A new role for glutathione in the regulation of root architecture linked to strigolactones

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**Abbreviations**: BSO: Buthionine sulfoximine; -ECS: -glutamyl cysteine synthetase; GSH: total glutathione; LR: lateral root; LRP: Lateral root primordium; MS: Murashige and Skoog; SLs: strigolactones.

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**SUMMARY**

Reduced glutathione (GSH) is required for root development but its functions are not characterised. The effects of GSH depletion on root development were therefore studied in relation to auxin and strigolactone (SL) signalling using a combination of molecular genetic approaches and pharmacological techniques. Lateral root density was significantly decreased in GSH synthesis mutants (*cad2-1, pad2-1,* *rax1-1*) but not by the GSH synthesis inhibitor, buthionine sulfoximine (BSO). BSO-induced GSH depletion did therefore not influence root architecture in the same way as genetic impairment. Root glutathione contents were similar in the wild type seedlings and *max3-9* and *max4-1* mutants that are deficient in SL synthesis and in the SL signalling mutant, *max2-1*. BSO-dependent inhibition of GSH synthesis depleted the tissue GSH pool to a similar extent in the wild type and SL synthesis mutants, with no effect on LR density. The application of the SL analogue GR24 increased root glutathione in the wild type, *max3-9* and *max4-1* seedlings but this increase was absent from *max2-1*. Taken together, these data establish a link between SLs and the GSH pool that occurs in a MAX2-dependent manner.

**INTRODUCTION**

Reduced glutathione (GSH) has wide range of functions in plants including antioxidant defence and biotic and abiotic stress responses, as well as defence against heavy metals, and the formation of conjugates with metabolites and xenobiotics (Noctor et al. 2011, 2012). GSH is a key component of cellular redox homeostasis and signalling and it has important roles in secondary metabolism and sulphur metabolism. GSH is required for cell proliferation and is recruited into the nucleus early in the cell cycle (García-Giménez et al. 2013). Genetic evidence has demonstrated that GSH is also essential for root growth (Vernoux et al. 2000) but the mechanisms involved remain poorly characterised.

The GSH synthesis pathway in plants as in other organisms involves two ATP-dependent enzymes that produce GSH from glutamate, cysteine, and glycine, via the intermediate, -EC. The first enzyme, glutamate-cysteine ligase (also called -glutamylcysteine synthetase (-ECS) is encoded by a single gene (*GSH1*) in Arabidopsis. -ECS is localised to plastids in Arabidopsis (Wachter et al. 2005). Knockout mutations in the *GSH1* gene produce an embryo-lethal phenotype (Cairns et al. 2006). Mutants with less severe defects in the *GSH1* gene have been useful in the characterization of GSH functions in plants. The *rootmeristemless1* (*rml1*) mutant, which has less than 5% of wild-type glutathione contents, shows a marked phenotype because of an early arrest of root apical meristem (Vernoux et al. 2000). The *rml1* mutant shows a much less severe shoot phenotype, because of redundancy between glutathione and thioredoxins (TRX) in the control of shoot apical meristem functions (*NTRA*, *NTRB*; Reichheld et al. 2007). The functions of GSH in plants have also been studied using buthionine sulfoximine (BSO), which is considered to be a specific inhibitor of -ECS. For example, BSO-dependent inhibition of GSH synthesis in Arabidopsis was used to confirm that GSH is required for root meristem development (Koprivova, Mugford & Kopriva 2010).

In addition to *rml1,* a number of other mutants with defects in the *GSH1* gene are available, with varying levels of tissue glutathione accumulation. For example, the *cad2-1* mutant has15-30 % of wild type GSH(Cobbet et al. 1998). The glutathione pool in the *rax1-1* mutant is decreased by between 50-80% of the wild type (Ball et al. 2004) and by 80% in the *pad2-1* mutant (Parisy et al. 2007).The *cad2* mutant was identified by its enhanced sensitivity to cadmium. The *rax1* was identified by the altered expression of the gene encoding the cytosolic ascorbate peroxidise, *APX2*. The *pad2* mutant has decreased camalexin contents and shows enhanced sensitivity to fungal pathogens (Howden et al., 1995; Cobbett et al., 1998; Ball et al., 2004; Parisy et al., 2007). Mutations in the *GSH2* gene have also been very useful in elucidating the functions of glutathione in plants (Pasternak et al. 2007; Au et al. 2012).

