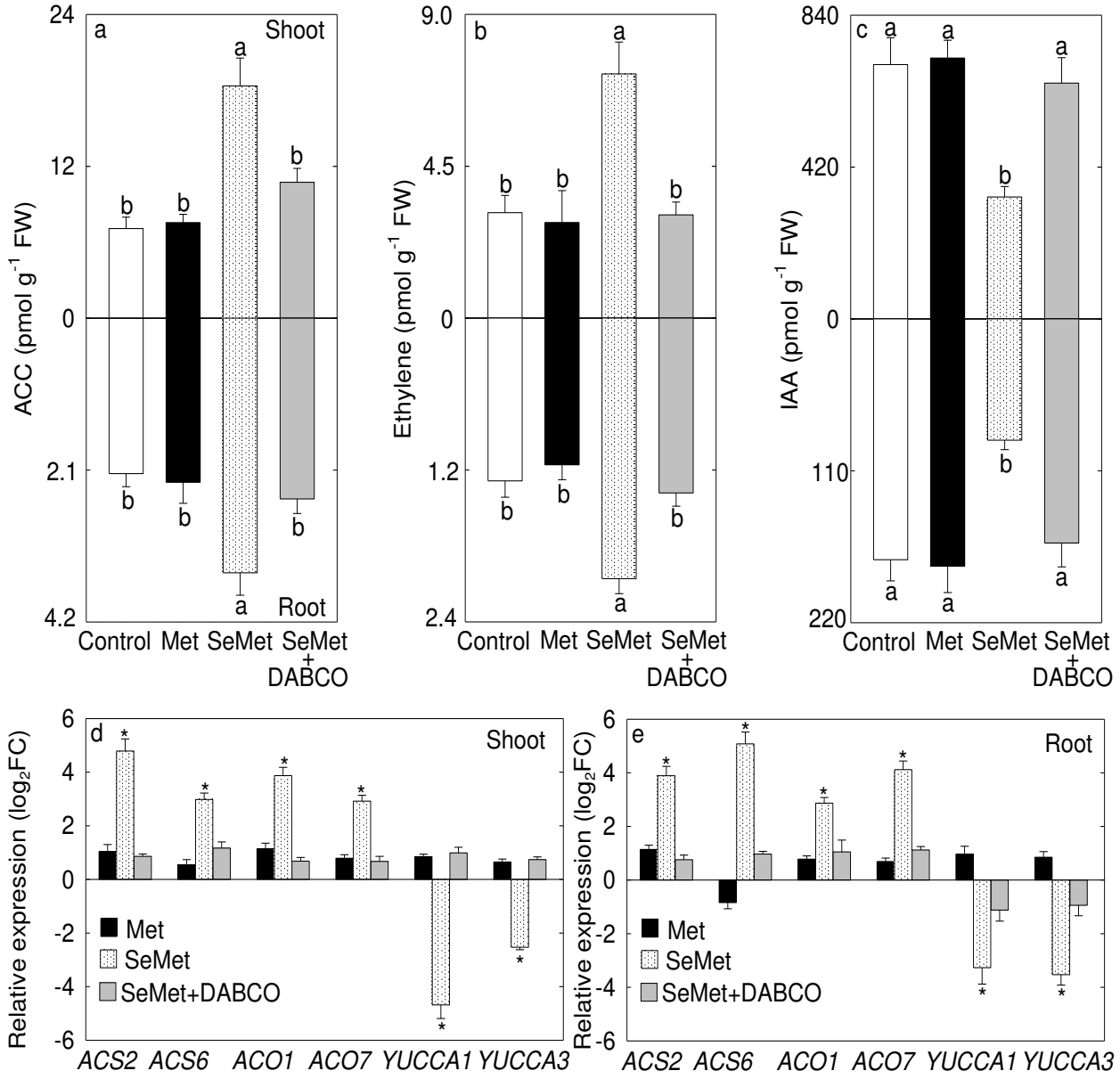

Fig. 4. Concentrations of ACC, ethylene, IAA as well as the expression of genes related with ethylene and IAA biosynthesis in shoot and root of rice seedlings treated with Met, SeMet and SeMet+DABCO. **a** ACC concentration. **b** Ethylene concentration. **c** IAA concentration. **d** Gene expression in shoot. **e** Gene expression in root. Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Asterisks indicate values determined by the Student's *t*-test to be significantly different from the control ($P < 0.05$). Data are mean ± standard error of three separate experiments, with five replicates, for concentrations of ethylene, ACC and IAA, and three replicates for gene expression.

