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Strigolactones enhance competition between shoot branches by dampening auxin transport

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SUMMARY

Strigolactones (SLs), or their derivatives, were recently demonstrated to act as endogenous shoot branching inhibitors, but their biosynthesis and mechanism of action are poorly understood. Here we show that the branching phenotype of mutants in the *Arabidopsis* P450 family member, *MAX1*, can be fully rescued by strigolactone addition, suggesting that MAX1 acts in SL synthesis. We demonstrate that SLs modulate polar auxin transport to control branching and that both the synthetic SL GR24 and endogenous SL synthesis significantly reduce the basipetal transport of a second branch-regulating hormone, auxin. Importantly, GR24 inhibits branching only in the presence of auxin in the main stem, and enhances competition between two branches on a common stem. Together, these results support two current hypotheses: that auxin moving down the main stem inhibits branch activity by preventing the establishment of auxin transport out of axillary branches; and that SLs act by dampening auxin transport, thus enhancing competition between branches.

KEY WORDS: Strigolactone, Auxin transport, Shoot branching, *Arabidopsis*

INTRODUCTION

An extraordinary plasticity in postembryonic development allows plants to adapt their form according to the prevailing environmental conditions (reviewed by Leyser, 2009). A striking example is the degree of shoot branching. Genotypically identical plants can develop as a single unbranched shoot, or as a highly ramified bush with secondary and higher order branches. Shoot branches arise through the activity of secondary shoot apical meristems laid down in the axils of leaves produced by the primary shoot apical meristem. Axillary meristems can arrest as buds after the formation of a few unexpanded leaves. Such buds may remain dormant, or they may reactivate to produce a shoot with the same developmental potential as the primary shoot. The diversity of shoot system architectures and their plasticity is due in part to differential activity of axillary buds, and the sensitivity of bud activation to environmental inputs.

Bud activation is controlled by a network of interacting plant hormones that move systemically through the plant (Leyser, 2009). Prominent among them is auxin, synthesized in young expanding leaves at the shoot apex and actively transported down the plant (Ljung et al., 2001; Thimann and Skoog, 1933). This pathway for auxin movement is termed the polar auxin transport stream (PATS). The PATS is dependent on auxin efflux carriers of the ABCB and PIN families (Zazimalova et al., 2010). Of particular relevance is PIN1, which is basally localised in xylem-associated cell files in the stem, providing directionality to the PATS (Gälweiler et al., 1998). Auxin in the PATS inhibits bud activity, contributing to the phenomenon of apical dominance, in which the primary apex inhibits the activity of buds in the axils of leaves below it (Thimann and Skoog, 1933). Removal of the apex removes the auxin source, triggering bud activation. Application of auxin to the decapitated stump prevents bud activation. However, auxin acts indirectly, without entering the buds (Booker et al., 2003; Hall and Hillman, 1975). To account for this, a second messenger to relay the auxin signal into the bud has been proposed.

There are two, non-exclusive, candidates for this role. The first is cytokinin, which can directly promote bud activation and is transported acropetally in the transpiration stream (Sachs and Thimann, 1967; Bangerth, 1994). Cytokinin biosynthesis is downregulated by auxin, suggesting that auxin inhibits buds partly by reducing their cytokinin supply (Li et al., 1995; Tanaka et al., 2006; Nordström et al., 2004). The second candidate is strigolactone (SL) or a derivative (Gomez-Roldan et al., 2008; Umehara et al., 2008), which moves acropetally and inhibits branching. SLs were identified as the branch-inhibiting signal previously demonstrated to exist through the analysis of mutants with increased branching in pea [*ramosus* (*rms*)], petunia [*decreased apical dominance* (*dad*)] and *Arabidopsis* [*more axillary growth* (*max*)]. The branchiness of a subset of these mutants can be rescued by grafting to wild-type roots, suggesting a defect in the synthesis of an upwardly mobile branch inhibitor (Beveridge et al., 1997; Morris et al., 2001; Napoli, 1996; Simons et al., 2007; Turnbull et al., 2002; Sorefan et al., 2003; Booker et al., 2005). Where measured, these mutants have reduced SL levels and their branching phenotypes can be restored to wild type by exogenous SL application (Gomez-Roldan et al., 2008; Umehara et al., 2008).