The GSH synthesis mutants generally have a shorter primary root than the wild type (Bashandy et al. 2010; Koprivova et al. 2010) but the link between GSH and root architecture has not been fully explored. The *cad2-1* and *pad2-1* mutants have fewer lateral (LR) roots than the wild type (Bashandy et al. 2010) and it was suggested that GSH depletion limits auxin transport by decreased expression of PIN-formed proteins (Bashandy et al. 2010; Koprivova et al. 2010). However, no detailed characterisation of the role of GSH in LR development has been reported to date.

The formation of LRs occurs in the differentiation zone of the root that is distant from the root apical meristem (Esau 1965; De Smet et al. 2006). Its initiation requires the activation of cell division from the pericycle layer, which leads to the production of a LR primordium (LRP), within which an active LR apical meristem is formed (Malamy & Benfey 1997). The generation of each LRP is regulated by a network of interacting pathways that affect different phases of the developmental process (Malamy & Benfey 1997; De Smet et al. 2006). Only a limited number of pericycle cells, called founder cells, become active to form each LRP (De Smet et al. 2006). The activation process occurs in a well defined spatial order such that once activated, the founder cells undergo a series of anticlinal and periclinal divisions that can be grouped into different stages that are designated I (initiation) to VIII (emergence), based on the cell layers formed (Malamy & Benfey 1997).

Auxin plays a key role during LRP development (Benkova et al. 2003; Casimiro et al. 2003; Fukaki & Tasaka 2009). Auxin is not only indispensable for the initiation process that enables the pericycle cells to re-enter the cell cycle (Casimiro et al. 2003) but it is also required for the correct control of cell division and patterning throughout the entire developmental process (De Smet, Signora & Beeckman 2003; Benkova & Bielach 2010). The presence of auxin during LR formation is often demonstrated by the expression of markers for auxin accumulation and response such as DR5::GUS (Benkova et al. 2003; Dubrovsky et al. 2008). Other hormones such as ethylene, abscisic acid (ABA) and cytokinins are also involved in the control of LR formation. Ethylene stimulates LR formation while cytokinins act antagonistically to auxin and ethylene (Aloni et al. 2006; Fukaki & Tasaka 2009). Like cytokinins, ABA blocks LR development (De Smet et al. 2003; Fukaki & Tasaka 2009). However, the ABA pathway, which is independent of auxin, plays an inhibitory role in the stages just prior to LR emergence (De Smet et al. 2003).

Recently it has been described that also strigolactones (SLs) are involved in the LR formation process, as the synthetic SL, GR24, inhibits LRP formation both at the initiation stages (Kapulnik et al. 2011) and at later stages of development (Ruyter-Spira et al. 2011).

Strigolactones (SLs) are important hormones that control plant development and plant interactions with the environment (Gomez-Roldan et al. 2008; Leyser 2009; Umehara et al. 2008; Dun, Brewer & Beveridge 2009). They are synthesized from carotenoids and act as signalling molecules in response to metabolic and environmental triggers (Dun et al. 2009; Domagalska & Leyser 2011). SLs function downstream of auxin in the control shoot branching by diminishing polar auxin transport to inhibit bud outgrowth (Gomez-Roldan et al. 2008; Umehara et al. 2008; Hayward et al. 2009; Crawford et al. 2010). However, SLs and auxin interact in a dynamic feedback loop, in which each hormone regulates the levels of the other (Bennet et al. 2006; Hayward et al. 2009).

SLs have been identified in species such as Arabidopsis, pea, rice and petunia that share a common synthesis and signalling pathway (Xie, Yoneyama & Yoneyama 2010). Mutants in either SL synthesis or signalling exhibit an altered branching phenotype. While our current knowledge of the SL synthesis remains incomplete, the biosynthetic pathway is considered to involve a -carotene isomerase (D27) and two carotenoid cleavage dioxygenases (CCDs), which are called CCD7 and CCD8 or MAX3 and MAX4 respectively in *Arabidopsis thaliana* (Gomez-Roldan et al. 2008; Umehara et al. 2008; Hayward et al. 2009; Waters et al. 2012)*.* A subsequent oxidation by a cytochrome P450 (MAX1) results in the production of a mobile compound that requires an F-box protein (MAX2) and a  hydrolase (D14) to elicit its effects on shoot branching (Stirnberg, van de Sande & Leyser 2002; Stirnberg, Furner & Leyser 2007; Gomez-Roldan et al. 2008; Umehara et al. 2008; Hayward et al. 2009; Xie et al. 2010; Hamiaux et al. 2012).

