Running head: Strigolactones and root system architecture

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Physiological effects of the synthetic strigolactone analog GR24 on root system architecture in Arabidopsis: Another below-ground role for strigolactones?

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ABSTRACT

In the present study, the role of the recently identified class of phytohormones, strigolactones, in shaping root architecture was addressed. Primary root lengths of strigolactone deficient and insensitive Arabidopsis plants were shorter than those of wild type plants. This was accompanied by a reduction in meristem cell number which could be rescued by application of the synthetic strigolactone analog GR24 in all genotypes except in the strigolactone insensitive mutant. Upon GR24 treatment, cells in the transition zone showed a gradual increase in cell length, resulting in a vague transition point and an increase in transition zone size. PIN1/3/7-GFP intensities in provascular tissue of the primary root tip were decreased, whereas PIN3-GFP intensity in the columella was not affected.

During phosphate-sufficient conditions, GR24 application to the roots, suppressed lateral root primordial development and lateral root forming potential, leading to a reduction in lateral root density. Moreover, auxin levels in leaf tissue were reduced. When auxin levels were increased by exogenous application of NAA, GR24 application had a stimulatory effect on lateral root development instead. Similarly, under phosphate-limiting conditions, endogenous strigolactones present in wild type plants stimulated a more rapid outgrowth of lateral root primordia when compared with strigolactone-deficient mutants. These results suggest that strigolactones act through the modulation of local auxin levels and that the net result of strigolactone action is dependent on the auxin status of the plant. We postulate that the tightly balanced auxin-strigolactone interaction is the basis for the mechanism of the regulation of the plants' root to shoot ratio.

INTRODUCTION

Strigolactones, exuded from plants, have been known for a long time to act as germination stimulants for seeds of root parasitic plants such as *Orobanche* and *Striga* spp. (for review see Bouwmeester et al. 2003 and 2007). As root parasitic plants consume a large proportion of the host plants' solutes, they cause wilting and early plant death. Initially, the discovery that strigolactones are also involved in the symbiotic interaction with arbuscular mycorrhizal fungi (Akyiama et al., 2005) was believed to provide an explanation for why the host plants' capacity to produce strigolactones was not lost during evolution. Because arbuscular mycorrhizal fungi are potent providers of nutrients such as phosphate (Pi) and nitrogen to their host, the observation that Pi starvation induced strigolactone biosynthesis in host plants' roots was not surprising (Lopez-Raez et al., 2008; Yoneyama et al., 2007). The recent discovery that strigolactones, or closely related compounds, also act as phytohormones inside the host plants and are involved in the inhibition of axillary bud outgrowth (Gomez-Roldan et al., 2008; Umehara et al., 2008), is an additional explanation why plants continue to produce these fatal germination stimulants: plants use the strigolactones to adjust their shoot architecture to the ever changing environmental conditions. Indeed, Pi starvation was shown to reduce the number of shoot branches (Cline 1991) which was recently proven to be related to increased strigolactone production observed under these conditions (Kohlen et al., submitted)

The discovery that strigolactones are the same as, or are at least closely related to this branching inhibiting signal (BIS), which is a major player in the process of apical dominance, unexpectedly merged two worlds of research and provides new mutual tools and insights. Early studies on BIS revealed that it concerns a mobile, long-distance signal, which moves acropetally (Turnbull et al., 2002; Sorefan et al., 2003; Booker et al., 2005). In Arabidopsis, the carotenoid dioxygenases *MORE AXILLARY GROWTH3* (*MAX3*, *AtCCD7*), *MAX4* (*AtCCD8*) and the cytochrome P450 *MAX1* (*AtCyp711A1*) are involved in the biosynthesis of BIS (Turnbull et al., 2002; Sorefan et al., 2003; Booker et al., 2004, 2005; Auldridge et al., 2006) whereas the F-box and Leu-rich repeats containing protein MAX2 probably acts either in signal perception or transduction (Stirnberg et al., 2002, 2007; Booker et al., 2005). Plants mutated in any of the *MAX* genes all display increased numbers of shoot branches. This mutant phenotype can be rescued by the application of the synthetic strigolactone analog GR24 (Gomez-Roldan et al., 2008, Umehara et al., 2008) in a MAX2 dependent manner.

Besides strigolactones, auxin is another phytohormone that is essential during the process of shoot branching control in apical dominance. In contrast to strigolactones, auxin moves basipetally in the main stem and indirectly controls axillary bud outgrowth (Booker et al., 2003). In a recent study by Hayward and co-workers (2009) it was shown that auxin positively regulates the expression of the strigolactone biosynthetic genes *MAX3* and *MAX4*. Reduced local endogenous auxin levels, in NPA treated or decapitated plants, resulted in a reduction of the expression levels of these genes. In addition, Bainbridge et al. (2005) showed that auxin can locally induce *MAX4* expression in the root tip. Strigolactone deficient *max* mutant plants carrying the auxin reporter gene *DR5-GUS*, were shown to have relatively high GUS intensities in vascular tissue of the lower stem (Bennett et al., 2006), suggesting elevated auxin levels. Recently, Prusinkiewicz et al. (2009) indeed demonstrated the presence of increased auxin levels in the polar transport stream of *max4*. Furthermore, an increase in polar auxin transport capacity, elevated mRNA levels of the polar auxin efflux carrier *PIN1* and higher pPIN1-GUS activity in *max* mutants have been reported by Bennet et al. (2006). The combination of these results suggests that strigolactones and auxin tightly interact and modulate each other's levels and distribution through a feedback mechanism (Hayward et al., 2009).

Also root growth and -branching are tightly regulated processes co-ordinately controlled by several plant hormones of which auxin is playing a key role. Shoot derived auxin is delivered to the root tip through the polar transport stream which is facilitated by proteins of the PIN family. In the columella root cap, auxin is redistributed laterally towards the epidermal and cortical cell layers where acropetal auxin transport towards the elongation zone establishes a local auxin gradient regulating cell division and elongation. Finally, auxin is returned to the polar transport stream again (Blilou et al., 2005, for review and schematic representation see Leyser 2006). During this process of, PIN activity dependent, auxin re-circulation inside the root tip, LR initiation is triggered by the local accumulation of auxin in root pericycle cells adjacent to the xylem vessels (Casimiro et al., 2001; De Smet et al., 2006, 2007; Dubrovsky et al., 2008, Lucas et al., 2008). Subsequent tightly regulated cell division will then lead to LR primordial development and finally to emergence of a young LR from the parent root. In contrast to LR initiation, LR development is supported by auxin coming directly from the aerial part of the plant (Bhalerao et al., 2002). To allow and sustain lateral auxin influx from the polar auxin transport stream into the developing lateral root, PIN1 polarity is rearranged. This dynamic repolarization is mediated by endocytic recycling of the PIN1 protein (Jaillais et al., 2007). The subsequent establishment of a proper auxin gradient inside the developing

LR primordia, which is also mediated by members of the PIN protein family, is crucial for correct LR development (Benkova et al., 2003). Finally, at the stage when an autonomous meristem is formed, the LR primordium is able to produce its own auxin and becomes independent of auxin from the shoot (Casimiro et al., 2003).
Because auxin and auxin transport play such crucial roles in defining root system architecture (RSA) and strigolactones have been suggested to play a role in regulating auxin fluxes, we investigated the contribution of this new plant hormone to root developmental processes. In the present study we describe the effect of application of the synthetic strigolactone GR24 on primary and lateral root development in relation to auxin in both strigolactone deficient and insensitive Arabidopsis mutants carrying the auxin reporter construct *DR5-GUS*. We report that GR24 application has a dual effect on primary root length, lateral root development and lateral root initiation, of which the net result is dependent on the auxin status of the plant. Our results suggest that these effects are mediated through a reduction of auxin fluxes in the vascular tissue, leading to changes in the distribution of auxin levels in the root tip and a reduction of free auxin levels in the aerial parts of the plant. Finally, we hypothesize that strigolactones are responsible for the changes in RSA of Arabidopsis plants growing under Pi-limiting conditions.

RESULTS

Application of the strigolactone analogue GR24 leads to a MAX2-dependent increase in primary root length

Growth and development of roots of 8, 11 and 14 day old Arabidopsis seedlings of the strigolactone biosynthesis mutants *max1-1* and *max4-1*, the strigolactone signaling mutant *max2-1* and their corresponding wild type (Col0) was examined. Plants were grown in the presence of different concentrations, ranging from 1.25 to 10 µM, of the synthetic strigolactone analogue GR24. This concentration range was based on the level of GR24 required for a complete rescue of the branched phenotype of the *max1, 3 and 4* mutant plants (results not shown). Application of 1.25 µM GR24 resulted in increased primary root lengths in 8 days old wild type plants and *max4*, but not in *max1* and *max2* (Fig 1A). GR24 concentrations above 2.5 to 5 µM inhibited primary root elongation in a MAX2 independent manner. In 11 and 14 day old plants, the increase in root length was not observed anymore (data not shown). To explore the possiblity that under optimal growing conditions a general high rate of primary root growth could obscure the specific root growth stimulating effect of

GR24, plants were subsequently grown under less favorable conditions. Because carbohydrate starvation leads to a decrease in primary root length which is not mediated through reduced meristem activity (Jain et al., 2007) sucrose was omitted from the medium.Under these conditions, all genotypes including *max1*, but not *max2*, showed a clear response to GR24 (2.5 µM) treatment further confirming that the response to GR24 is mediated through *MAX2* (Fig. 1B). Moreover, roots of untreated *max* mutant plants were significantly shorter than those of untreated wild type plants. This correlated with a lower number of cortical cells in the primary root meristem (Fig. 1 C) and a higher DR5-GUS intensity in primary root tips of *max* mutant plants containing the auxin reporter construct DR5-GUS (Fig. 1 D and E). Finally, GR24 application at 2.5 µM resulted in an increase in meristem cortical cell number in all genotypes except *max2* (Fig. 1 C).

GR24-mediated increase in primary root length is accompanied by an increase in meristem and transition zone sizes

To further investigate the effect of GR24 on meristem cells as well as on cells present in the transition zone (the region between meristem and elongation zone), the number and length of all root cortical cells in one cell file extending from the 10th cell above the quiescent centre until the elongation zone were determined in wild type plants. By plotting the cell number of individual plants against their cumulative cell length, an impression of root cell dynamics in response to different concentrations of GR24 is obtained (Fig. S1 A-C). Application of 2.5 µM GR24 resulted in a tremendous increase in the number of cells in this region (Fig. 2A). When 10 µM GR24 was applied, the meristem and transition zones only showed a minor increase in cell number when compared with untreated plants (Fig. 2A). For both GR24 concentrations, cells in the transition zone showed a strikingly slow and irregular increase in cell length before they reached their final stabilized elongated state (Fig.S1 B-D). Cells in the meristem zone of plants treated with 2.5 µM GR24 were shorter than in untreated plants, whereas 10 µM GR24 treatment resulted in a small increase in meristem cell length (Fig. 2B). Both GR24 concentrations resulted in decreased cell lengths in the transition zone. Despite these reduced cell sizes, the increased cell numbers of the transition zone finally gave rise to an increase in transition zone size (Fig. 2C). Also, meristem size was increased by both GR24 treatments. With the higher doses of GR24 (5-10 µM), root curvature was induced in some plants (Fig. S1E).

The localization of the transition point, as well as the size of the meristem and transition zone, is largely controlled by the local establishment of an auxin gradient. High auxin levels stimulate cell proliferation whereas low auxin levels favor cell elongation. The establishment of the auxin concentration gradient in the cortical meristem and transition zone is regulated by auxin efflux facilitating proteins of the PIN family. These proteins jointly control the recirculation of auxin in the root tip (Blilou et al., 2005).

To explore a potential effect of GR24 on the levels and distribution of the PIN1, PIN2, PIN3 and PIN7 proteins, which are involved in this auxin circulation process, we examined 6 day old wild type plants carrying constructs encoding the respective PINpromoter-PINprotein-GFP fusion proteins. In the provascular region, application of 2.5, 5 (Fig. 3A-C) and 10µM GR24 resulted in a significant ($p<0.001$) reduction in GFP intensity in the PIN1 (38% reduction), PIN3 (50% reduction) and PIN7 (73% reduction) reporter lines.There was no significant effect on PIN2-GFP levels (data not shown). In the columella, treatment with 2.5 µM GR24 resulted in a minor reduction in PIN7-GFP intensity while the level of PIN3-GFP was not significantly affected.Interestingly, upon GR24 treatment PIN3-GFP signal was observed in a larger number of columella cells which also displayed irregular shapes (Fig. 3B). Although the latter was also occasionally observed in GR24 treated non-transformed plants, the incidence was higher in transgenic plants carrying the *pPIN3:PIN3-GFP* construct. Finally, root curvature was found to be associated with this distortion of columella cells.