The genes defined by the graft-rescuable mutants encode two plastidic carotenoid cleavage dioxygenases, CCD7/RMS5/DAD3/MAX3 (Booker et al., 2004; Johnson et al., 2006; Drummond et al., 2009) and CCD8/RMS1/DAD1/MAX4 (Sorefan et al., 2003; Snowden et al., 2005), and a cytochrome P450 family member,

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MAX1 (Booker et al., 2005). Mutations in orthologous rice *CCD* genes confer similar high branching phenotypes along with pronounced dwarfism and the genes are named *HIGH TILLERING DWARF1* (*HTD1*)/*DWARF17* (*D17*), and *DWARF10* (*D10*), respectively (Zou et al., 2006; Arite et al., 2007). Additional components, *D27* and *D14/HTD2/D88*, have been identified in rice (Lin et al., 2009; Arite et al., 2009; Gao et al., 2009; Liu et al., 2009), and further mutants with similar phenotypes are represented in the various mutant collections. These include *rms4/max2/d3*, which is not graft-rescuable and acts substantially cell autonomously in *Arabidopsis* (Beveridge et al., 1996; Booker et al., 2005; Stirnberg et al., 2007). RMS4/MAX2/D3 is an F-box protein likely to function in SL signal transduction (Stirnberg et al., 2002; Johnson et al., 2006; Ishikawa et al., 2005). Consistent with this idea, exogenous addition of SL cannot suppress branching in these mutants, and where measured, their SL levels are at least wild-type (Gomez-Roldan et al., 2008; Umehara et al., 2008).

There is strong evidence that SLs are involved in auxin-mediated bud inhibition. Where tested, SL mutant buds are resistant to apically supplied auxin (Beveridge et al., 2000; Sorefan et al., 2003; Bennett et al., 2006). Furthermore, in most cases, the transcription of *CCD7* and *CCD8* is upregulated by auxin (Sorefan et al., 2003; Foo et al., 2005; Johnson et al., 2006; Arite et al., 2007; Hayward et al., 2009), and grafting experiments suggest that the branchiness of some auxin-signalling mutants is partly due to SL deficiency (Hayward et al., 2009). These data suggest the attractive hypothesis that auxin moving in the PATS upregulates SL biosynthesis, and the SLs move acropetally into buds and repress their growth (Brewer et al., 2009). In this model, growth repression is a local bud event, and consistent with this idea, direct application of SLs to buds of pea and *Arabidopsis* inhibits their outgrowth.

However, an alternative model for bud-suppression by SL has been proposed in which, rather than acting only locally in buds, SLs act systemically by modulating auxin transport (Bennett et al., 2006; Prusinkiewicz et al., 2009). According to this model, auxin does not require a second messenger that moves into buds to inhibit their activity. Instead, auxin in the PATS suppresses bud activation by preventing the establishment of auxin transport out of the bud into the main stem, which is postulated to be a requirement for bud activity (Li and Bangerth, 1999; Prusinkiewicz et al., 2009). Consistent with this idea, there is a strong correlation between the ability of branches to transport auxin, PIN polarisation in branch stems, and branch activity (Morris, 1977; Prusinkiewicz et al., 2009).

Auxin export from new leaves at the bud shoot apex (an auxin source), into the stem (an auxin sink), is likely to proceed by canalisation, and may be necessary to allow phyllotactic patterning and hence leaf initiation to proceed (Bayer et al., 2009). According to the canalisation hypothesis, an initial flux of auxin from a source to a sink is upregulated by positive feedback to establish files of cells with highly polarised accumulation of PIN auxin exporters. Such cell files therefore act as auxin transport canals, and may subsequently differentiate into vascular strands (Sachs, 1981; Sauer et al., 2006). If buds must export auxin to be active, then auxin exported from active apices, moving in the PATS in the main stem, would reduce the sink strength of the stem for auxin and prevent canalised auxin export from inactive buds.

Computational modelling supports the plausibility of this mechanism (Prusinkiewicz et al., 2009), and it is consistent with phenotypic analysis of SL mutants, which show increased branching, increased polar PIN accumulation in the PATS and increased auxin transport. The SL mutant branching phenotypes can be rescued by reducing auxin transport levels to wild type by treatment with low doses of auxin transport inhibitors (Bennett et al., 2006; Lazar and Goodman, 2006; Lin et al., 2009). This is particularly striking because higher levels of auxin transport inhibition typically promote branching. These data are consistent with the idea that SLs act by limiting PIN accumulation on the plasma membrane, dampening canalisation and thus preventing bud activation. In this model, SL is not a straightforward second messenger for auxin, relaying information between the stem and bud. Rather it acts systemically to dampen auxin transport and enhance competition between shoot apices for common auxin transport pathways on the main stem (Prusinkiewicz et al., 2009).