In addition to regulating shoot and root branching, SLs also have a strong influence on cellular redox homeostasis (Woo et al. 2004). For example, the *ore9* mutant, which is deficient in MAX2, exhibits delayed senescence and is more tolerant to oxidative stress than the wild type (Woo et al. 2001; Stirnberg et al. 2002; Woo et al. 2004). However, the precise nature of the interaction between SLs and redox metabolites is unknown. Our aim in the present study was to characterise the relationship between GSH and SL synthesis and signalling in the control of root architecture.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

Seeds of wild type (Columbia; Col-0) *Arabidopsis thaliana*, strigolactone signalling mutant (*max2-1*)*,* strigolactone synthesis mutants (*max3-9, max4-1*) were kindly provided by Dr. Ottoline Leyser. The glutathione synthesis mutants (*cad2-1, pad2-1,* *rax1-1*) were obtained from the ABRC stock centre <http://abrc.osu.edu/>). All the mutants are on the Columbia background(*Col-0*). Seeds were surface sterilized, immersed in ethanol 75% for 1 min, then in sodium hypochlorite 4% for 5 min and then rinsed three times with sterilised water. They were then placed on 12 cm square plates with ½ strength MS medium solidified with 0.8% agar (Agar agar, Fisher Scientific) and supplemented with 0.01% myo-inositol (Sigma-Aldrich), 0.05% MES buffer (pH 5.7) (Alfa Aesar) and 1% sucrose (Sigma-Aldrich) and grown vertically for 3 days. Then seedling were transferred to new plates and grown for 5 more days supplemented with GR24 (2 M, dissolved in acetone), BSO (Sigma-Aldrich) (0.25 mM, dissolved in water), NAA (Sigma-Aldrich) (0.1 M, dissolved in DMSO) and combinations of 0.1 M NAA with GR24 (2 M).

All plates were cold stratified for 2 days and then placed to a plant growth cabinet with 16 hours day photoperiod and 21°C.

Three independent biological replications with 5 plates per treatment and genotype and 8 seeds per plate were used.

To screen the effects of glutathione on auxin-dependent signalling in relation to SL signalling or availability, the DR5::GUS marker line (Ulmasov et al. 1997) was crossed into the *max2-1*, *max3-9* and *max4-1* mutant lines and made homozygous for both the marker and the mutation.

**Root system architecture measurements**

The root length and number of lateral roots (LR) formed per treatment was analyzed on 8 days old seedlings. Photos were taken and the root length was measured using ImageJ software. Lateral root density (LRD) was calculated as the division between the number of visible lateral roots and the main root length for each root analysed.

**Glutathione measurement**

Glutathione was assayed by spectrophotometry in a FluoStar Omega plate reader (BMG Labtech GmbH, Ortenberg, Germany) as described in Queval & Noctor (2007). Glutathione levels were determined as described by Noctor & Foyer (1998) and Foyer et al. (2008). Plant material was homogenized in 1 M HClO4 with a precooled mortar and pestle and liquid N2. The mixture was clarified by centrifugation at 14000 *g* for 10 min at 4°C and the pH of the supernatant was adjusted to between pH 5.5 and pH 6.0 by the addition of 2.5 M K2CO3 (Foyer et al. 1983). Total glutathione was analyzed using dithio-bis-2-nitrobenzoic acid (DTNB) and glutathione reductase in the presence of NADPH as described by Noctor & Foyer (1998). Three independent biological samples were analysed per treatment and genotype.

**Root staging**

The stages of the development of the LR primordium were determined essentially as described by Malamy & Benfey (1997). The stages of primordia development were classified as described by Péret et al. (2009) as follows: stage I (single layered primordium composed of up to ten small cells of equal length formed from individual or a pairs of pericycle founder cells), stage II (periclinal cell division forming an inner and an outer layer), stages III, IV, V, VI and VII (anticlinal and periclinal divisions create a domeshaped primordium), stage VIII (emergence of the primordium from the parental root) and grouped for an easy analysis.

**Gus staining**

The β-Glucuuronidase (GUS) stain was performed according to the methods of Jefferson, Kavanagh & Bevan 1987 and Malamy & Benfey 1997, with some modifications. Briefly, 8 day-old seedlings were harvested and placed in NT buffer (100 mM Tris and 50 mM NaCl, pH 7). This buffer was replaced by GUS buffer (2 mM K3[Fe(CN)6] dissolved in NT buffer and 2 mM X-gluc dissolved in DMSO) and stained for 2 or 16 hours at 37 °C. The reaction was stopped by replacing the GUS buffer by 70% ethanol, roots were cleared as described by Malamy & Benfey (1997) and then photographs were taken. The two different incubation times was due an over staining of the roots at 16 hours.