GR24 decreases LR density through a suppression of LR outgrowth and a reduction in LR forming potential

GR24 also affected lateral root density (LRD) and total lateral root forming potential (LRFP). The latter is defined as the sum of emerged lateral roots plus lateral root primordia (LRP). At concentrations of 2.5 and 5 uM GR24, the biosynthetic *max* mutans – *max1* and *max4* – and wild type Arabidopsis showed a significant reduction in LRD, but not *max2* (Fig. 4A). A MAX2 independent decrease in LRD was observed at the highest concentration of 10 µM GR24. LRFP was not affected at the lower concentrations of 1.25 and 2.5 µM GR24. However, at 5 µM GR24 a clear MAX2 dependent decrease was observed (Fig. 4B). Finally, like LRD, also LRFP was negatively affected in the *max2* mutant line when 10 µM GR24 was applied.

Because at 2.5 µM GR24 LRD was decreased while LRFP was not affected, it seems likely that at this concentration the reduction in LRD is caused by a reduction in LRP outgrowth. To

explore this assumption and assess whether LR outgrowth was affected randomly or if it concerned the suppression of a specific LRP developmental stage, all LRP in the primary roots of all genotypes under investigation were classified and counted according to the histological scale of Malamy and Benfey (1997). For each stage, the number of LRP was expressed as a percentage of the total LRFP. GR24 treatment resulted in a significantly higher accumulation of LRP stage V in all genotypes except *max2* (Fig. 4C-F). 2.5 µM GR24 almost completely suppressed the developmental transition of LRP stage V into stage VI and abolished LR formation in both wild type and *max1*, but much less so in *max4* and it did not affect *max2*.

LR development is primarily determined by auxin signaling (see review of Woodward and Bartel, 2005). To explore if the suppressive effect of GR24 on LRP outgrowth is mediated through the modulation of auxin levels and/or auxin distribution patterns in LRP, the percentage of LRP associated with GUS staining of the auxin reporter gene construct DR5-GUS present in GR24-treated and untreated *max4* plants was determined. Although LRP with low (or absent) DR5-GUS levels were observed in untreated *max4* plants (Fig. 5A), GR24 treatment resulted in a strong increase in the percentage of non-DR5-GUS stained stage V LRP in *max4* (Fig. 5B). Wild type plants (producing endogenous strigolactones) also exhibited more non-GUS stained stage V LRP than *max4* (Fig. 5C-E), suggesting that the effect of GR24/endogenous strigolactones on LR outgrowth is mediated through a reduction in free auxin levels hereby leading to an arrest in LRP development. Arrested stage V LRP (Fig. 5E) were not able to form the polarized central cell files which are characteristic for stage VI LRP. Because PIN1 activity contributes to the formation of an auxin gradient which is directing cellular organization in developing LRP (Benkova et al., 2003), we studied PIN1-GFP abundance in GR24 treated and untreated wild type stage V LRP expressing *pPIN1:PIN1-GFP*. Figure 5 (F and G) shows that GR24 treatment resulted in decreased PIN1-GFP intensities.

The effect of GR24 on LRP development and outgrowth is dependent on the auxin status of the plant

To investigate the auxin mediated nature of strigolactone action, the effect of GR24 in the presence of high exogenous levels of auxin was investigated. Wild type plants were grown for 5 days on vertical plates and subsequently transferred to plates containing 10 µM of the naturally occurring auxin IAA, or 2.5 µM of the synthetic auxin NAA, either supplemented

with or without 5 or 10 μM GR24. Roots were evaluated after 12, 24, 72 hours and 8 days. NAA and IAA treatment strongly stimulated the initiation of LRP. GR24 treatment did not affect the timing of LRP initiation. All LRP in all treatments readily developed into lateral roots without showing any sign of deviating cellular organization (Fig. 5H), indicating that the previously observed inhibitory effect of GR24 on LR development is absent if enough auxin is supplied to the developing LRP. Treatment with 5 μM GR24 did not reduce PIN1-GFP intensities in developing LRP of NAA (2.5 μM) treated plants (data not shown), suggesting that the previously observed reduction in PIN1-GFP intensity is a secondary effect caused by reduced auxin levels. Only when 10 μM GR24 was applied, a minor decrease in pPIN1:PIN1-GFP intensity was observed. Still, a physiological effect of 5 μM GR24 on LRP development was observed. In contrast to the previous observations showing an inhibitory effect of GR24 application on LRP development, simultaneous application of 2.5 μM NAA and 5 μM GR24 resulted in a stimulation of LRP development. Moreover, after 8 days of treatment, GR24 application resulted in significantly longer roots (0.5 instead of 0.4 mm ($P<0.02$)) in both NAA- and IAA-treated plants (Fig. 5I). Surprisingly, also LRD increased from 41 to 64 LR/cm ($P<0.0005$) as a result of GR24 application.

These results suggest that the physiological response upon GR24 treatment is mediated through a modulation of local auxin levels and is therefore dependent on the auxin status and/or sensitivity of the plant. To test this hypothesis, the effect of GR24 on lateral root development was investigated in *max* mutant and wild type plants grown under Pi starvation. Pérez-Torres et al. (2008) showed that the increase in LR formation in Pi-starved Arabidopsis seedlings is, at least in part, mediated by an increase in auxin sensitivity of root cells. Plants were grown for 5 days under Pi-sufficient conditions and then transferred to plates containing a limiting (20 μM) Pi concentration supplemented with or without 2.5 μM GR24. The distribution of the different LRP stages was determined 12 days after germination. In wild type plants GR24 application resulted in a reduction in the proportion of emerged lateral roots (Fig. S2A). However, GR24 failed to reduce this proportion in the *max* mutants (Fig. S2B and C). This is also reflected by the absence of a clear effect of GR24 on the accumulation of stage V LRP in these latter genotypes, which did occur in the wild type. Apparently, the combination of higher initial DR5-GUS intensities as observed in *max* mutant roots, either suggesting elevated auxin levels or increased auxin sensitivity, and the increased auxin sensitivity induced by Pi deprivation (Lopez-Bucio et al., 2002, Perez-Torres et al., 2008), together lead to a loss of the inhibitory effect of GR24 on LR development.

GR24 induced suppression of LRP outgrowth is partially mediated through decreased shoot derived auxin levels

LRP development is stimulated by shoot derived auxin. To explore whether the GR24 mediated reduction in DR5-GUS levels in LRP, leading to the suppression of LRP development, could have been mediated through reduced auxin levels in the aerial parts of the plants, DR5-GUS intensities in the rosette leaves and cotyledons of transgenic DR5-GUS *max* and wild type plants, were studied. Plants were grown for 12 days on MS agar plates containing 0, 1.25, 2.5, 5 or 10 µM GR24. All GR24 concentrations tested, decreased DR5-GUS intensities in the leaf margins and the tissue surrounding the hydathodes (Fig.6 A and B) which are the primary sites of auxin production in young arabidopsis plants (Aloni et al., 2003). The observed reduction was dependent on the presence of MAX2 (Fig. 6C). Besides decreased DR5-GUS levels around the auxin biosynthesis sites, vascularization between hydathodes and leaf veins was also negatively affected (Fig. 6A and B). Quantification of auxin in leaf material from *max2* and *max4* mutant lines using LC-MS/MS showed a 79% reduction in auxin levels of *max4* (14.44 to 2.98 pg/mg FW, student's T-test p=0.013) upon GR24 treatment, while no significant reduction was observed when *max2* was treated with GR24. Finally, the leaf surface area was decreased by application of 2.5 µM GR24 in a MAX2 dependent way (Fig. S3).

Endogenous strigolactones stimulate lateral root outgrowth during Pi-limiting conditions

Because the high levels and potential ectopic localization of GR24 may obscure the true effects that endogenous strigolactones have on root system architecture, the relevance of endogenous strigolactones in determining RSA was investigated. Therefore, LR development in 12 day old *max1*, *max4* and wild type plants grown under Pi-sufficient and Pi-limiting conditions was studied. Under Pi-sufficient conditions, LRFP was equal for all genotypes (data not shown). However, *max4* plants showed a significantly ($P<0.05$) higher LRD than wild type plants, implying that during Pi-sufficient conditions the endogenous strigolactones of wild type plants had a suppressive effect on LR development (Fig. S4A).
Pi-limitation however, in wild type resulted in an almost two fold decrease in LRP of stage V and a more than four fold increase in LR density (Fig. 7A, Fig. S4B). In the *max* mutants Pi limitation induced accumulation of stage V LRP instead DR5-GUS mediated visualization of

the auxin status of all stages of LRP in wt and *max4* plants grown under Pi-limiting conditions, revealed that the majority of the accumulated stage V LRP of *max4* plants consisted of intensely stained cells (Fig. 7B and C).

DISCUSSION

Strigolactones play a role in shaping root architecture

In the present study a novel role for strigolactones in determining root architecture through an effect on primary root growth and LR development is described. When arabidopsis plants were grown in the presence of the synthetic strigolactone analogue GR24, primary root length was enhanced. For lateral root initiation and development a suppressive effect was observed. Interestingly, this suppressive effect was reduced when plants were grown under Pi deficiency, a condition known to enhance auxin sensitivity (Lopez-Bucio et al., 2002, Perez-Torres et al., 2008). Moreover, when exogenous auxin was applied, GR24 stimulated LR outgrowth which further supports that strigolactones and auxin interact in this process. The fact that the development of LRs in wild type plants was enhanced during Pi limiting conditions, but not in the strigolactone deficient *max* mutant plants, demonstrates that endogenous strigolactones play an important role in determining root architecture (fig. 7).In addition to this, wild type and *max* mutant plantsshow differences in root length. More specifically, *max* mutants have shorter primary roots containing fewer cortical meristem cells (Fig. 1B and C) while they have a higher DR5-GUS intensity in the provascular region (Fig. 1D). Interestingly, *max* mutant plants also have smaller leaves (Fig S3). Although leaf size is not a root characteristic, it probably reflects higher auxin levels (Ljung et al., 2001), which actually makes this observation relevant. Because LRP development is dependent on auxin sources derived from upper parts of the plant (Bhalerao et al., 2002) this could explain why *max* mutant plants tend to have a higher LR density. Although, this was only significant for *max4* and not *max1* and *max2* (Fig. 4C-F), the majority of stage V LRP in *max* mutant plants is associated with a clear DR5-GUS signal, showing the presence of a properly formed auxin maximum. It is likely that most of these stage V LRP will develop into LRs. Therefore, the relatively small difference in LR density observed between young wild type and *max* mutant plants is likely to become larger during later stages of plant development.

Strigolactones are involved in the regulation of primary root growth

Arabidopsis plants, grown in the presence of different concentrations of GR24, showed an increase in primary root length at lower levels of GR24 (1.25 and 2.5 M) and a decrease at higher levels of GR24 (Fig. 1A). This decrease was also observed in *max2*. Because a high dosis of GR24 also affects the entire plant's appearance and fitness this is likely to be the result of general toxicity. Still, a high dose of GR24 does not disturb the GR24 specific effect on meristem and transition zone size (Fig. 2) and could explain why the primary root length in *max2* shows a stronger decrease at 5 µM GR24 when compared to the other genotypes. Unexpectedly, *max1* did not show the initial increase in primairy root length upon 1.25 µM GR24 application as was observed in *max4* and wild type plants (Fig. 1A). This may be due to a sofar unexplained reduced sensitivity to GR24 which was also observed when a concentration range of GR24 was used to rescue the branching phenotype of the *max1*, *max3* and *max4* mutants (data not shown). Because root length was significantly reduced in all genotypes at 5, resp.10 µM GR24, indicating a general, *MAX2* independent, response, a putative *MAX2* dependent increase in *max1* root lengths at higher GR24 levels could have been masked. Although Kohlen et al. (submitted) show that the arabidopsis *max1* mutant is compromised in strigolactone levels, it could be that MAX1 is also involved in more downstream hydroxylation steps, and is able to modify the synthetic strigolactone GR24 increasing its biological activity.