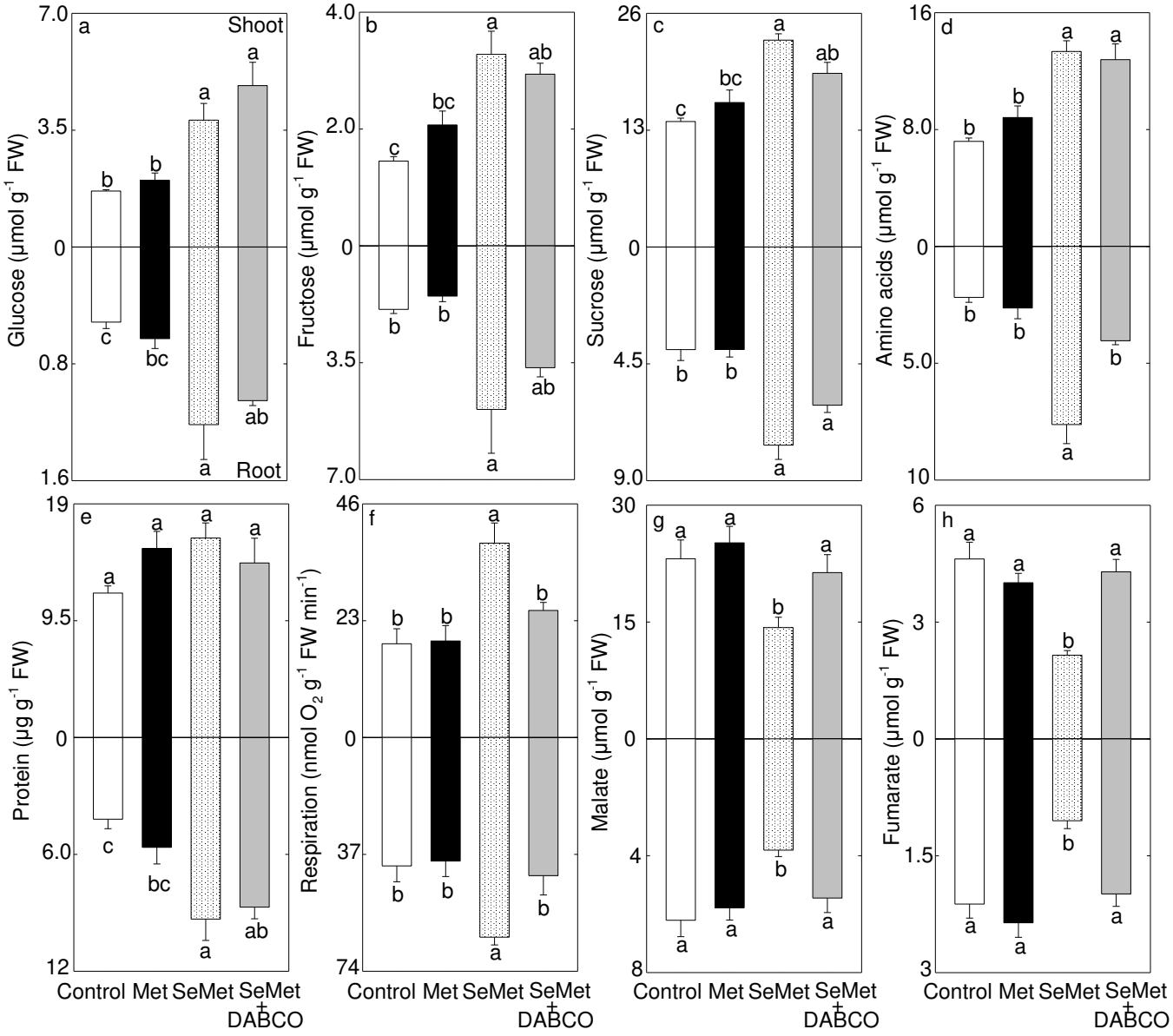

Fig. 5. Concentrations of metabolites and respiration rate in shoot and root of rice seedlings treated with Met, SeMet and SeMet+DABCO. **a** Glucose. **b** Fructose. **c** Sucrose. **d** Total amino acids. **e** Total protein. **f** Respiration rate in the dark. **g** Malate. **h** Fumarate. Bars followed by the same letters do not differ statistically at the 5% level by Tukey test. Data are mean ± standard error of three separate experiments, with five replicates each.