These models of SL action have been inferred largely from analysis of SL mutant phenotypes and the expression of SL pathway genes. With the discovery of SLs as bioactive compounds in the pathway, and the availability of synthetic SLs such as GR24, the mode of action of SLs can be analysed more directly. Here we present the results of experiments aimed at testing current hypotheses for SL-mediated bud inhibition.

MATERIALS AND METHODS

Plant lines and plant growth

All lines are in the Col-0 background. Experiments involving *max1* used *max1-1*, and *max4*, *max4-1*. For *max2*, *max2-3* was used, except for Figs 4 and 6 where *max2-1* was used. Seeds were sown onto F2 compost treated with Intercept 70WG (both Levington Horticulture; www.scottsprofessional.co.uk), stratified at 4°C for 2 days and transferred to the greenhouse or plant growth room with 16 hour light, 8 hours dark photoperiods and temperatures ranging from 16 to 24°C. For axenic growth, seeds were sterilised as described in Bennett et al. (Bennett et al., 2006), and stratified at 4°C for several days. The seeds were sown into 500 ml Weck jars (Weck, Germany) containing 50 ml *Arabidopsis thaliana* salts (ATS) solution (Wilson et al., 1990) with 1% sucrose, solidified with 0.8% agar, supplemented with hormones as required.

Hormones and other treatments

GR24 (LeadGen Labs, Orange, CT, USA) was dissolved in acetone, NAA (naphth-1yl-acetic acid) in ethanol, and dexamethasone (DEX) in dimethyl sulphoxide (DMSO) or ethanol.

Auxin transport assays

Polar auxin transport was measured in inflorescence stems, as described in Bennett et al. (Bennett et al., 2006), with some modifications for Figs 1 and 2. Stem segments were incubated with radiolabeled auxin, ± GR24, for 6 hours (Lewis and Muday, 2009). Radioactivity was counted using Microscint-20 (PerkinElmer). For DEX treatment, 30 mM DEX was mixed into lanolin to a final concentration of 1 mM. DEX-lanolin was applied to the basal 5 mm of the main inflorescence stem of 39-day-old *Arabidopsis* plants. Seventy-two hours later, 15 mm segments above the site of application were excised and used for transport assays.

DEX>>MAX1, max1

To generate *DEX>>MAX1*, full-length *MAX1* cDNA was amplified with primers 5′-GGGACTAGTAGATGAAGACGCAACATCAA-3′ and 5′-CCCACTAGTTCAAAGATCTTCTTCAGAAATAAGCTTTTGTTCGAATCTTTTGATGGTTCTGA-3′ (contains 1×MYC tag) and cloned into *Spe*I-cut pTA7002 (Aoyama and Chua, 1997). The construct was transformed into *max1-1* plants by floral dipping (Clough and Bent, 1998). Several independent single insert lines were obtained, all of which behaved similarly. DEX-dependent induction of *MAX1* expression was assessed by RT-PCR. Four-week-old soil-grown plants were treated with 30 μM DEX for 12 hours. Basal 2 cm stem segments were harvested from at least ten plants. RT-PCR was performed as described in Bennett et al. (Bennett et al., 2006). Phenotypic rescue was assessed in soil-grown *DEX>>MAX1, max1* plants watered with 0.1% ethanol (mock) or 10 μM dexamethasone. For quantitative analysis of rosette branching *DEX>>MAX1*, *max1* plants were grown under sterile conditions in Weck jars with or without 30 μM DEX. The number of rosette branches was counted 35 days after germination.

Microscopic observation of PIN1:GFP

Wild-type, *max1-1* and *max2-3* plants homozygous for the *PIN1::PIN1:GFP* transgene cassette (Benkova et al., 2003) were used. The most basal part (15 mm) of the primary inflorescence stem from soil-grown 6-week-old plants were longitudinally sectioned by hand with a razor blade and immersed in ATS medium, supplemented with 0.1% acetone or 5 μM GR24. After 6 hours, sections were mounted in the same medium, and observed using a Zeiss Axiovert 200M-LSM 510 Meta confocal laser scanning microscope. The GFP signal was excited at 488 nm, collected with a 505-550 nm bandpass filter, and recorded along with a transmitted light image. For each condition, at least 12 specimens were observed. The data shown are representative of three biological replicates. Quantification of the GFP signal was performed using ImageJ. In one confocal image from each of the three biological replicates for this experiment, regions of four putative xylem parenchyma cells were manually selected according to the shape of a cell and position relative to vessels. The mean intensity in each region was measured and graphed.