**Protein extraction and determination of GUS activity by fluorometric method.**

Protein extracts were prepared from 8 days old seedlings using 120 roots in 350 μL of buffer containing 50 mM phosphate buffer pH 7, 10 mM 2-βmercapthoethanol (Fluka), 10 mM Na2-EDTA (Sigma-Aldrich), and 0.1% Triton X-100 (Sigma-Aldrich). The ground plant tissue was centrifuged at 14000 rpm twice at 4°C for 10 min to remove insoluble material and protein content was measured according to Bradford (1976). GUS activity, expressed as units of GUS protein relative to the total amount of soluble extracted protein (units GUS protein mg−1), was determined as described by Breyne et al. (1993) in a Fluorstar Optima plate reader (BMG Labtech GmbH, Ortenberg, Germany). Briefly, 10uL of diluted protein (2 µg, 1 µg, 0.1 µg and 0.01 µg) , 240 µL of protein extraction buffer and 10 µL of 4 mM 4-Methylumbelliferyl-β-D-glucuronide hydrate (4-MUG, Sigma-Aldrich) were added to each well of the black plates (NUNC polysorp 96). The calibration was done with β-glucuronidase (GUS) enzyme (Sigma-Aldrich) and extraction buffer was used as blank. After 10 min incubation in dark at 37°C the measurement started, using 355 and 450 nm as excitation and emission filters respectively, measuring every two minutes for 30 minutes. Three independent biological samples were analysed per treatment and genotype.

**Data analysis**

Data represent mean ± standard error of the mean (SEM) of three independent biological replicates. Statistical analysis was performed using SPSS by one-way analysis of the variance (ANOVA) and Tukey’b post hoc test to analyse the difference among all the treatments and genotypes with a significant level of p<0.05.

**RESULTS**

**Glutathione deficiency affects Arabidopsis root architecture**

To study the relationship between root architecture and GSH, we measured primary root length and LR density in the *cad2-1, pad2-1* and *rax1-1* mutants that possess between 20 and 50% of the wild type GSH content. After 8 days of growth, the roots of *cad2-1*and *rax1-1* presented a significantly shorter primary root compared to the wild type, but this was not the case for *pad2-1* (Figure 1a). The *cad2-1, pad2-1* and *rax1-1* mutants had a lower number of LRs leading to lower LR densities in all the mutant genotypes compared to the wild type (Figure 1b,c). These data indicate a clear link between glutathione and root development.

Next, we investigated whether we could obtain the same phenotype via the addition of the glutathione synthesis inhibitor, buthionine sulfoximine (BSO). The addition of 0.25 mM BSO resulted in a decrease in the root GSH pool of over 70% (Figure 2a). After 8 days of growth (5 in the presence of BSO) a decrease in the primary root length was observed (Figure 2b). The number of LRs was also decreased but the LR density was similar to the wild type (Figure 2c). We next examined whether BSO could influence LR development. To do so, the stages of the primordia development were analysed in 8-day old seedlings essentially as described by Malamy & Benfey, (1997) in the absence and presence of BSO. No differences were found between mock and treated plants, as the same number of lateral root primordia was found at each developmental stage (Figure 2d). This finding indicates a clear effect of BSO on the main root length, but no effect on lateral root development.

**Glutathione depletion reduces the auxin accumulation in the hypocotyls, but not in the roots.**

An earlier study of *cad2-1* and *pad2-1* mutants suggested that GSH deficiency caused a reduction in auxin transport (Bashandy et al., 2010) and a decrease in the expression of PIN proteins (Bashandy et al., 2010; Koprivova et al. 2010). We therefore analysed the effects of BSO on the auxin response using the expression of the auxin response reporter, DR5::GUS in the root tip. No changes in DR5::GUS expression were detected in the roots of 8 days old seedlings compared with untreated plants (Figure 3a-b), after 5 days of treatment with BSO. In agreement, no significant changes in GUS activity were detected in the protein extracts derived from those roots (Figure 3c). Conversely, we have found that BSO reduced the DR5::GUS expression in the hypocotyls of wild type plants (Figure 3c-d).

**The synthetic strigolactone GR24 increase glutathione in a MAX2 dependent way.**

Like GSH synthesis mutants, SLs modify root architecture (Kapulnik et al. 2011; Ruyter-Spira et al. 2011). It has previously been shown that treatments with low levels of GR24 (1.25 M) increase primary root length in wild type plants and also in the SL synthesis mutant *max4* but not in the *max2* SL-signalling mutant (Ruyter-Spira et al. 2011). Higher GR24 concentrations (2.5-10 M) caused a MAX2-independent decrease in main root length (Ruyter-Spira et al. 2011). We therefore checked the effectiveness of GR24 in the regulation of root architecture. The addition of 2M GR24 significantly decreased the LR density in the *Col-0* plants and in the *max3-9* and *max4-1* mutants, but not in *max2-1,* confirming the role of MAX2 as an important SL signalling component (Supplementary figure 1). We have observed a decrease in the length of the main root and in the LR density following the addition of 2 µM GR24 that occurred in a MAX2-dependent manner.