GR24 mediated changes in root meristem patterning are indicative of altered auxin concentration gradients

The effect of GR24 on primary root growth is accompanied by a GR24 concentration dependent change in both cell number and cell length of cells located in the root meristem and transition zones (Fig. 2). Since high auxin levels are known to stimulate cell division whereas low auxin levels favor cell elongation these GR24 induced responses probably reflect the auxin mediated nature of strigolactone action. Local auxin concentrations are established by the combined action of five auxin efflux proteins of the PIN family, jointly regulating auxin fluxes circulating in the primary root tip (Blilou et al 2005). Therefore, auxin transport is a major contributor to root meristem patterning (Sabatini et al., 1999, Friml et al., 2003). Prusinkiewicz et al (2009) suggested that strigolactones act by modulating PIN protein cycling between the plasma membrane and endomembrane system hereby regulating the allocation of PINs to the plasma membrane, which is part of a process called polarization. A

GR24 mediated reduction in PIN protein cycling would then result in a decrease in auxin transport capacity in vascular tissue of both root and shoot, as these are the main sites of *MAX2* expression (Stirnberg et al., 2007). Indeed, Crawford et al. (2010) recently demonstrated that both endogenous strigolactones and GR24 are able to reduce basipetal auxin transport. This could finally explain the changes in root meristem patterning we observed in the present study. Increased auxin levels in the cortical meristem zone were also observed in an in silica study in which reduced auxin transport was enforced by simulating a reduction in vascular *PIN* expression (Grieneissen et al., 2007). Although the underlying cause for reduced auxin transport in that study may differ from the present study, it illustrates that a GR24 mediated reduction in auxin transport is likely to be involved. Interestingly, in the same modeling work, Grieneisen et al. (2007) also show that the absence of lateral epidermal PINs, responsible for the auxin reflux to the main polar transport stream, results in a more spread out and uneven auxin distribution. This is provoking the loss of a clear transition point separating the meristem zone from the elongation zone which was also observed in the present study. The latter may thus be the consequence of a negative effect of GR24 on the efficiency of these lateral epidermal PINs.

Biological relevance of the GR24 mediated changes in root meristem patterning

The contribution of the GR24 mediated increase in transition zone size to the total increase in primary root length is relatively high (Fig. 2 C). Expansion of the transition zone is also observed in radicle growth during seed germination (Sliwinska et al., 2009). Interestingly, in Arabidopsis, the strigolactone biosynthetic gene *CCD8* is specifically expressed in the root cortical and epidermal cells of the transition-elongation zone upon auxin treatment (Bainbridge et al., 2005). Moreover, also *MAX2* expression is elevated in this part of the root (Brady et al., 2007). Therefore, strigolactone mediated modulation of the lateral auxin reflux, which occurs in this particular region, could be responsible for the increase in transition zone size during radicle growth. It is not unlikely that this process is at the basis of the germination of seeds of most plant species, including parasitic plants. Still, the underlying mechanism for exogenous strigolactone dependency of parasitic plant germination remains an intriguing issue.

Interesting is the GR24 induced lateral expansion of PIN3 protein localization to adjacent cells in the root cap. The disturbed cellular organization in this region furthermore suggests

irregular cell divisions (Fig. 3B). If strigolactones are involved in PIN protein cycling as suggested by Prusinkiewitcz et al. (2009), a GR24 mediated distortion of PIN3 polarization, could have resulted in non-regular auxin fluxes and ectopic PIN3 distribution. Asymmetric lateral distribution of the PIN3 protein, leading to auxin asymmetry in the elongation zone, is characteristic for the gravitropical response (Friml et al., 2002), and the induction of root curvature (Ottenschläger et al., 2003). This may explain the observed induction of root curvature in the present study (Fig. S1E). In tomato, Koltai et al. (2009) also observed asymmetric root growth with high levels of GR24 (27 μM). It would be of interest to explore whether strigolactones are involved in stimulating directional growth of the parasitic plants'radicle towards the host root. Assymetric perception of exuded strigolactones by the parasitic plants' radicle might lead to a single sided reduction in PIN cycling efficiency and subsequent auxin accumulation. As in gravitropism this would result in the redirection of radicle growth, in this case towards the strigolactone source, the host root.

GR24 application leads to a reduction of auxin levels in leaf tissue

GR24 reduces the intensity of the auxin reporter DR5-GUS, and auxin levels in young expanding rosette leaves in a MAX2 dependent way. This is accompanied by a decrease in the number of vascular connections between the auxin production sites surrounding the hydathodes and the major leaf veins. Also, a reduction in leaf size was observed which is a known consequence of a reduction in auxin content (Ljung et al., 2001). Because *MAX2* is expressed in vascular tissue throughout the entire plant, it is likely that the putative GR24 mediated reduction in PIN1 cycling as suggested by Prusinkiewicz et al. (2009) also occurs in vascular tissue of the leaf and stem resulting in a decrease in auxin transport capacity. Besides this effect, a GR24 induced reduction in PIN1 cycling could also be responsible for a reduction in the auxin induced, PIN polarization dependent, canalization properties responsible for the formation of new vascular tissue (Sachs 2000, Sauer et al. 2006, Prusinkiewicz et al. 2009). These effects would lead to a local and temporal accumulation of auxin, finally provoking a negative feedback on free auxin levels, explaining the observed reduction in DR5-GUS intensities and auxin levels in leaf tissue of GR24 treated plants. In a study applying the auxin transport inhibitor naphtylphthalamic acid (NPA) to young Arabidopsis plants, Ljung et al. (2001) also observed a feedback inhibition of auxin biosynthesis in expanding leaves and cotyledonsIn our study, a reduction in both auxin biosynthesis and auxin transport capacity, would subsequently lead to a reduction in the auxin

supply to the root system and influence primary root growth and meristem patterning and reduce LRP initiation and development.
Combining the results of Brewer et al (2009), showing that strigolactones act downstream of auxin, and the results of the present study in which we show that strigolactones in their turn are able to modulate auxin levels, we postulate that strigolactones and auxin are likely to operate in a tightly regulated feedback circuit.

GR24 application influences LRP development

GR24 significantly reduced LR density which is the combined result of suppressed LRP development and reduced total LRFP (Fig. 4). These processes are dependent on different auxin fluxes (Lucas et al. 2008). LR initiation is stimulated by auxin circulating in the root tip (Casimiro et al. 2003), being the net result of auxin influx from aerial parts of the plant and the dynamics of auxin (re)fluxes and production in the root tip itself. In contrast, LRP development is solely dependent on auxin sources directly derived from aerial parts of the plant. Auxin is delivered to the LRP through polar auxin transport (Bhalerao et al. 2002) and is subsequently imported into the developing LRP through repolarization of the PIN1 protein allowing lateral auxin influx. Interestingly, the results of the present study indicate that GR24/strigolactones are able to modulate all these processes mentioned above. Since it was observed that GR24 reduces auxin levels in aerial parts of the plant, it is likely that the amount of auxin reaching the majority of the LRP is not sufficient to sustain subsequent LRP development beyond stage V (Fig. 8 left part of graph).

The GR24 mediated reduction of PIN-GFP levels in LRP is a secondary effect

When similar levels of GR24, which were previously found to reduce PIN1-GFP intensities in the LRP, were applied in the presence of exogenous auxin, PIN1-GFP intensities were not affected. This suggests that GR24 is not directly affecting *PIN* expression, but that the decrease in PIN-GFP levels are a consequence of reduced auxin levels. Indeed, *PIN* gene expression is known to be auxin inducible (Reinhardt et al., 2003, Peer et al., 2004) ., The lack of MAX2-GUS staining in LRP as shown by Shen et al. (2007), suggesting the absence of MAX2 expression, would imply that GR24 is not able to act directly inside LRP.
Although no changes in PIN1-GFP levels were observed, simultaneous application of GR24 and auxin still affected LRP development. Interestingly however, under these conditions

GR24 had a stimulatory effect on LRP development instead. Because it is hypothesized that a reduction in PIN cycling is the direct effect resulting from strigolactone action (Prusinkiewicz et al., 2009), it is likely that the lateral auxin influx into the LRP, which is facilitated by re-polarization of PIN1 proteins located in vascular tissue of the root, is disturbed by GR24 treatment. This would lead to a reduction in the supra-optimal auxin levels inside the NAA treated LRP, provoking a shift towards the auxin optimum which is stimulating maximum LRP development (Fig. 8 right part of graph). Apparently, this auxin reduction was too low to be reflected by decreased PIN1-GFP intensities. These results again demonstrate that strigolactones act through the modulation of auxin levels and that the net result of strigolactone action is dependent on the auxin status of the plant.

GR24 has a dual effect on LR initiation depending on the auxin status of the plant

LR initiation starts with the auxin induced division of pericycle founder cells. Auxin reaches these cells through the PIN protein mediated lateral auxin reflux at the level of the transition zone in the root tip (Casimiro et al., 2001, De Smet et al., 2007). Within the founder cells, a fraction of the auxin reflux accumulates until a LR initiation threshold is reached (Lucas et al., 2008). GR24 has a dual effect on LR initiation depending on the auxin status of the plant. During normal physiological conditions low levels of GR24 application do not affect LR initiation. As described above, under these conditions the GR24 mediated decrease in meristem cell size suggests increased auxin levels in the epidermal/cortical meristem zone. When these locally increased auxin levels are combined with a slight reduction in the rate of lateral auxin reflux, which is transporting auxin from the epidermal/cortical cell layers back to the polar transport stream in provascular tissue, net levels of accumulated auxin in founder cells will remain unchanged. Only higher doses of GR24, further reducing the apical auxin supply, hereby reducing the auxin levels in epidermal/cortical cell layers, will lead to a reduction in LR initiation. These results agree with the root branching model, as proposed by Lucas et al.(2008), which explains differences in root branching by local changes in auxin transport.

When plants are grown in the presence of NAA, LR initiation is significantly enhanced by GR24 application. A constant high exogenous auxin supply, combined with a GR24 induced reduction in lateral cellular auxin efflux, will result in an increase in auxin accumulation in pericycle founder cells. Interestingly, increased LR initiation was also observed for the

pin2pin3pin7 triple mutant (Laskowski et al., 2008) which may partially mimic the effect of GR24 application under exogenous NAA administration.

Implications for the role of endogenous strigolactones under natural conditions at the whole-plant level.

Our results show that exogenous application of the strigolactone analogue GR24 affects both primary and lateral root growth, as well as LRP initiation and development, either in a positive or negative way. We furthermore show that GR24 down-regulates auxin levels in the leaf. Because a concentration range of auxin yields an optimum response curve for primary and lateral root development, the net effect of GR24 action depends on the auxin status of the plant (Fig. 8). The true action of strigolactones on root architecture in nature can best be deduced from phenotypical differences between wild type and strigolactone deficient mutants. *Max* mutant plants show a higher DR5-GUS intensity in their primary root tips when compared to wild type plants. Therefore, it is likely that under Pi-sufficient conditions, endogenous strigolactones in wild type plants suppress LRP development through a further reduction in already sub-optimal auxin level/sensitivity. This would be analogous to GR24 application during Pi-sufficient conditions (Fig. 8 left part of graph). However, under Pi-limiting conditions the opposite is observed (Fig. 7A). In this situation, LR development in wild type plants is enhanced. The mechanism leading to this result is likely to be similar to the situation in which GR24 application in the presence of exogenous NAA was shown to enhance LR outgrowth (Fig. 5I and Fig. 8 right part of graph).
Strigolactones are also known to suppress bud outgrowth (Gomez-Roldan et al., 2008; Umehara et al., 2008). Because strigolactone production is enhanced under Pi deficiency in tomato (Lopez-Raez et al., 2008), red clover (Yoneyama et al., 2007) and Arabidopsis (Kohlen et al., submitted), the desirable response of reduced shoot branching under low Pi conditions would be achieved. Recent results in our lab, using the Arabidopsis *max* mutants demonstrate that low Pi induced strigolactone biosynthesis is indeed responsible for the reduction in shoot branching under low Pi conditions (Kohlen et al., submitted). This results in enhanced carbon allocation to the roots sustaining an increase in root branching to expand the exploratory capacity of the root system (Bates and Lynch, 1996; Lopez-Bucio et al., 2002; Sanchez-Calderon et al., 2005). In the present study, we demonstrate that, in addition to controlling shoot architecture, endogenous strigolactones also play an important role in stimulating lateral root development under Pi-limiting conditions. This is in contrast to Pi-

sufficient conditions, during which endogenous strigolactones limit the outgrowth of LRP.Therefore, we postulate that the major role of strigolactones in plant development lies in the coordinated, balanced control of the root to shoot branching ratio under continuously changing environmental conditions.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of the *max1-1*, *max2-1* (Stirnberg et al 2002), *max4-1* (Sorefan et al., 2003) and their parental Col-0 wild type lines either carrying (Bennet et al., 2006) or not carrying the *DR5-GUS* transgene were kindly provided by Prof. O. Leyser (The University of York, UK). Seeds of the *pPIN1/2/3/7*::*PIN1/2/3/7:GFP* lines (Benkova et al., 2003, Friml et al., 2003) were kindly provided by Prof. J.Friml (Gent University, Belgium). Before sowing on MS plates, seeds were surface sterilized in 10% (w/v) chlorine bleach and then washed with 70% (w/v) ethanol and sterile distilled water. Seeds were imbibed on wet filter paper at 4°C for 2-4 days and plated on Murashige and Skoog (1962) (MS)/agar plates (0.5 x MS salts supplemented with 1 x Gamborg's B5 vitamin mix, 0.8% [w/v] agar (Daichin), without (unless stated otherwise) sucrose at pH 5.8). Plants were grown either on horizontal (for leaf surface measurements) or on near vertical plates (for root system architecture analysis) in a climate chamber under a 22°C/18°C 16 hour light/8 hour dark regime (80 $\mu molm^{-2}\ s^{-1}$).
Pi starvation experiments were conducted by transferring plants pre-grown (5 days) on Pi-sufficient (1.25 mM) MS plates to low Pi (20 μM) MS plates. Lateral root induction by high levels of NAA (2.5 and 10 μM) or IAA (10 μM) was performed according to Himanen et al. (2002) with the exception that, during the 5 day period of pre-growing, NPA was omitted from the medium. All experiments were repeated at least three times.