Bud hormone response assays

Bud hormone response assays were performed as described by Chatfield et al. (Chatfield et al., 2000) for axenically grown plants, and by Ongaro et al. (Ongaro et al., 2008) for soil-grown plants. For long-term auxin treatments, the synthetic auxin NAA was used because of the instability of the natural auxin, IAA.

Statistical analysis

Comparison of two sample means was performed using the two-tailed Student's *t*-test. Dose-response relationships were analysed using Williams' test (Williams, 1971; Williams, 1972) and Shirley-Williams' test (Shirley, 1977; Williams, 1986). Data sets with multiple control groups were analysed using Tukey's test and the Dwass-Steel test (Neuhäuser and Bretz, 2001). Branch numbers were compared using non-parametric methods, based on the assumption that they do not always follow the normal distribution.

RESULTS

Auxin transport inhibition by GR24

If SLs act systemically by dampening auxin transport, then treatment of stem segments with SLs may reduce their ability to transport auxin. To test this prediction, we measured the amount of radio-labeled auxin transported over a 6-hour period along 1.5 cm *Arabidopsis* bolting stem segments in the presence of increasing concentrations of GR24. GR24 was able to reduce auxin transport in wild-type stem segments in a dose-dependent manner by up to 30% in this experiment (Fig. 1A). Concentrations as low as 1 nM significantly reduced auxin transport, and maximum inhibition was achieved at 100 nM. Higher concentrations had no further impact. This is in striking contrast to known pharmacological inhibitors of auxin transport such as N-1-naphthylphthalamic acid (NPA). In this assay, 1 μM NPA reduced auxin transport to only 9±1% of the untreated control.

To test further the relationship between auxin transport inhibition by SL and the *MAX* pathway, we examined the ability of GR24 to inhibit auxin transport in *max* mutant backgrounds. GR24 was effective at reducing auxin transport in wild type and *max* biosynthetic mutants, exemplified by *max4*, but auxin transport in *max2* stems was unresponsive, remaining at its characteristically elevated level even in the presence of 10 μM GR24 (Fig. 1B). The response in *max4* was smaller than that of the wild type. This is to be expected, as the GR24 and radiolabeled auxin were added simultaneously, such that some auxin would be transported before the GR24 could take effect. The results demonstrate that GR24 acts in a MAX2-dependent manner to reduce polar auxin transport in isolated stem segments.

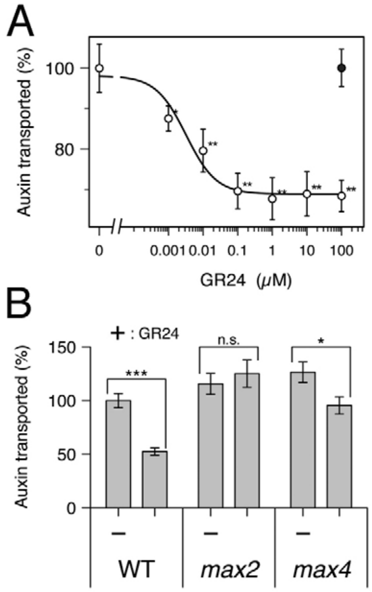

Fig. 1. Effect of GR24 on polar auxin transport. (**A**) Dose-response of basipetal indole-3-acetic acid (IAA) transport to GR24. Wild-type *Arabidopsis* inflorescence stem segments were incubated in ATS containing 1 μM [^{14}C]IAA, either alone or with increasing concentrations of GR24. The mean amount of radiolabeled auxin transported over a period of 6 hours for each GR24 dose is shown. These were compared with the vehicle control group using a Williams' test; *, $P<0.05$; **, $P<0.01$. Basipetal IAA transport in the presence of 1 μM NPA and acropetal IAA transport without drugs were 9±1% and 14±1%, respectively. The closed circle represents a 100 μM abscisic acid treated group, which showed no significant difference ($P>0.05$, Student's *t*-test) from the vehicle control group. (**B**) The effect of 10 μM GR24 on basipetal IAA transport in wild-type, *max4* and *max2* stems, measured as in A. Comparisons between the vehicle control group and the group treated with 10 μM GR24 were made using Student's *t*-test; n.s., not significant ($P>0.05$); *, $P<0.05$; ***, $P<0.001$. In both A and B, the mean ± s.e.m. of 16 segments are shown. WT, wild type.