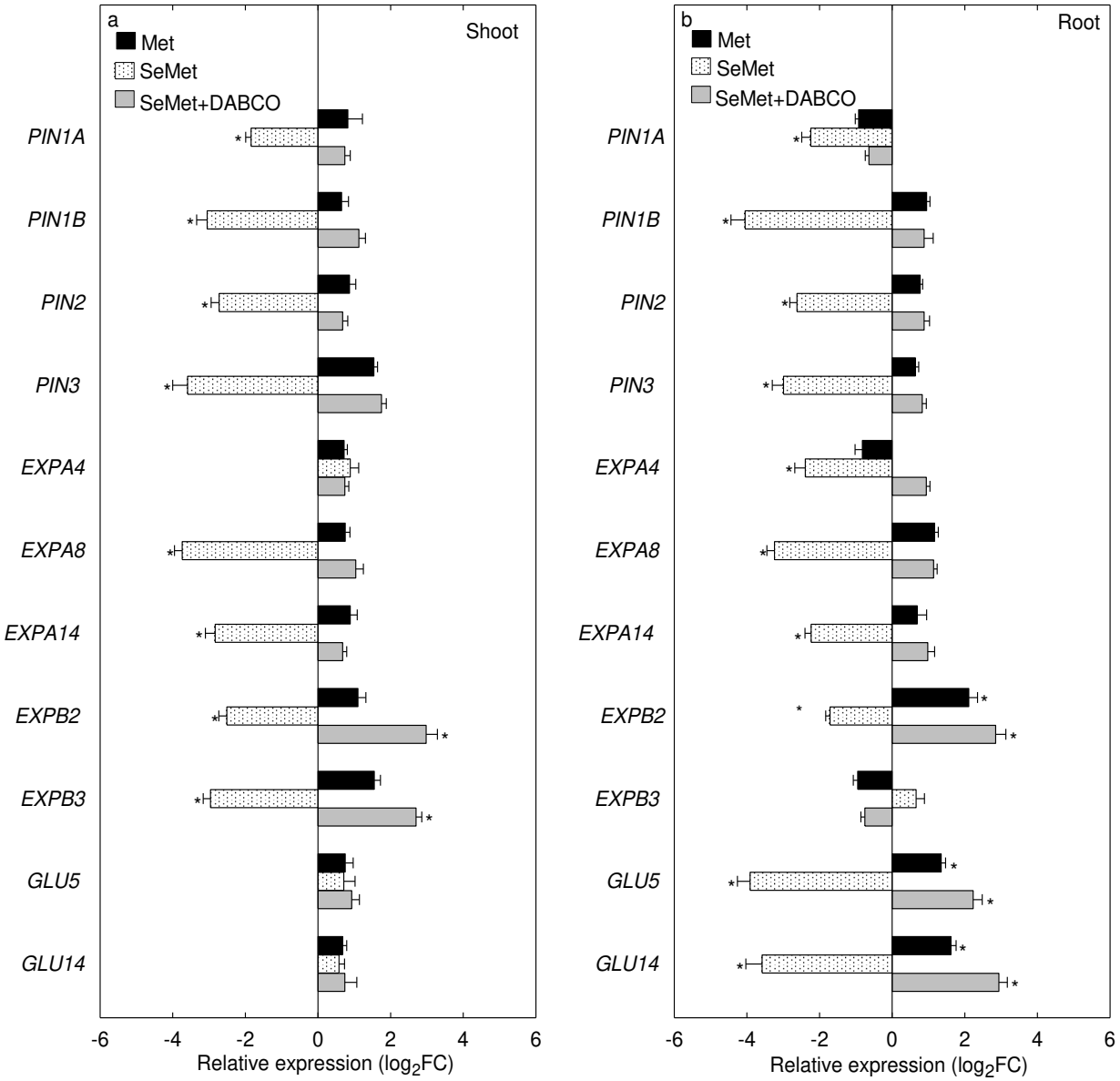

Fig. 6. Changes in gene expression in shoot and root of rice seedlings treated with Met, SeMet and SeMet+DABCO. **a** Relative expression levels of genes in shoot. **b** Relative expression levels of genes in roots. Asterisks indicate values determined by the Student's *t* test to be significantly different from the control ($P < 0.05$). Data are mean ± standard error of three separate experiments, with three replicates each.

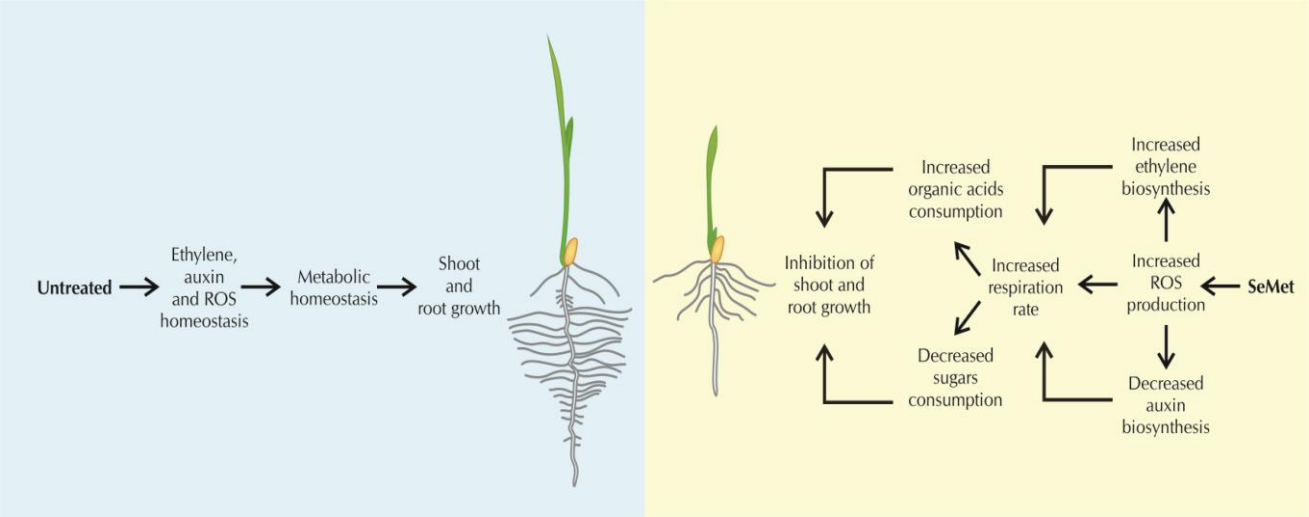

Fig. 7. Proposed model of SeMet-dependent regulation in rice seedling growth. Diagrams of rice seedlings untreated (left) and treated (right) with SeMet. Treatment of rice seedlings with SeMet increases ROS accumulation, which may inhibit auxin biosynthesis while increase ethylene production. These combined effects might result in the stimulation of seedling respiration, which is positively correlated with organic acids consumption, but negatively with sugars consumption, thereby decreasing seedling growth.