To determine the relationship between root glutathione contents and SLs, we analysed the GSH content of SL synthesis and signalling mutants and the effects of the addition of GR24 on the root GSH pool. The levels of root GSH were similar in all the genotypes under control conditions. However, while the addition of GR24 led to a significant increase in root GSH in the *Col-0*, *max3-9* and *max4-1* mutants, it had no significant effect on the GSH levels of the *max2-1* mutant roots(Figure 4). These results indicate that the GR24-dependent increase in the GSH pool requires MAX2 signalling.

**Effects of BSO in the *max* mutants**.

To further explore the link between endogenous SLs and GSH, the effect of BSO was analysed in *Col-0* and *max* mutants. The plants were grown for 3 days under control conditions and then for 5 more days either in the absence (0) or presence of 0.25mM of BSO. The treatment with BSO decreased the total root glutathione pool in all genotypes by about 70% (Figure 5a). The BSO depletion of the root glutathione pool is therefore independent of MAX2.

Next, we investigated whether the effect of BSO had a differential effect on root architecture in the different *max* mutants compared to *Col-0*. All the genotypes showed a similar BSO-dependent reduction in primary root length (Figure 5b) and BSO had no effect on LR density comparing each control and treated genotype (Figure 5c). As previously described, *max2-1* had a higher LR density than the other genotypes. No changes in the DR5::GUS expression and activity were detected in the roots of the *max* backgrounds in the presence of BSO just as we observed in *Col0* (Supplementary Figure 2), and no significant differences in the GUS activity from root extracts. However, in contrary to wild type plants, no changes were found in the expression in the hypocotyls treated with BSO compared to the untreated plants (Supplementary Figure 2).

**Effects of GR24 in the glutathione synthesis mutants (*cad2-1, pad2-1* and *rax1-1*)**.

The GSH synthesis mutants all show altered root architecture compared to wild type under control conditions. We therefore decided to check the effect of GR24 on root architecture in the GSH synthesis mutants. In all cases the addition of GR24 reduced primary root length in a similar manner to that observed in *Col-0* (Figure 6a). The lateral root density decreased upon application of GR24 in the GSH synthesis mutants to a lower extent compared to *Col-0* (Figure 6b). However, since the *cad2-1* and *rax1-1* mutants have a relatively lower root density under control conditions, these data should be handled with care.

**Auxin effects on lateral root density requires SLs**

Previous studies have linked GSH to auxin signalling and transport (Agusti et al. 2011; Bashandy et al. 2010; Kapulnik et al. 2011; Koltai, 2012; Koprivova et al. 2010; Hayward et al. 2009; Ruyter-Spira et al. 2011). In order to explore the relationships between the effects of auxin, SLs and GSH in the control of root architecture, 3 day-old WT and *max* mutant seedlings were grown for 5 more days in either the absence or presence of 0.1 M NAA and in combination with 2 GR.

The addition of low levels (0.1 M) of NAA had no effect on the root GSH contents (Figure 7a) but had a pronounced effect on root architecture, with a significant increase in the LR density in all the genotypes (Figure 7b). However, the NAA-dependent increase in the LR density was not the same in all genotypes. The *max2-1* mutant was less responsive to presence of NAA, showing an increase in LR density in the presence of NAA of 3.5 times compared to 5.7 times in *Col-0*. In the absence of NAA *max2-1* had a higher LR density than the wild type. In the presence of NAA *max2-1* had a similar LR density to the wild type suggesting a decrease in net sensitivity. The *max3-9* and *max4-1* mutants had a lower LR density than the wild type in the presence of NAA, showing a 4.9 and 4.3 fold increase in LR density in the presence of NAA respectively. compared with 5.7 times in *Col-0*. In the absence of auxin these mutants had similar LR densities (Figure 7b). These results again suggest a decrease in net sensitivity SLs biosynthesis and signalling are therefore required for the auxin response mechanism.

When seedlings were grown for 5 days in the presence of 0.1M NAA and 2M GR24 the root GSH content was higher in the *Col-0*, *max3-9* and *max4-1* genotypes than controls (Figure 7a), similar to our earlier observations with GR24 treatment alone (Figure 4). However, in the presence of 2M GR24, the auxin-dependent increase in LR density was less marked in all genotypes relative to the auxin treatment alone, with the lowest fold increase in *max2-1* (with 2.8 times compared with 3.4-3.4 of wild type and *max3-9* and  *max4-1*) (Figure 7b).