Auxin transport inhibition by endogenous MAX pathway activity

To determine whether endogenous SLs have a similar effect, we put the *MAX1* gene under the control of a DEX-inducible promoter (Aoyama and Chua, 1997) and introduced it into the *max1* mutant background. When 4-week-old soil-grown plants were treated with 30 μM DEX, upregulation of *MAX1* transcript accumulation was observed (Fig. 2A). The weak *MAX1*-specific preinduction amplicon probably represents endogenous transcript containing the *max1-1* point mutation (Booker et al., 2005), or leaky expression of the transgene. Prolonged growth with DEX resulted in restoration of wild-type branching (Fig. 2B,C), indicating successful restoration of MAX pathway activity.

Using this system, we tested the effect of MAX activity on stem auxin transport. DEX was applied to the base of the bolting stem for 72 hours before segments were excised from immediately above the treated region and auxin transport through the segments was measured. Treatment with DEX resulted in reduced auxin transport in comparison with untreated controls (Fig. 2D). These results suggest that endogenously produced SLs can act in the stem to modulate auxin transport. Furthermore, because there was no difference in branching as a

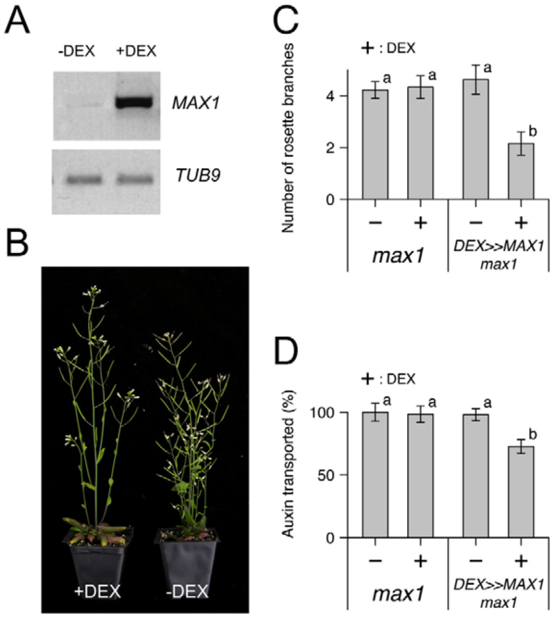

Fig. 2. Effect of endogenous strigolactones on polar auxin transport. (**A**) DEX induces *MAX1* expression in *DEX>>MAX1, max1* plants. Four-week-old plants were treated with 30 µM DEX for 12 hours. *MAX1* expression before and after DEX treatment was assessed by semi-quantitative RT-PCR (top). Normalisation of cDNA was performed with *TUBULIN9*-specific primers (bottom). (**B**) DEX restores wild-type shoot branching in *DEX>>MAX1, max1* plants. Plants were watered with 0.1% ethanol (–DEX) or 10 µM dexamethasone (+DEX). (**C**) Quantification of DEX-dependent rescue of *DEX>>MAX1, max1* rosette branching. Plants were grown under sterile conditions in Weck jars on ATS medium containing 0.1% ethanol (–DEX) or 30 µM dexamethasone (+DEX). The number of rosette branches was counted 28 days after germination. Means ± s.e.m. of 10-16 plants are shown. (**D**) Effect of induced endogenous strigolactone on basipetal IAA transport. The most basal part of the main inflorescence of *max1* or *DEX>>MAX1, max1* plants was treated for 72 hours with lanolin alone or with lanolin containing 1 mM DEX. The stem segment just above the treatment was assayed as in Fig. 1. Means ± s.e.m. of 12 segments are shown. The different letters in C and D denote significant differences at $P<0.05$ in mean values, as determined using the Dwass-Steel test and Tukey's test, respectively.