β-Glucuronidase (GUS) staining

Histochemical GUS staining was performed according to Stomp (1992). The GUS activities were visualized by incubating the seedlings with the GUS substrate X-Gluc for 13 hours at 37 °C. After clearing overnight in 70% ethanol the plants were stored in 4°C prior to imaging.

Root system architecture measurements

Pictures of the root systems grown on MS plates were taken with a digital camera (Canon EOS 350d) and were proportionally enlarged and printed to measure primary root lengths using a curvimeter. Images of NAA/IAA treated plants were taken with a digital camera connected to a stereo microscope at 5 x magnification. LRP developmental stages were counted and evaluated using a Nikon Optiphot microscope equipped with Nomarski optics at 10x magnification. Roots were cleared for 2 to 16 hours in a drop of Hoyer's solution (7.5 g gum arabic, 100 g chloral hydrate, 5 mL glycerol in 30 mL water) on a microscope slide. LRP developmental stages were classified according to the system of Malamy and Benfey (1998). The number of root meristem cells was determined by counting cortical cells in one cell file, starting from the quiescent centre until the first cell showing signs of rapid elongation using confocal microscopy.

Confocal microscopy

Roots of seedlings expressing GFP were incubated for 10 min in 1 μM propidium iodide (PI) in growth medium prior to imaging, washed and coverslip mounted for imaging on an Axiovert 200M with a Zeiss 510 META confocal laser scanning microscope (Carl Zeiss, Jena, Germany). Representative root tips closest to the coverslip were selected for imaging with a 10x (NA 0.3) or 20x (NA 0.4) Fluar objective. Imaging was done in a reproducible manner starting with similar sample preparation, to image acquisition settings and data processing, for all experiments. Samples were excited with 5% of a 488 nm laser (emission from a 30mW Ar tube) for GFP excitation, and 80% of a 543nm laser (emission from a 1 mW HeNe tube) for PI excitation. Optical sections of roots and subsequent zseries were made using DM 488/543, EM 505-530 (GFP in green) and EM LP 615 (PI in white). Transmission images were simultaneously collected.

Single midplane optical sections were selected and compared, while lateral root primordia could most accurately be counted from zseries, flat projected for maximal pixel value. Image

analysis was done using Zeiss LSM Image Examiner (version 3.5), ImageJ v8-32 and Adobe Photoshop CS2 (Adobe Systems Inc., Mountain View, California).

Stereo microscope

Roots were imaged on an Zeiss stereo Discovery (A12) with a Plan S 1.0X FWD 81mm (1 to 100x) objective. Images were taken with a AxioCam MRc5 (5 MPix camera, Zeiss) and analyzed using AxioVision 4.6 software

IAA extraction of Arabidopsis leaf material

For IAA analysis, 200 mg of root or shoot tissue was ground in a mortar with liquid nitrogen. The samples were extracted with 1 ml of cold methanol containing [Phenyl $^{13}C_6$]-IAA (0.1 nmol/ml) as internal standard in a 2 ml eppendorf tube. The tubes were vortexed and sonicated for 10 min in a Branson 3510 ultrasonic bath (Branson Ultrasonics, Danbury, CT, US) and placed overnight in orbital shaker at 4ºC. The samples were centrifuged for 10 min at 11500 rpm in an Heraeus Fresco 17 centrifuge (Thermo Scientific, US) at 4ºC after which the organic phase was transferred to a 4 ml glass vial. The pellets were re-extracted with another 1 ml of methanol. The combined methanol fractions were further purified by anion exchange column (Grace Pure Amino 500mg/3ml SPE) as previously described (Chen et al., 1988), dried in a SpeedVacuum Savant SPD121P (Thermo Scientific, US) and the residue dissolved in 200 µl of acetonitrile:water:formic acid (25:75:0.1, v/v/v). Before LC-MS/MS analysis, samples were filtered through Minisart SRP4 0.45 µm filters (Sartorius, Germany)

IAA detection and quantification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Analysis of IAA in Arabidopsis leaf extracts was performed by comparing retention times and mass transitions with those of IAA standard using a Waters Xevo tandem quadruple (TQ) mass spectrometer (Waters, Milford, MA, USA) using the settings as previously described by Kohlen et al. (submitted) and the gradient described for ABA by López Ráez et al. (2010). Multiple reaction monitoring (MRM) was used for IAA quantification. Parent-daughter transitions were set according to the MS/MS spectra obtained for the standards IAA and [Phenyl $^{13}C_6$]-IAA. Transitions were selected based on the most abundant and specific fragment ions for which the collision energy (CE) was optimized. For identification, the

MRM transitions *m/z* 176>103 at a CE of 30 eV and 176>130 at 16 eV; and for [Phenyl $^{13}C_6$]-IAA, the transitions *m/z* 182>109 at 28 eV and 182>136 at 16 eV were selected. Cone voltage was set to 18 eV. IAA was quantified using a calibration curve with known amount of standards and based on the ratio of the area of the MRM transition 176>130 for IAA to the MRM transition 182>136 for [Phenyl $^{13}C_6$]-IAA. Data acquisition and analysis were performed using MassLynx 4.1 software (Waters, USA). The summed area of all the corresponding MRM transitions was used for statistical analysis.

Leaf surface quantification

25 seedlings were grown horizontally for 12 days on 9 cm wide petridishes in triplicates. Images were taken using a digital camera (Nikon D80 with Nikkor AF-S 60mm f/2.8 G Micro ED) connected to a computer, using Nikon camera control pro software version 2.0. Image analysis was performed using ImageJ based on segmentation by color-thresholding using visual scripting for ImageJ according to Joosen et al (2010).

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FIGURE LEGENDS

Figure 1. GR24 affects primary root length in a concentration dependent way.
A, Primary root lengths of 8 day old *max1-1*, m*ax2-1*, *max4-1* and wild type (wt) Arabidopsis plants grown on vertical MS plates containing 0.5% sucrose and different levels of GR24. B, primary root length of 12 day old plants grown on vertical MS plates containing no sucrose and 0 or 2.5 μM GR24. Data are means ± SE (n= 16-20). C, Cortical meristem cell number, expressed as the number of cells in one cell file extending from the quiescent centre to the first elongated cell, of 7 day old Arabidopsis plants grown on vertical MS plates containing 0.5% sucrose and 0 or 2.5 μM GR24. Data are means ± SE (n= 5). Marks indicate a statistically significant difference compared with untreated (*) or wild type plants (x) as determined by Student's t-test ($p<0.05$). D and E, Nomarski images of GUS stained primairy roots of *max4* (D) and wild type (E) plants containing the DR5-GUS reporter construct. Scale bars represent 0.5 mm.

Figure 2. Application of GR24 affects cortical root cell number and length of the meristem and transition zone in a concentration dependent way.
A, Average cortical cell number present in a cell file starting from the quiescent centre throughout the meristem and transition zone. B, Average length of cortical cells in meristem and transition zone. C, Meristem and transition zone size. Measurements were performed using 7 day old wild type plants grown in the presence of different concentrations of GR24. Data are means ± SE (n=12-16). Asterisks indicate a statistically significant difference between treated and untreated plants as determined by Student's t-test ($p<0.05$).

Figure 3. PIN-GFP protein levels and localization in roots of GR24 treated and untreated Arabidopsis plants. A-C, Confocal midplane sections of roots of untreated and 5 μM GR24 treated 6 day old plants carrying the *pPIN1:PIN1-GFP* (A), *pPIN3:PIN3-GFP* (B) and *pPIN7:PIN7-GFP (C*) transgene. The upper arrow indicates the pro-vascular region, the lower arrow indicates the localization of the columella. Propidium iodide stained cell walls are represented in white. The intensity of the GFP signal was quantified by converting RGB pixels to brightness values using the program ImageJ (Abramoff et al., 2004).

Figure 4. GR24 treatment decreases lateral root density (LRD) (A) and total lateral root forming potential (LRFP) (B) and delays lateral root development (C-F). LRFP is defined as the sum of all lateral roots plus lateral root primordia. *Max1-1, max2-1, max4-1* and wild type (wt) plants were grown either on a range of GR24 levels (A-B) or on a fixed concentration of 2.5 µM GR24 (C-F), on vertical MS plates with (A-B) or without (C-F) 0.5% sucrose and were evaluated at 14 (A-B) or 12 (C-F) days after germination. Data are means ± SE (n= 20-25). LR developmental stages were characterized according to the scheme of Malamy and Benfey (1997).

Figure 5. GR24 treatment delays lateral root development. LR developmental stages were characterized according to the scheme of Malamy and Benfey (1997). Wild type (WT) (A), *max1-1* (B), *max4-1* (C) and *max2-1* (D) plants were grown on vertical MS plates with or w/o 2.5 µM GR24 for 12 days. The y-axis represents the % of each developmental stage out of the total LRFP. Data are means ± SE (n= 15-20).

Figuur 6. GR24 treatment results in decreased intensities of the auxin reporter DR5-GUS in aerial parts of the plant. A and B, Close-up of a leaf of an untreated (B) and GR24 treated (C) *max4-1* plant. Arrows point at the locations where developing vasculature is either present (A) or absent (B). C, GUS staining of the leaves of 12 day old *max4-1* and *max2-1* plants carrying the *DR5-GUS* transgene grown in the presence or absence of 2.5 µM GR24 showing that the decrease in GUS intensities is dependent on MAX2.

Figure 7. Endogenous strigolactones in wild type plants allow a more rapid development of LRP into LRs during phosphate limiting conditions. A, Graph showing fold change in LRP V- and LR density of 12 day old wild type (WT), *max4-1* and *max1-1* plants as a response to Pi limiting conditions relative to sufficient Pi conditions. Plants were pre-grown for 5 days on vertical MS plates containing sufficient Pi levels (1.5 mM) after which all plants were transferred to either Pi deficient (20 µM Pi) or Pi sufficient MS plates. B and C, DR5-GUS distribution in LRP of wild type (WT) (B) and *max4-1* (C) plants carrying the *DR5-GUS* transgene grown under Pi limiting conditions showing that the majority of the highly accumulated *max4-1* stage V LRP is associated with extremely high GUS intensities. Data are means ± SE (n=15-20).

Figure 8. Schematic representation of the putative mechanism of GR24 action in root system architecture.

Bell shaped auxin response model for the auxin mediated effect of the synthetic strigolactone GR24 on LR development. (1) Under sufficient phosphate (Pi) conditions, GR24 application has an inhibitory effect on LR development mediated through a reduction in auxin levels in the polar auxin transport stream (PAT) coming from the shoot. (2) In the presence of exogenous auxin LR development is increased instead. Under these conditions a GR24 mediated reduction of auxin levels reaching LRP, through reduced auxin import into the developing LRP, results in an auxin concentration closer to the auxin optimum, hereby increasing the LR developmental rate.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Application of GR24 affects cortical root cell dynamics of the proliferation and transition zone in a concentration dependent way.
A-C, Cumulative root cortical cell length as a response to different concentrations of GR24 as measured in one cell file starting from the 10th cell above the quiescent centre plotted against the cell number. Lines represent the values of individual 7 day old Arabidopsis wild type plants. The linear, left part of the curves, reflects the meristematic zone in which cells have a relatively constant length.The change in the slope is indicative for the transition from meristematic to elongation zone. D, Primary root images (confocal microscopy) of propidium iodide stained, untreated and 2.5 resp. 10 μM GR24 treated 7 day old plants. EZ=elongation zone, TZ= transition zone, MZ= meristem zone, RC= columella root cap as observed for the untreated plant. Arrows indicate the approximate start of the transition zones. E, GR24 induced root curvature in 6 day old Arabidopsis seedlings grown on 1x MS plates in the presence of 5 μM GR24.