Taken together, the observations suggest that the SL mutants have a lower sensitivity to NAA than *Col-0* in terms of regulation of LR density. The addition of exogenous SLs together with NAA decreased the effect of NAA alone in a MAX2-independent manner. Moreover, the addition of NAA did not change the GR24 dependent increase in GSH concentration.

**DISCUSSION**

The results presented here provide evidence of a novel function for GSH that is linked to auxin and SLs signalling in the control of root architecture. Moreover, the data demonstrate that BSO-dependent inhibition of GSH synthesis does not produce the same phenotype in terms of root architecture as that observed in mutants that are defective in GSH synthesis. LR density was not changed by BSO, whereas it was significantly decreased in all the GSH synthesis mutants. The differences in these observations may be explained by differences in the intracellular distribution of GSH in the mutants compared to that occurring in cells where GSH synthesis is blocked by BSO. Previous evidence has indicated that some intracellular compartments are more resistant to BSO-dependent GSH depletion than others (Markovic et al. 2009). Moreover, root meristem development could be restored in BSO-treated plants by the addition of dithiotreitol (Koprivova et al. 2010) but not in the *rml1* mutant, which exclusively required GSH to restore root growth (Vernoux et al. 2000).

The application of the SL analogue GR24 influences the root glutathione pool in a MAX2-dependent manner. While root glutathione contents were similar in the *max* mutants and the wild type, the addition of GR24 led to an increase in root glutathione in the wild type plants and also in the roots of the SLs synthesis mutants (*max3-9* and *max4-1*). The GR24-dependent increase in root glutathione was absent in SL signalling mutant, *max2-1*. These data establish a link between exogenous SLs and the GSH pool that occurs in a MAX2-dependent manner.

GSH depletion might alter the response of root architecture to GR24, as illustrated by our observations in the GSH synthesis mutants (*cad2-1*, *pad2-1* and *rax1-1*) that have much lower GSH levels than the wild type plants (Cobbet et al. 1998; Ball et al. 2004; Parisy et al. 2007; Koprivova et al. 2010). The percentage of reduction in LR density is smaller in the GSH-deficient mutants than in wild type but these mutants have a lower LR density relative to the wild type in the absence of GR24. It is therefore difficult to state whether or not they have an altered sensitivity to GR24. Nevertheless, these results suggest that the GR24-dependent stimulation of root glutathione accumulation might be important in the SL-dependent inhibition of LR development.

BSO depleted the total glutathione pool in wild type and *max* mutants in a similar extent. The length of the primary root was decreased in the presence of BSO, as described previously (Koprivova et al. 2010). BSO treatment had no effect on LR density in any of the genotypes. BSO-dependent glutathione depletion did not alter LR density or the staging of the roots, indicating that inhibition of GSH synthesis per se does not impair LR formation. In contrast, all of the GSH-deficient mutants were responsive to GR24, suggesting that mechanisms other than GSH synthesis, such as the intracellular partitioning of GSH, might be crucial to the GR24-dependent regulation of LR density.

Earlier studies indicated that glutathione depletion impairs the growth of the primary root and blocks auxin transport (Bashandy et al. 2010; Koprivova et al. 2010). The *max* mutants have higher auxin transport capacity (Bennet et al. 2006) and higher levels of auxin in the stem than the wild type (Hayward et al. 2009). Moreover, the MAX pathway is responsible of the regulation of PIN protein localization and expression and hence auxin transport (Bennet et al. 2006). The data presented here from an analysis of 25 different plants per genotype show that the *max* mutants did not have a visibly higher level of expression of the auxin responsive reporter DR5::GUS in the roots under control conditions. Previous studies reported a higher level of DR5::GUS expression in *max4* compared to the wild type (Ruyter-Spira et al. 2011). However, no, changes in PIN accumulation were found in the roots of Col0 after treatment with GR24 (Shinohara, Taylor & Leyser, 2013). Inhibition of GSH synthesis by BSO did not change the auxin responsive reporter DR5::GUS expression in the roots. Crucially, DR5::GUS expression was decreased in the vasculature of the hypocotyls of the wild type plants but not in the *max* mutants. These findings demonstrate that GSH depletion limits the expression of auxin-responsive genes in the vasculature of the hypocotyls but not in the main root tip. In addition, the influence of GSH on auxin-responsive gene expression was MAX-dependent, as no effect was observed in the *max* mutants.