Supplementary material

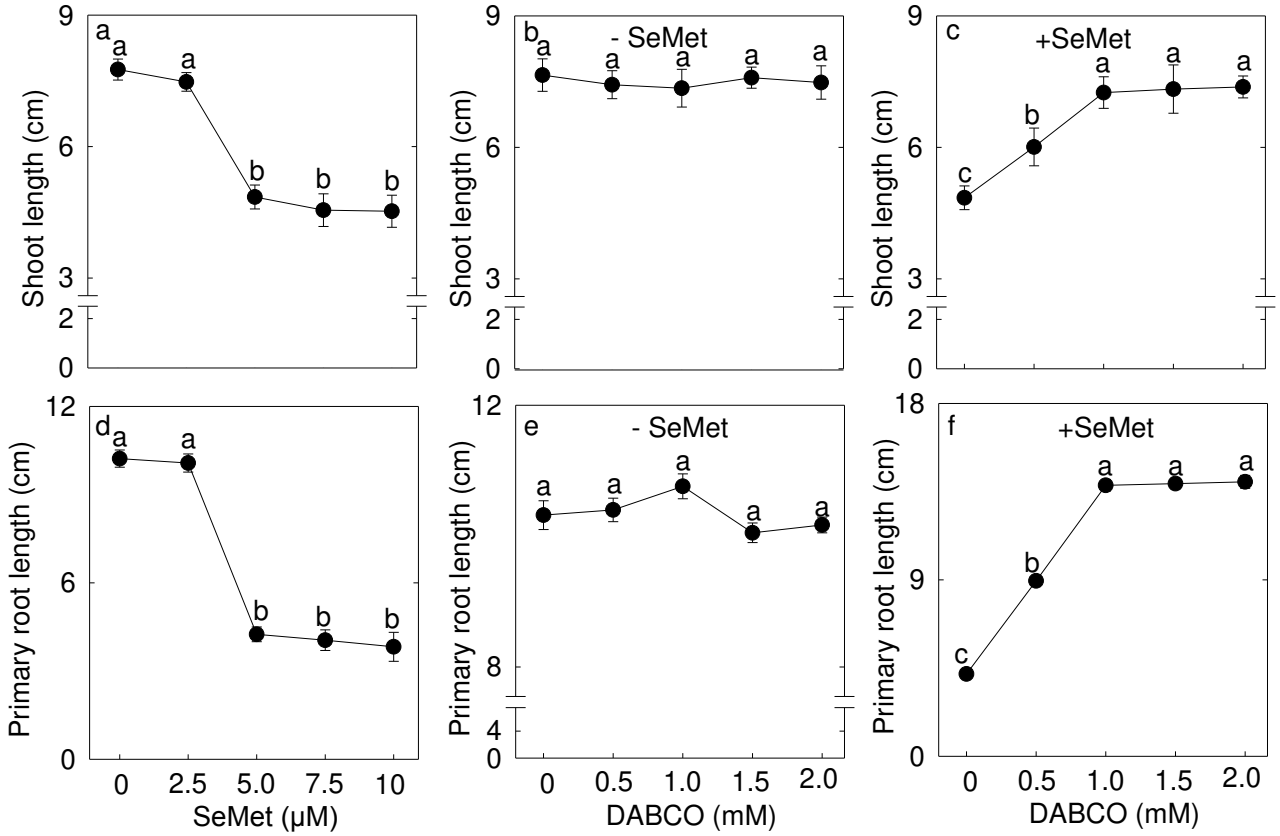

Fig. S1 Comparative analysis of shoot and root growth in rice seedlings treated with SeMet, DABCO and SeMet+DABCO. Length of shoot and primary root were determined 5 days from the start of seedling incubation in half-strength Hoagland's solution alone or also containing SeMet, DABCO and SeMet+DABCO. **a** SeMet dose-response curve for shoot length. **b** SeMet dose-response curve for primary root length. **c** DABCO dose-response curve for shoot length. **d** DABCO dose-response curve for primary root length. **e** Effect of DABCO supplied in SeMet (5 μM) solution on shoot growth. **f** Effect of DABCO supplied in SeMet (5 μM) solution on root growth. Values with the same letter do not differ statistically at the 5% level by Tukey test. Data are means ± standard error of three separate experiments, with five replicates each.