Figure S2. The inhibitory effect of GR24 application on LRP development is decreased in plants grown under phosphate limiting conditions. Wild type (WT) (A), *max1-1* (B) and *max4-1* (C). Plants were pre-grown on vertical MS plates containing sufficient levels (1.5 mM) of Pi. After 5 days, plants were transferred to Pi limiting (20 μM) MS plates supplemented with or without 2.5 μM GR24. When plants were 12 days old, LR developmental stages were characterised according to the scheme of Malamy and Benfey (1997). The y-axis represents the % of each developmental stage out of the total LRFP. Data are means ± SE (n= 15-20).

Figure S3. GR24 treatment results in a decrease in leaf surface.
Leaf surface was decreased by GR24 application in a *MAX2* dependent way. Data are means ± SE (n=50-75).

Figure S4. The effect of endogenous strigolactones on lateral root development in Pi sufficient (A) and Pi limiting conditions (B). A-B, Density (LRP per cm primary root) for each category of LRP developmental stage, characterised according to the classification scheme of Malamy and Benfey (1997), of 12 day old wild type (wt), *max1-1* and *max4-1* plants carrying the *DR5-GUS* transgene. Plants were pre-grown for 5 days on vertical MS

plates containing sufficient Pi levels (1.5 mM) after which all plants were transferred to either Pi deficient (20 μM Pi) or Pi sufficient MS plates. Arrows indicate the decrease in LRP V and the increase in LR density which is specific for wild type plants. Data are means ± SE (n=15-20). Asterisks indicate significant ($P<0.05$) differences between *max* mutant and wild type plants as determined by Student's t-test.

Figure 1.

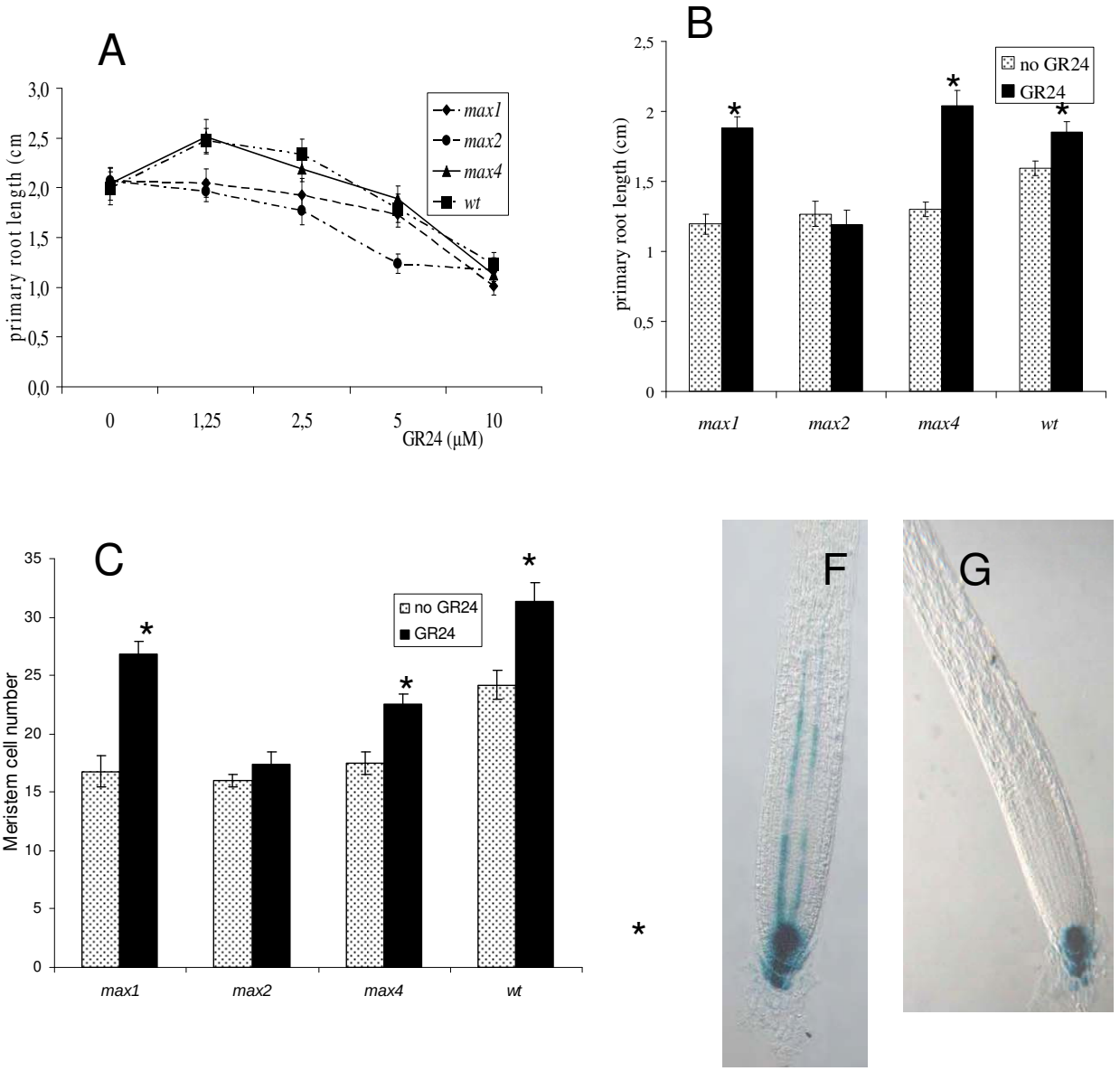

A
primary root length (cm
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
1,25
2,5
5
10
GR24 (µM)
max1
max2
max4
wt
B
primary root length (cm)
2,5
2
1,5
1
0,5
0
no GR24
GR24
*
max1
max2
max4
wt
C
Meristem cell number
35
30
25
20
15
10
5
0
no GR24
GR24
*
max1
max2
max4
wt
F
G
D
E
max4
wt

Figure 2I.

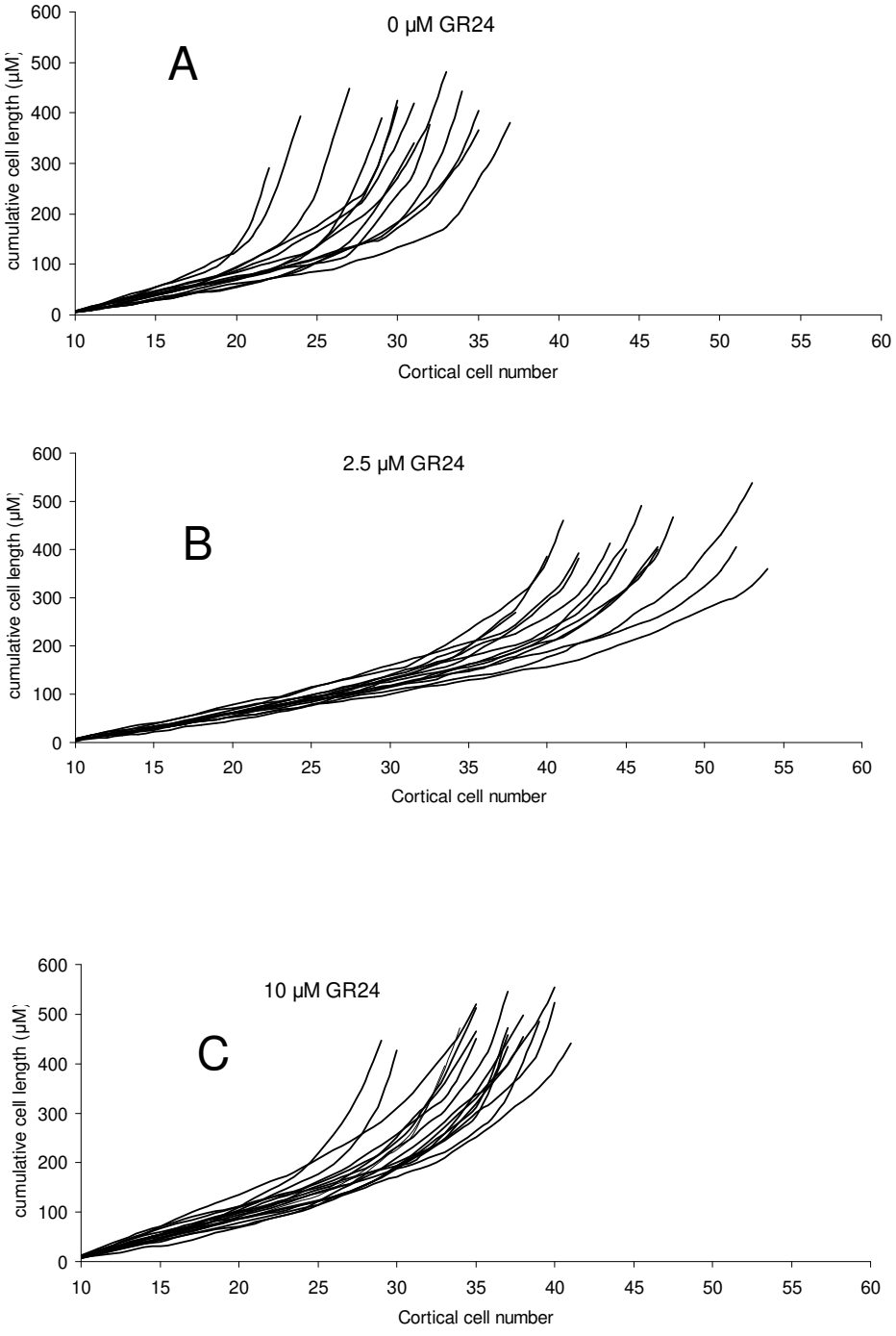
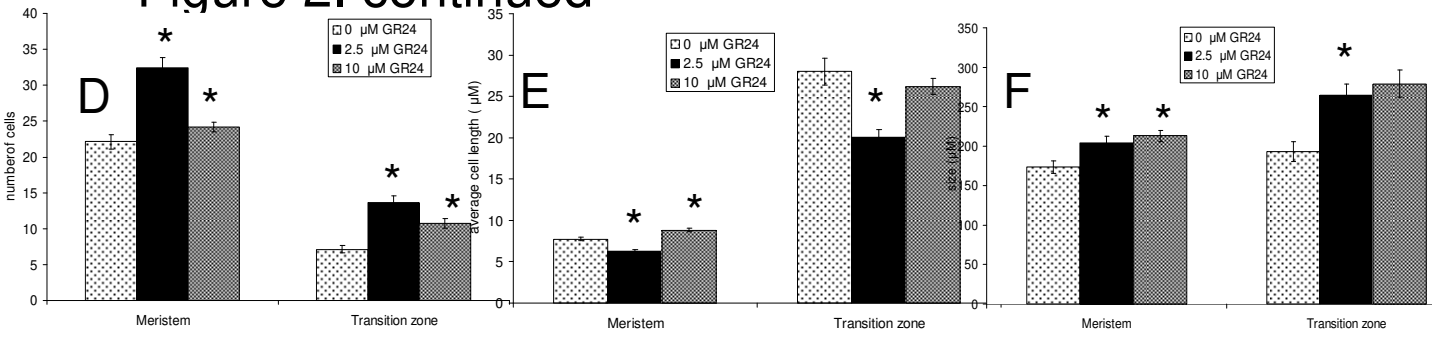
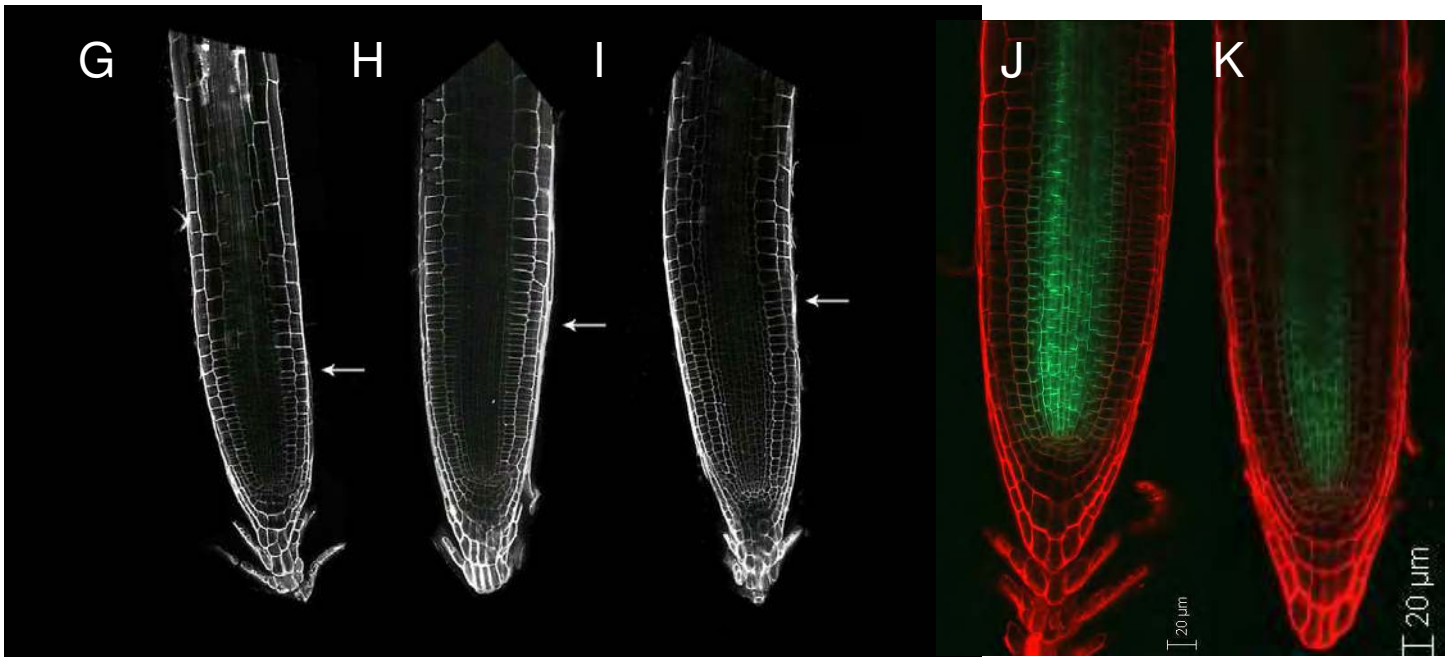
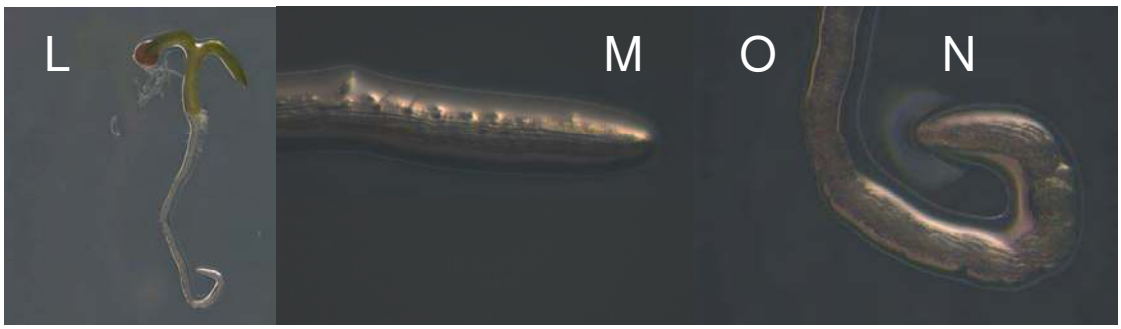
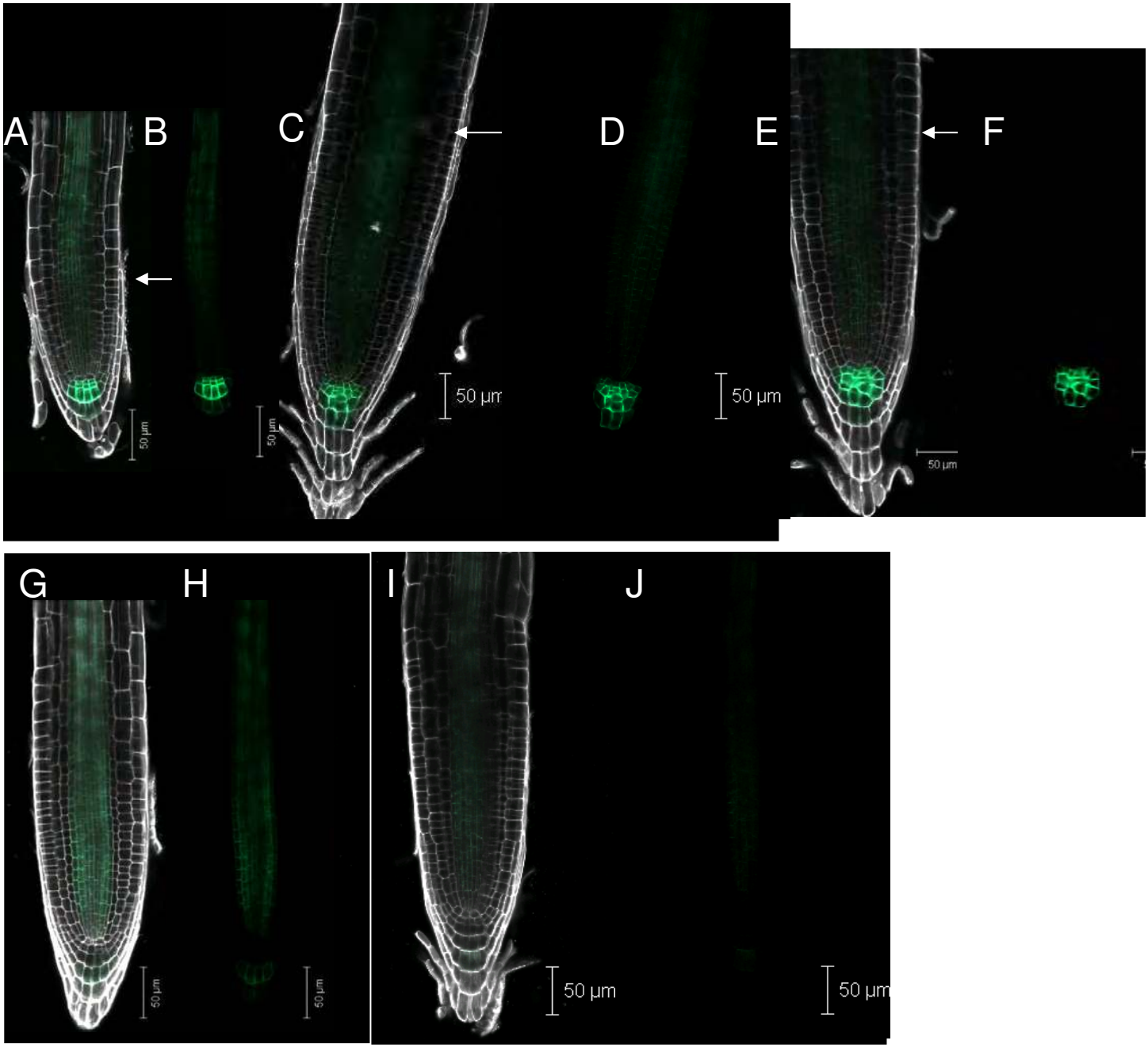