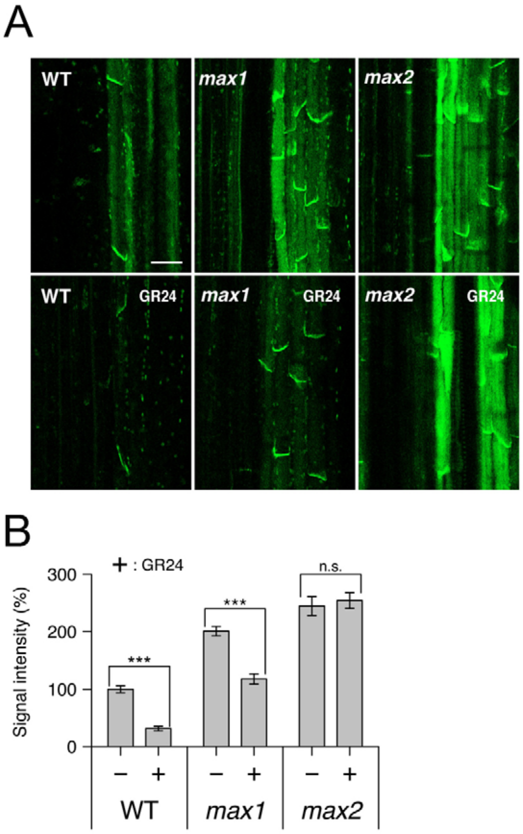

Fig. 3. Effect of GR24 on PIN1 protein accumulation. (**A**) Localisation of PIN1:GFP in longitudinal hand sections of 6-week-old basal inflorescence stems from wild-type (left), *max1* (middle) and *max2* (right) plants. Sections were incubated with 0.1% acetone (top) or 5 µM GR24 (bottom) for 6 hours before observation. Cells with GFP signals are xylem parenchyma cells. (**B**) Quantification of PIN1:GFP in xylem parenchyma cells. Means ± s.e.m. of 12 cells are shown. Comparisons between the vehicle control group and the group treated with 5 µM GR24 were made using Student's *t*-test; n.s., not significant ($P>0.05$); ***, $P<0.001$. Scale bar: 30 µm.

result of the short DEX treatment, the results demonstrate that the increased auxin transport observed in the *max* mutants is not a secondary consequence of branching.

GR24 reduces PIN1 accumulation in xylem parenchyma cells

We have previously shown that the increased auxin transport observed in *max* mutants is associated with increased accumulation of PIN1 in xylem parenchyma cells (Bennett et al., 2006). To test whether GR24 reduces PIN1 accumulation, we used plants carrying the well-characterised *PIN1::PIN1:GFP* transgene (Benkova et al., 2003). The basal 15 mm of bolting stems from wild-type, *max1* and *max2* plants homozygous for this transgene were halved longitudinally and incubated in ATS solution for 6 hours with or without 5 µM GR24. PIN1:GFP accumulation was assessed using confocal microscopy (Fig. 3A). The results parallel those obtained for auxin transport. PIN1:GFP overaccumulates in *max* mutants compared with wild type, consistent with previous observations (Bennett et al., 2006). GR24 treatment reduces PIN:GFP accumulation in wild-type and *max1* mutant plants, but not in the signalling mutant *max2*. Quantification of the fluorescence confirmed these results (Fig. 3B).

The mechanism by which SLs reduce PIN accumulation is unknown. The *max* mutants overaccumulate both *PIN1* transcripts and PIN1 protein (Bennett et al., 2006; Lazar and Goodman, 2006), and we have previously proposed that transcriptional upregulation may cause the *max* phenotypes (Bennett et al., 2006). To test this idea, we investigated the effect of overexpression of *PIN1* from the cauliflower mosaic virus 35S promoter using a previously described *35S:PIN1* line (Benkova et al., 2003), which is known to overproduce the PIN1 protein. There were no significant differences in either branching or stem auxin transport in this line (see Fig. S1 in the supplementary material). Thus, although SLs clearly modulate auxin transport and PIN1 accumulation in the stem, simply changing *PIN1* transcription is insufficient to account for this. This result, combined with the clear reductions in PIN1:GFP accumulation within 6 hours of GR24 treatment, suggests that a post-transcriptional mode of action may be involved.

Inhibition of bud growth by GR24 is context dependent

The results presented above support the idea that SLs modulate auxin transport by affecting PIN protein accumulation. This is consistent with the auxin transport canalisation model for bud activation (Bennett et al., 2006; Prusinkiewicz et al., 2009). However, the auxin transport effect of SLs could be independent of their direct local inhibition of buds. On the one hand, if SLs repress buds directly, then they should inhibit buds regardless of the presence of auxin in the main stem. If, on the other hand, they act by inhibiting auxin transport canalisation out of buds, then they should be much less effective without competing auxin in the main stem.