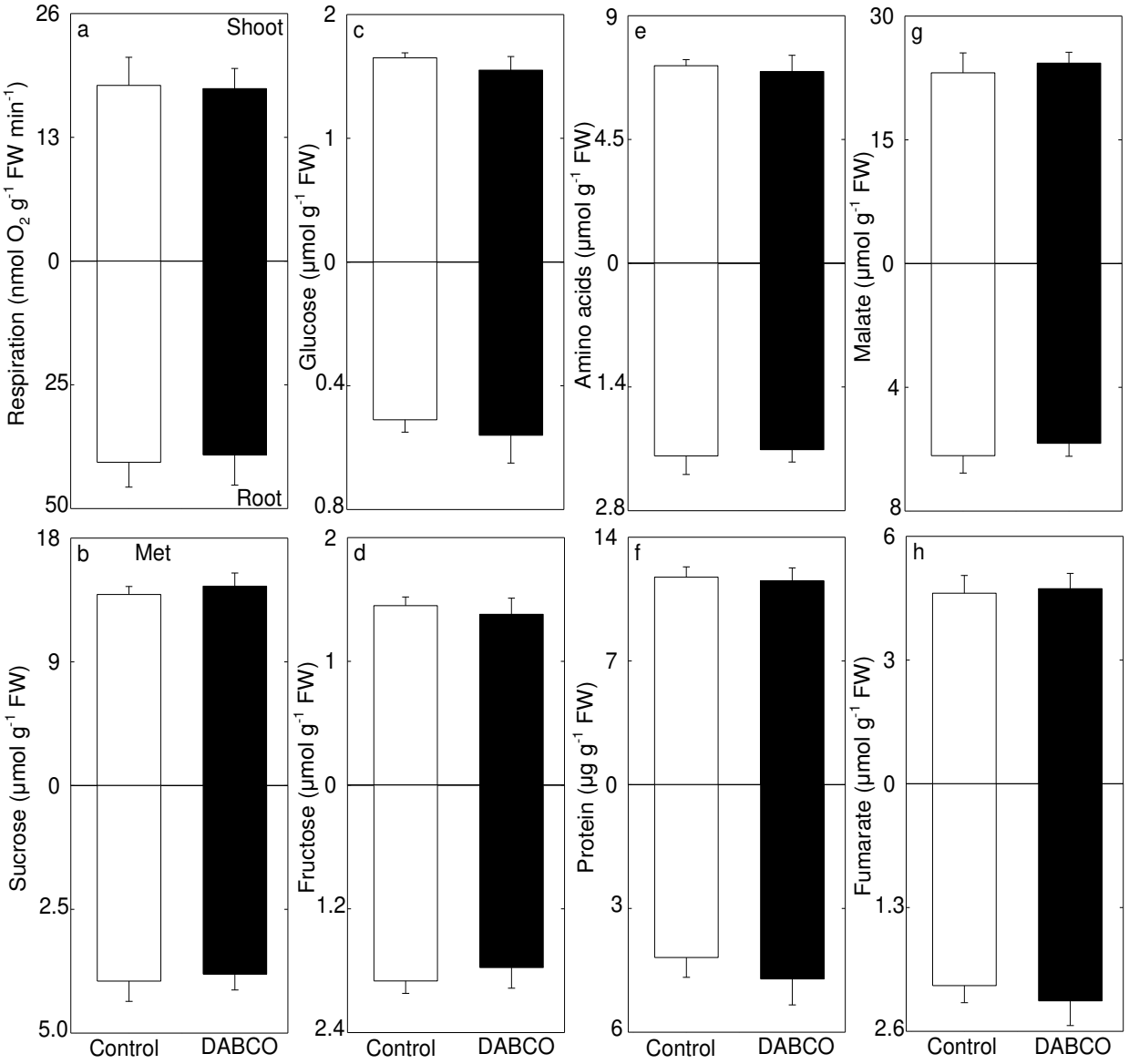

Fig.S2 Physiological and metabolic analyses of rice seedlings treated with DABCO. The analyses were assayed 5 days from the start of seedling incubation in half-strength Hoagland's solution (control) alone or also containing 1 mM DABCO. **a** Respiration rate in the dark. **b** Sucrose. **c** Glucose. **d** Fructose. **e** Amnino acids. **f** Protein. **g** Malate. **h** Fumarate. Data are means ± standard error of three separate experiments, with five replicates each.

Table S1 Primers sequences used for qRT- PCR analysis

Name	Primer sequences	
	Forward	Reverse
EXPB2	CAACCAGTACCCGTTCATGTCC	GTTGTTGGTGCACCGTATCTGG
EXPB3	TGCGGGTTCAAGAACACCAACC	GGGTGGTTGACGCATCTTATCTGG
EXPA4	TTGTACCGGAGTGATGGCCTTG	ACAGCCGCTAGCTACGACAAAG
EXPA8	TACACCTCCTCGGCTCAGTTCTAC	TGCCACGTCGATCAAAGCATAC
EXPA14	CCCGGTTATCTACCAAAGGGTTCC	AGTCGTGCCCGTTAATGGTAAAC
ACO1	TGGAGCAGCTGGATGATGCTTG	AGATGCCGTGGTTCAGGATCTC
ACO7	ATCGTCGTGTAGTACGCAGGGTTC	AGTTACCGTGATAACCACCCAACC
ACS2	TTTGGCGCCTTGACGGCCTC	AAAGGGAGCGCACCATGGCC
ACS6	CCGGGCGACACGTTCAGCTT	ACAGCGCGAACGGGTTCCAG
PIN1A	TCATCTGGTCGCTCGTCTGC	CGAACGTCGCCACCTTGTTC
PIN1B	TGCACCCTAGCATTCTCAGCA	CCCTCCTCCCAAATTCTACTTC
PIN2	CAGGGCTAGGAATGGCTATGT	GCAAACACAAACGGGACAA
PIN3	ATCCTGAGCACAGCGGTAAT	CAATGTCCGACAACAGGCTA
YUCCA1	TCATCGGACGCCCTCAACGTCGC	GGCAGAGCAAGATTATCAGTC
YUCCA3	GTGAGAACGGGCTCTACTCGGTCG	GCTTATGCATGACCGATGAACACG
GLU5	AACTCCTGCAACACTCTAAACAGC	ACGGTGATGCTGTTGTCGTACC