A
0 µM GR24
B
2.5 µM GR24
C
10 µM GR24
cumulative cell length (µM)
Cortical cell number
0
100
200
300
400
500
600
10
15
20
25
30
35
40
45
50
55
60

Figure 2I continued








A
B
C
D
E
F
50 µm
50 µm
50 µm
50 µm
50 µm
50 µm

G
H
I
J
50 µm
50 µm
50 µm
50 µm

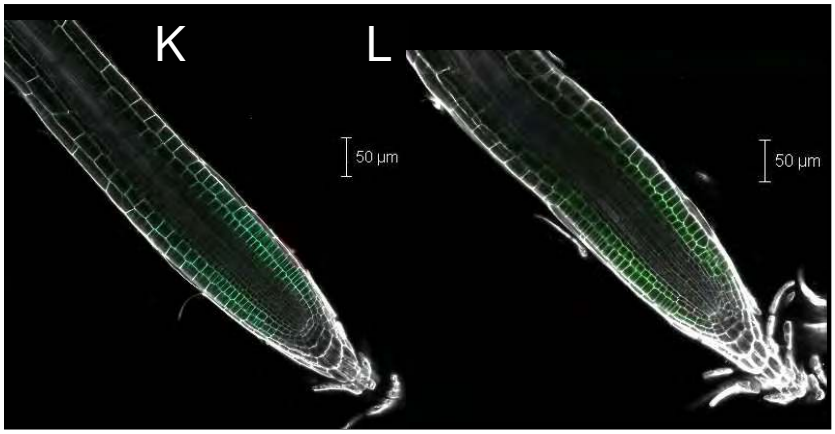

K
L
50 µm
50 µm

Figure 2II

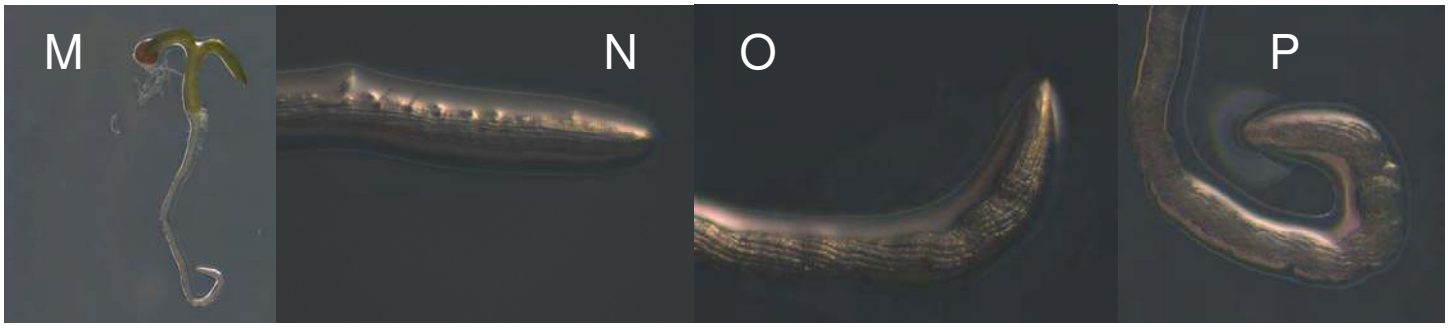
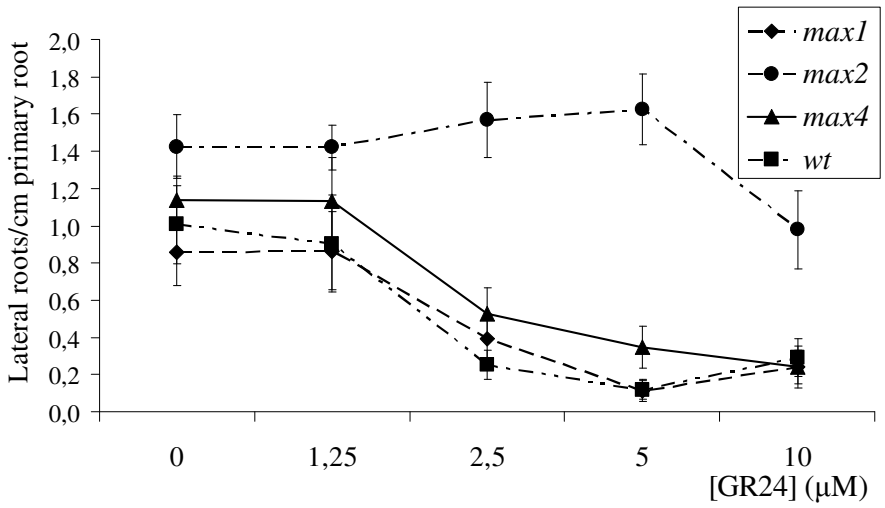

M
N
O
P

Figure 3.