The addition of low levels of auxin (NAA) had no effect on root glutathione levels. NAA decreased primary root length in a similar manner in all genotypes in line with previous observations. Exogenous addition of auxin reduces primary root length by decreasing cell elongation and reducing cell division (Grieneisen et al. 2007; Rahman et al. 2007; Ioio et al. 2008). However, the auxin-dependent increase in LR density was less marked in the *max* mutants than the wild type implying that SLs can contribute to the auxin-dependent increase in LR density. The combined addition of NAA and GR24 resulted in a decrease in LR density in all genotypes, including in the *max2* mutants compared to NAA treatment alone. These data might at first sight appear to be contradictory but such observations may result from the operation of negative feed-back loops. For example, GR24 can reduce MAX4 gene expression and possibly also endogenous SL levels (Mashiguchi et al. 2009; Rasmussen et al. 2013). GR24 has been reported to reduce the effects of auxins on root hair growth (Koltai et al. 2010) and on the adventitious roots in tomato (Kohlen et al. 2012) and Arabidopsis (Rasmussen et al. 2012). Further work on the spatio-temporal expression patterns of SL, auxin and LR markers in different genetic backgrounds is needed in order to resolve the nature of interactions between auxin and SL signalling during LR development.

The present analysis allows us to suggest links between auxin, SL and GSH that are summarised in Figure 8. The addition of exogenous SLs, GR24, increases root glutathione levels and blocks LR formation in a MAX2-dependent manner. However, the tissue glutathione level is independent of the endogenous levels of SLs, as all the genotypes possess similar levels (Figure 8a). Genetic depletion of glutathione (in *cad2-1, pad2-1,* *rax1-1*) produces a different root architecture phenotype to that observed when GSH synthesis is blocked pharmacologically (BSO). Hence, factors other than the rate of GSH synthesis are important in the GSH and SL interaction that regulates LR density. BSO-dependent inhibition of GSH synthesis depleted the tissue GSH pool to a similar extent in the wild type and SLs mutants, with no effect on LR density (Figure 8b).

Finally, we report an interesting new link between auxin and SLs, as the *max* mutants are less sensitive to auxin-induced effects on LR production. In the presence of low levels of NAA, LR density was increased by 5.7 in wild type plants relative to untreated controls. The NAA-dependent increase in LR density was smaller in all the *max* mutants (4.9-4.3 times in *max3-9* and *max4-,* respectively, and 3.5 times in *max2-1*; Figure 8c). The addition of GR24 together with NAA produced a net reduction in LR density compared to the treatment with NAA alone. The effect of exogenously added auxin seems to be modified by GR24 in a MAX2 independent manner (Figure 8d), since no differences were found in the fold increase between wild type and *max3-9* and *max4-1*, but a lower increase was found in *max2-1*. GR24 increased the root glutathione pool to a similar extent in the presence or absence of auxin. Therefore, SLs are needed for the signalling of the auxin in the control of LR development. These results demonstrate the complexity of the SL, auxin, GSH interaction, involving a number of the steps that modulate auxin-dependent mechanisms in the SL mutants, which have increased levels of auxin and an increased capacity for auxin transport compared to the wild type (Bennet et al. 2006).

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**Legends to Figures**

**Figure 1. Root architecture in mutants that are defective in glutathione synthesis.** Primary root length (a), number of visible lateral roots (b) and lateral root density (c) in wild type (*Col-0*) and *pad2-1, cad2-1* and *rax1-1* mutants. Data shown mean ± standard error of the mean (SEM) of three independent biological replications. In each graph, different letters indicate significant differences (p<0.05) (ANOVA and Tukey’b post hoc test).

**Figure 2: Effects of the glutathione synthesis inhibitor BSO on root glutathione contents and root architecture in wild type Arabidopsis.** Effects of 0.25 mM BSO on the glutathione content (a), primary root length (b), lateral root density (c) and the number of primordia lateral roots at the different stages (d). Data shown mean ± standard error of the mean (SEM) of three independent biological replications for (a), (b) and (c). In each graph, different letters indicate significant differences (p<0.05) (ANOVA and Tukey’b post hoc test). In panel (d), the mock plants are in white bars and the BSO treated ones in grey bars. The column labelled as “TOTAL” is the sum of all the other columns.

**Figure 3. Effects of the glutathione synthesis inhibitor on the expression of the auxin responsive reporter, DR5.** DR5::GUS expression in *Col-0* either in the absence (a,c) or presence of 0.25 mM BSO (b,d) and GUS activity measured in the root extracts (e). For (a-d) 25 plants were analyzed and a representative image is shown (scale bar = 0.6 for roots and 1 mm for hypocotyls). In (e) data shown mean ± standard error or the mean (SEM) of 3 independent biological repeat, with 120 roots per repeat and treatment and letters indicate significant differences (p<0.05) (ANOVA and Tukey’b post hoc test).