GLU14	AGCAGGACGACTTCACCTTCAG	TGCTCTGCAAGAATCCTTTGGTG
ACTIN	TGGATTGGAGGATCCATCTTGGC	CCTTGGCAATCCACATCTGCTG

GENERAL CONCLUSION

In this study, it was shown that the ability of selenium to regulate hormone biosynthesis and the physiological processes in rice, is dependent on the form of selenium supplied. The use of an inorganic form (selenite) alters the balance between auxin and ethylene by reducing the biosynthesis of these two hormones, which causes changes in the pattern of root growth. On the other hand, the use of an organic form (SeMet), inhibits the growth of rice seedlings, triggering the production of reactive oxygen species (ROS), with an increase in ethylene production, and reduction in auxin levels. In this sense, it is possible that the selenite is able to trigger a series of responses in the rice seedlings, even before being metabolized in seleno-amino acids. The direct application of a seleno-amino acid (SeMet) probably facilitates the formation of selenoproteins, which can increase ROS production, triggering different physiological responses in relation to those promoted by selenite. Therefore, this work highlights the potential role of organic and inorganic forms of Selenium in regulating the growth of rice seedlings, through alterations in the balance between auxin and ethylene. The elucidation of these effects may contribute to improving the strategies that attempt to increase the tolerance and accumulation of selenium in the rice crop.