A


Lateral roots/cm primary root
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
1,25
2,5
5
10
[GR24] (µM)
max1
max2
max4
wt

B

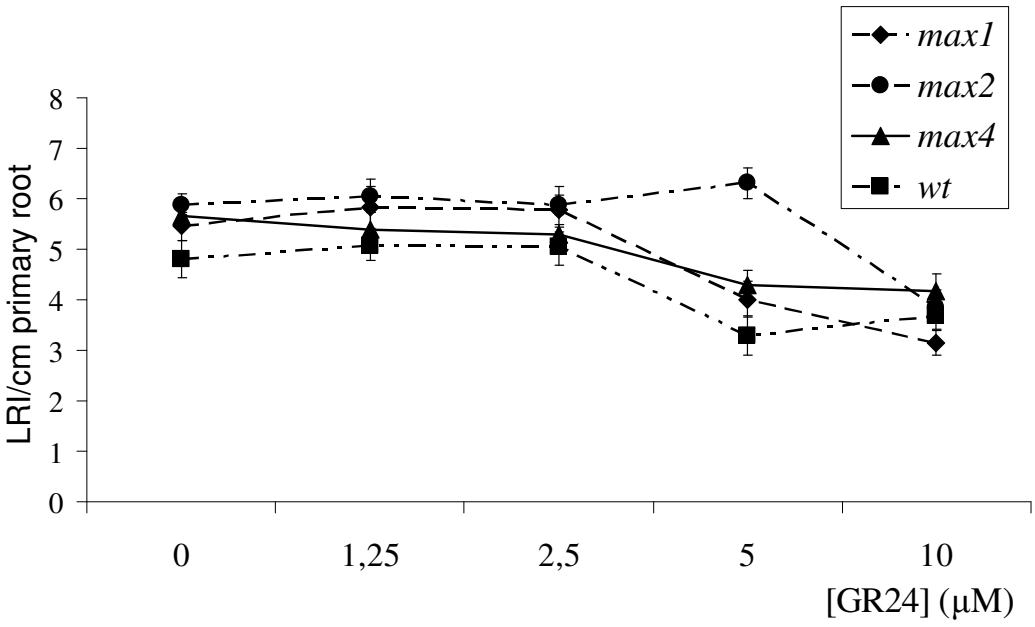

LRI/cm primary root
8
7
6
5
4
3
2
1
0
0
1,25
2,5
5
10
[GR24] (µM)
max1
max2
max4
wt

Figure 4

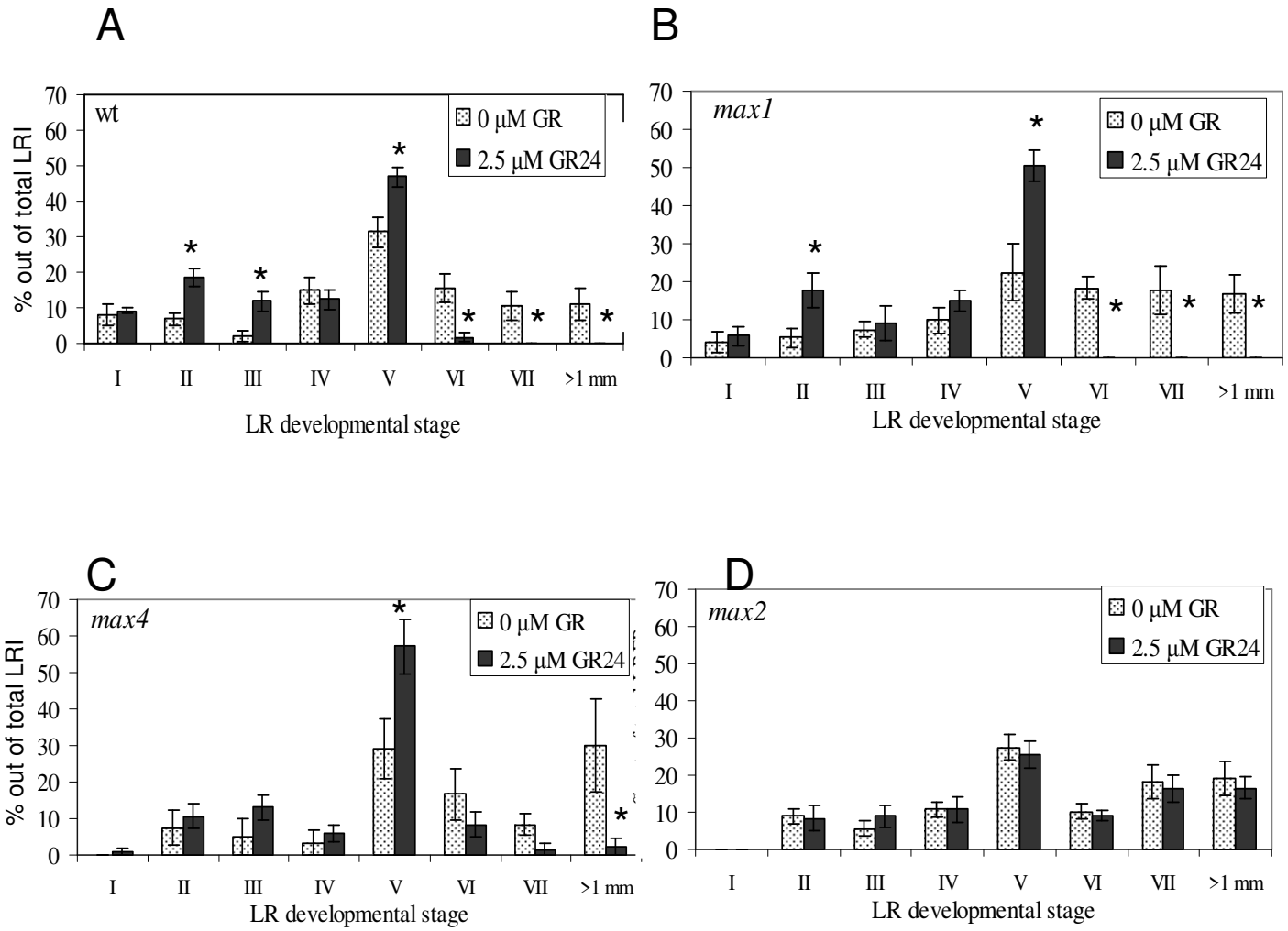
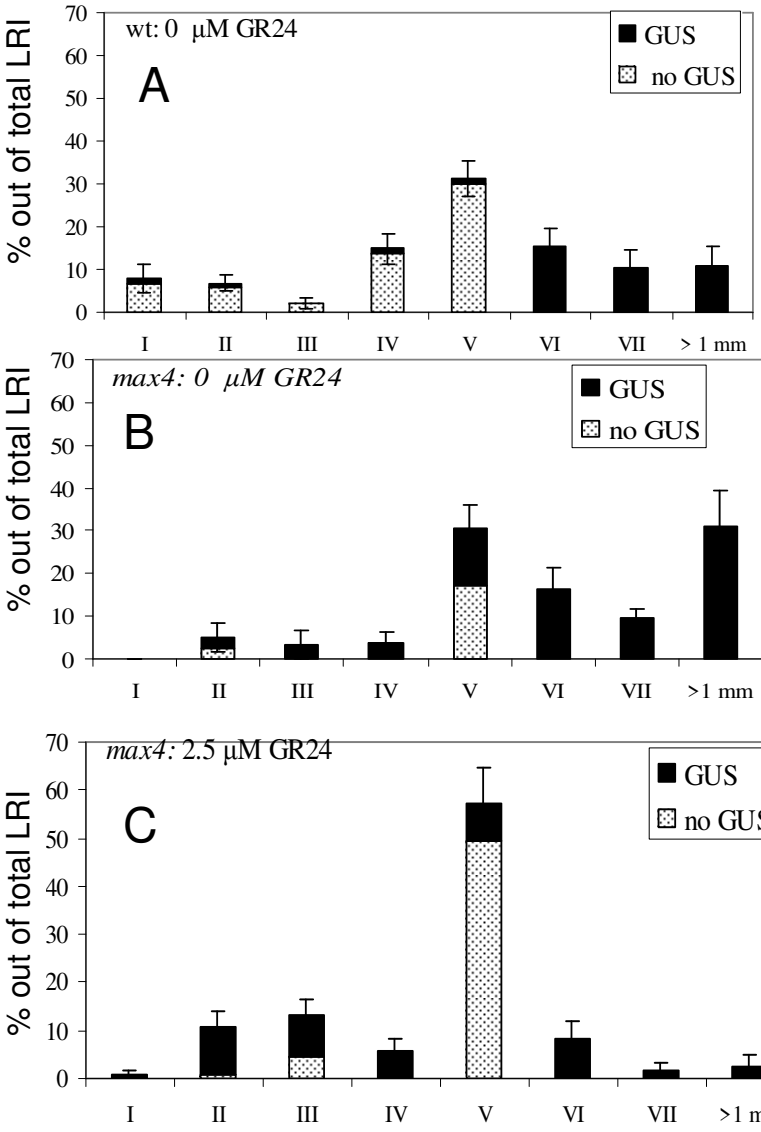
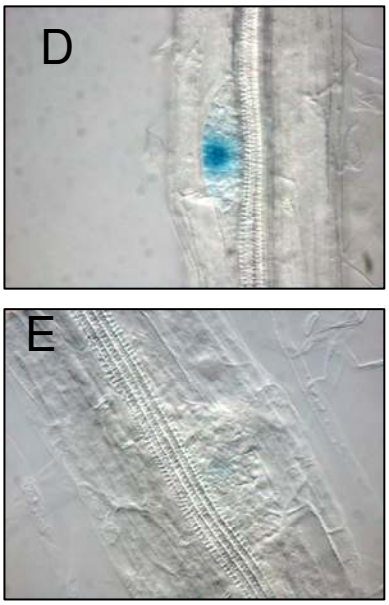

A
wt
0 µM GR
2.5 µM GR24
% out of total LRI
LR developmental stage
I
II
III
IV
V
VI
VII
>1 mm
B
max1
C
max4
D
max2

Figure 5


A
wt: 0 µM GR24
GUS
no GUS
% out of total LRI
70
60
50
40
30
20
10
0
I
II
III
IV
V
VI
VII
> 1 mm
B
max4: 0 µM GR24
GUS
no GUS
% out of total LRI
I
II
III
IV
V
VI
VII
>1 mm
C
max4: 2.5 µM GR24
GUS
no GUS
% out of total LRI
I
II
III
IV
V
VI
VII
>1 mm


D
E

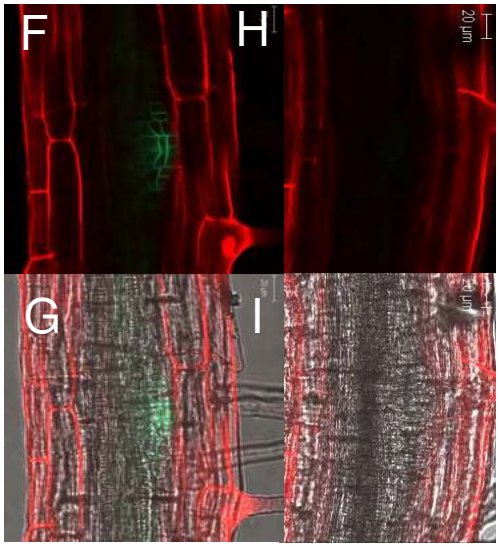
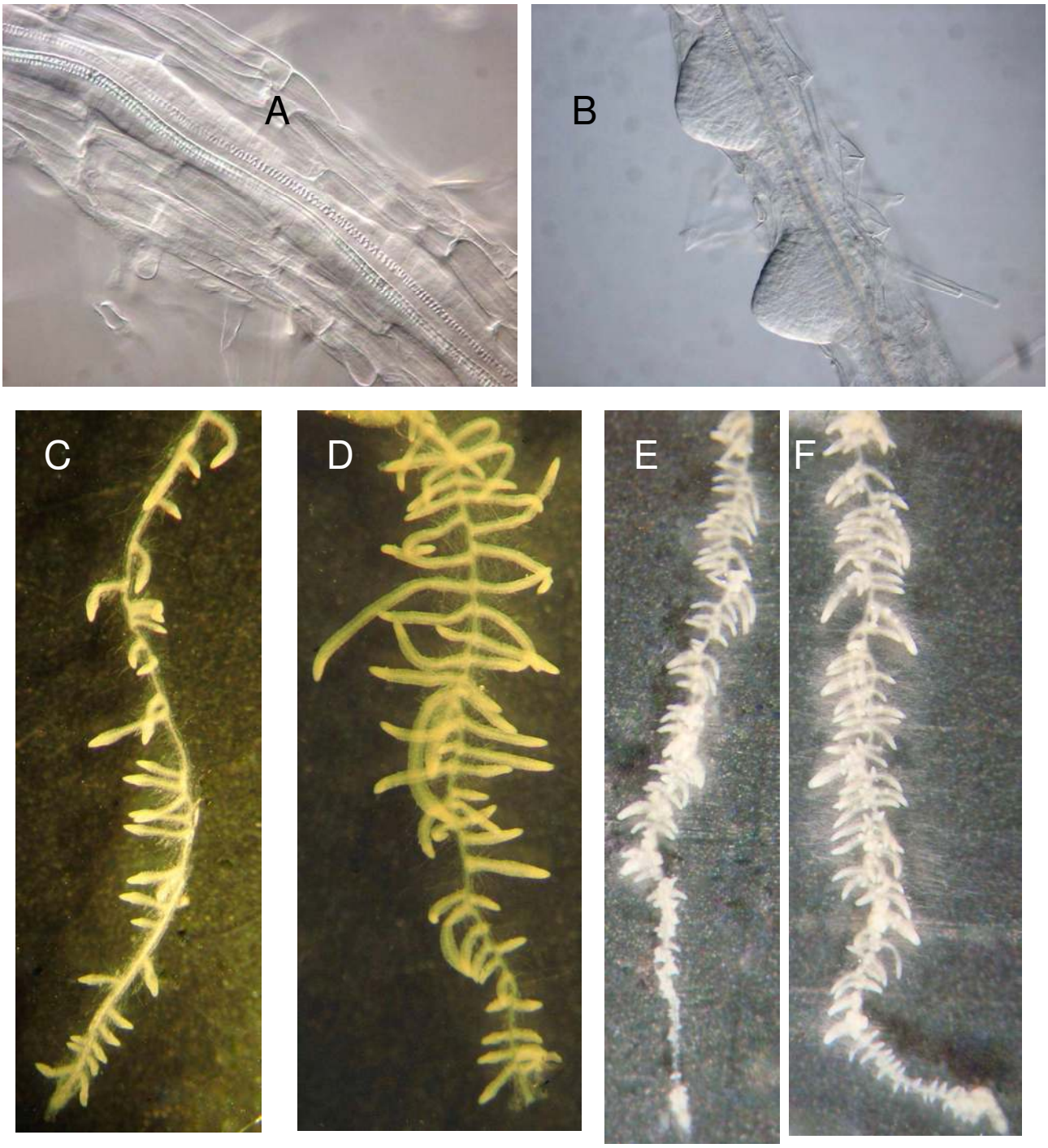