**Figure 4. Effects of the synthetic strigolactone GR24 on root glutathione contents in strigolactone synthesis and signalling mutants.** Effects of 2 µM GR24 in the glutathione content in in wild type (*Col-0*) and *max2-1, max3-9* and *max4-1* mutants. Data shown mean ± standard error of the mean (SEM) of three independent biological replications. In each graph, different letters indicate significant differences (p<0.05) (ANOVA and Tukey’b post hoc test).

**Figure 5. Effects of the glutathione synthesis inhibitor BSO on root glutathione contents and root architecture in strigolactone synthesis and signalling mutants.** Effects of 0.25 mM BSO on the glutathione content (a), primary root length (b) and the lateral root density (c) in wild type (Col-0) and *max2-1, max3-9* and *max4-1* mutants. Data shown mean ± standard error of the mean (SEM) of three independent biological replications. In each graph, different letters indicate significant differences (p<0.05) (ANOVA and Tukey’b post hoc test).

**Figure 6. Effects of the synthetic strigolactone GR24 on root architecture in mutants that are defective in glutathione synthesis.** Effects of 2 µM GR24 on the primary root length (a) and lateral root density (b) in the wild type (*Col-0*) and *pad2-1*, *cad2-1* and *rax1-1* mutants. Data shown mean ± standard error of the mean (SEM) of three independent biological replications. In each graph, different letters indicate significant differences (p<0.05) (ANOVA and Tukey’b post hoc test).

**Figure 7. Effects of auxin on glutathione contents and root architecture in strigolactone synthesis and signalling mutants.** Effects of 0.1 µM NAA and the combination of 0.1 µM NAA plus 2 µM GR24 on the glutathione content (a) and the lateral root density (b) in wild type (*Col-0*) and *max2-1, max3-9* and *max4-1* mutants. Data shown mean ± standard error of the mean (SEM) of three independent biological replications. In each graph, different letters indicate significant differences (p<0.05) (ANOVA and Tukey’b post hoc test). In (b) the numbers inside of the bars indicate the difference as fold times between treatment and control plants for each genotype.

**Figure 8. A summary model for the interactions between auxin, strigolactones and reduced glutathione (GSH) in the control of root architecture in Arabidopsis. (**a) Overall effects of exogenous and endogenous SLs and auxin on the root glutathione pool and on lateral root formation, (b) comparison of the effects of glutathione depletion by the GSH synthesis-inhibitor BSO and by genetic mutations in the pathway of GSH synthesis in root architecture, and effects of auxin (NAA) alone (c) and in combination with strigolactone (GR24) (d) on the fold change on the lateral root density of *Col-0* and the SLs mutants (*max2-1, max3-9* and *max4-1).*

**Supplementary figure 1. Effects of the synthetic strigolactone GR24 on glutathione content in strigolactone synthesis and signalling mutants.** Effects of the synthetic strigolactone (2 µM GR24) on the lateral root density (a), the length of the primary root (b) and the number of lateral roots (c) in wild type (*Col-0*) and *max2-1, max3-9* and *max4-1* mutants. Data shown mean ± standard error of the mean (SEM) of three independent biological replications. In each graph, different letters indicate significant differences (p<0.05) (ANOVA and Tukey’b post hoc test).

**Supplementary figure 2. Effects of glutathione synthesis inhibitor BSO on the expression of the auxin responsive reporter DR5.** DR5::GUS expression in roots (a-f) and hypocotyls (g-l) in *max2-1, max3-9* and *max4-1* backgrounds either in the absence (a, c, e, g, i, k) or presence of 0.25 mM BSO (b, d, f , h, j, l) and GUS activity in root extracts (m). For microscopy, 25 plants were analyzed per treatment and genotype and representative image is shown (scale bar = 0.6 mm for the roots and 1 mm for the hypocotyls) and for the GUS activity in root extracts data shown mean ± standard error or the mean (SEM) of 3 independent biological repeat, with 120 roots per repeat and no significant differences were found (ANOVA and Tukey’b post hoc test) **.**

**Supplementary figure 3. Effects of auxin plus the synthetic strigolactone GR24 on primary root length and the number of lateral roots.** Effects of auxin (0.1 µM NAA), and a combination of 0.1 µM NAA and 2 µM GR24 on primary root length (a) and the number of lateral roots (b) in wild type (*Col-0*) seedlings and *max2-1, max3-9* and *max4-1* mutants. Data shown mean ± standard error of the mean (SEM) of three independent biological replications. In each graph, different letters indicate significant differences (p<0.05) (ANOVA and Tukey’b post hoc test).