F
H
G
I

Figure 6



Mention in text:

Figure 7

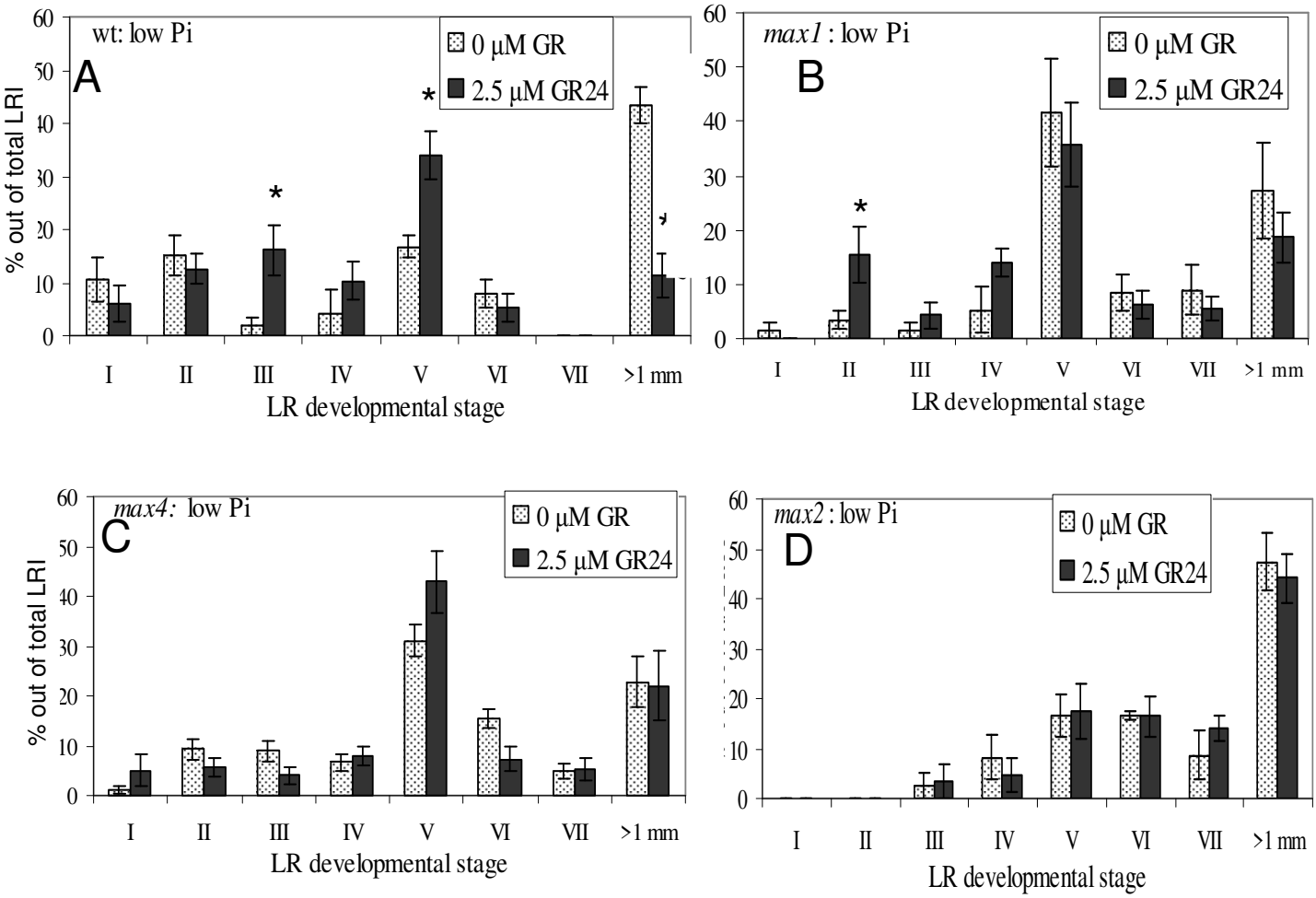
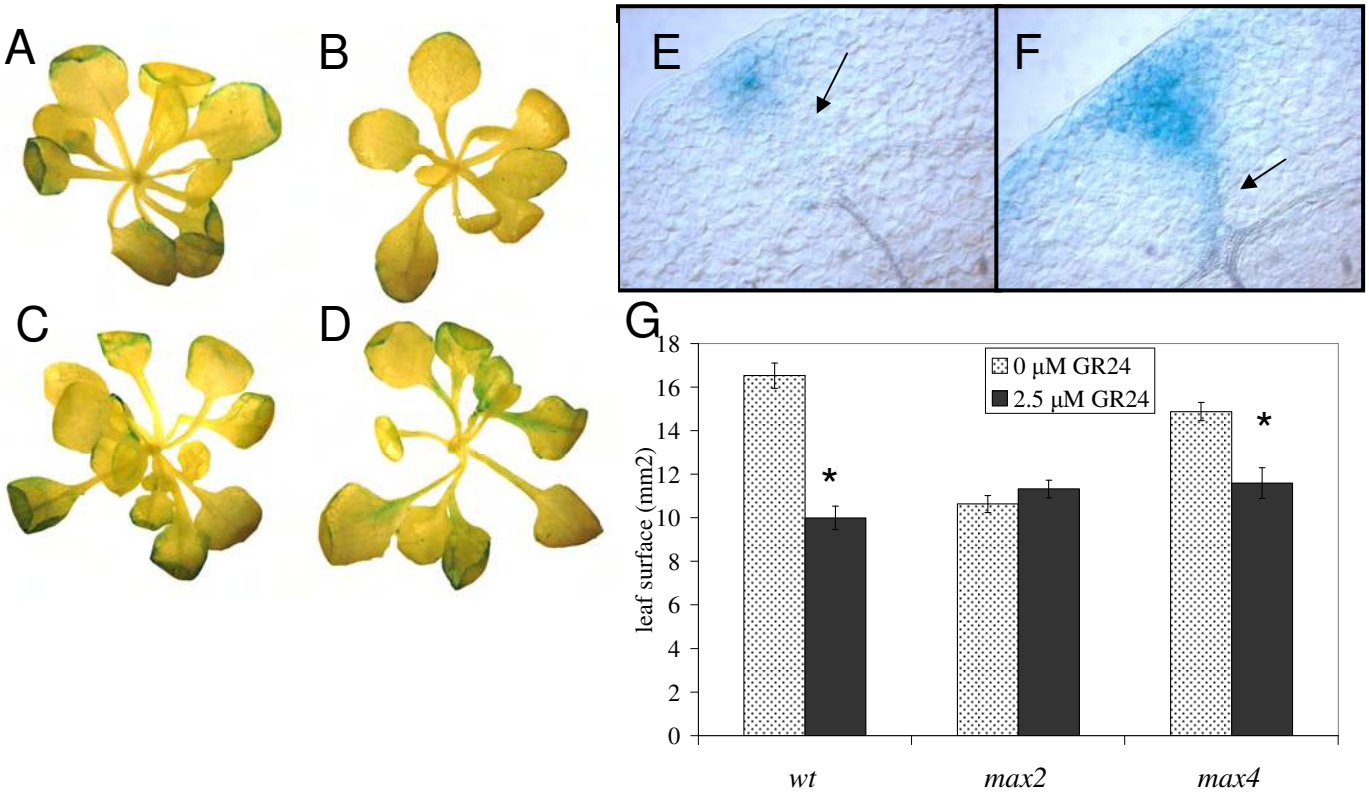

A
wt: low Pi
0 µM GR
2.5 µM GR24
% out of total LRI
LR developmental stage
I
II
III
IV
V
VI
VII
>1 mm
B
max1 : low Pi
C
max4: low Pi
D
max2 : low Pi

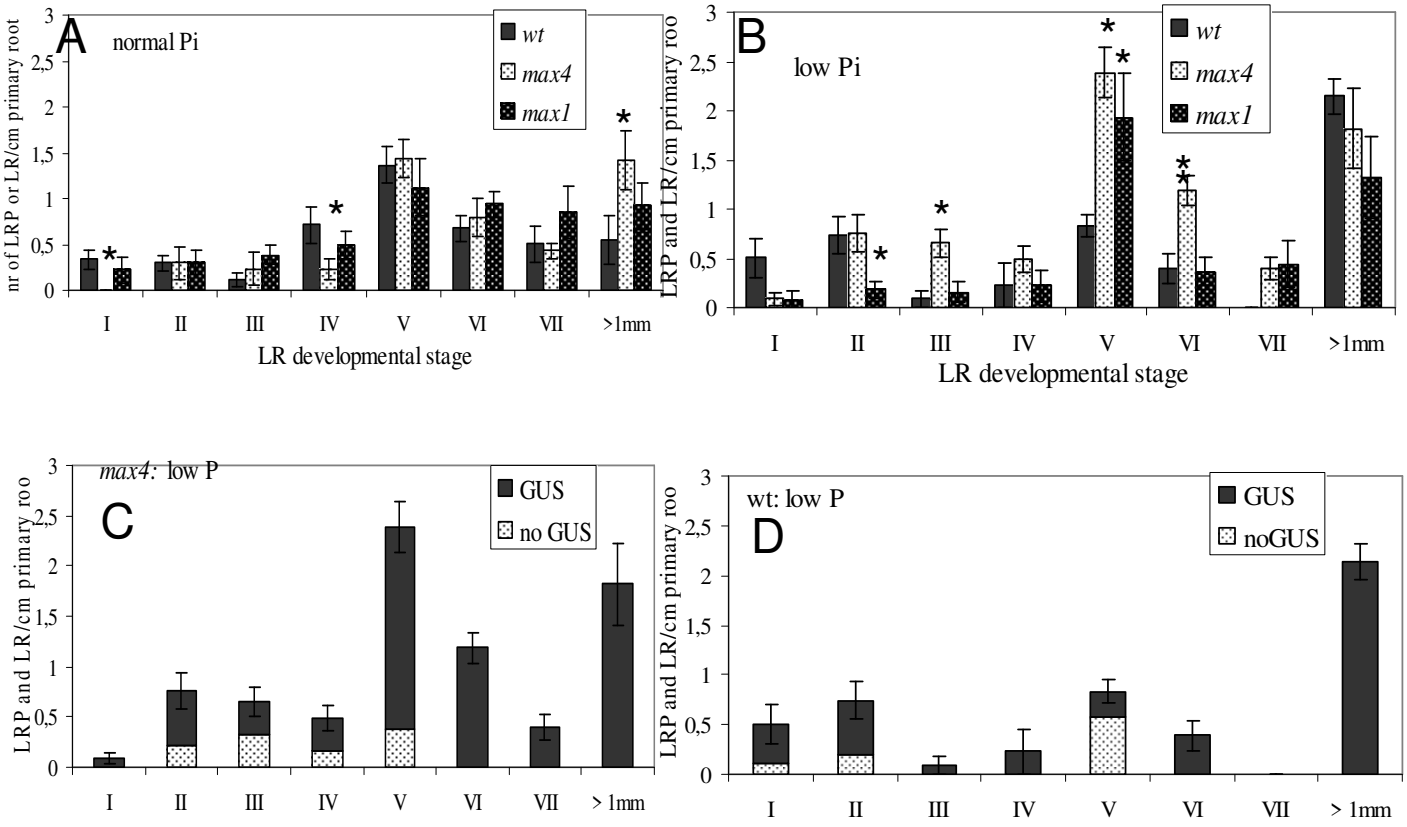
Figure 8



Replaced by A-D

IAA/IAA-conjugates
Figure +\- GR24

Figure 9.


A
normal Pi
wt
max4
max1
nr of LRP or LR/cm primary root
LR developmental stage
I
II
III
IV
V
VI
VII
>1mm
B
low Pi
LRP and LR/cm primary roo
C
max4: low P
GUS
no GUS
D
wt: low P
noGUS
> 1mm