To explore the relevance of competing auxin, we compared the response of buds in different auxin contexts to GR24 treatment. First we tested the ability of GR24 to repress branching in whole plants. We germinated seed of different *max* genotypes on agar-solidified media supplemented with different doses of GR24 (Fig. 4). Consistent with previous reports (Gomez-Roldan et al., 2008; Umehara et al., 2008), biosynthetic mutants in the MAX pathway responded to GR24 by bud repression, but the *max2* signalling mutant was unresponsive. The *max1* mutant, which had not previously been extensively tested, behaved as expected for an SL biosynthetic mutant. Higher concentrations were needed to repress branching than to repress auxin transport (compare Fig. 1A with Fig. 4). This could be due to breakdown of GR24 over the 5 weeks of the assay and/or reduced uptake via the roots in comparison to the cut surface of the stem segments used for the transport assays.

To investigate the effect of GR24 on solitary buds, we used an isolated node assay (Chatfield et al., 2000). Stem segments bearing a single cauline leaf with its associated bud were excised and placed between two agar slabs in a Petri dish. We have previously shown that basally supplied compounds can enter the bud and modulate its activity in this assay (Chatfield et al., 2000; Booker et al., 2003). We treated wild-type buds with a range of basally supplied GR24 concentrations. The buds were unresponsive, and activated at the same time, even in the presence of 20 μM GR24 (Fig. 5A). It should be noted that approximately 10% of buds treated in this way do show some delay in activation compared with untreated controls, but 90% are unaffected. By contrast, apical application of 1 μM naphth-1yl-acetic acid (NAA), a synthetic auxin, significantly delayed the activation of all buds by several days.

If SLs inhibit buds locally and directly, the lack of response of isolated buds to GR24 is difficult to explain. However, if SLs act by dampening auxin transport, reducing the ability of the bud to compete for auxin transport pathways in the main stem, then the result is as predicted, because in this assay there is no competing auxin source to prevent auxin transport canalisation out of the bud. A prediction from this interpretation is that GR24 should be effective in the presence of a competing auxin source. We therefore investigated the ability of 5 μM basal GR24 to inhibit bud activation in the presence of 0.5 μM apical NAA, which usually inhibits bud outgrowth for about 3 days (Chatfield et al., 2000). The results show that GR24 prolonged bud inhibition beyond the effect of apical auxin alone (Fig. 5B). To determine whether this effect is dependent on MAX2, we repeated the assay using *max2* mutant nodes. We have previously shown that *max2* mutants are resistant to apical auxin in this assay, although less so than the auxin signalling mutant *axr1* (Booker et al., 2005; Bennett et al., 2006). In contrast to wild type, basal GR24 had no effect on the timing of bud activation, even in the presence of apical auxin.

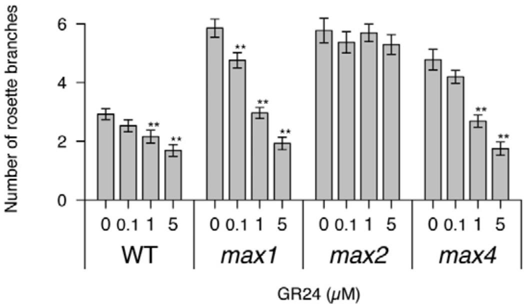

Fig. 4. Effect of GR24 on branching in whole plants. Plants were grown axenically in Weck jars on ATS medium containing the indicated concentrations of GR24. The number of rosette branches was counted at maturity. Means ± s.e.m. of at least 16 plants are shown. Double asterisks denote significant differences ($P<0.01$) from the vehicle control group compared by Shirley-Williams' test, otherwise there were no significant differences ($P>0.05$).

These results suggest a requirement for a competing auxin source for SL-mediated bud inhibition. This requirement has previously been called into question because application of GR24 to the most apical bud of peas inhibits its outgrowth even after decapitation of the primary apex, and hence removal of competing apical auxin sources (Brewer et al., 2009). However, more basal axillary buds were present on these plants and activated during the experiment. According to the canalisation model, basal auxin sources can also be effective competitors, as the auxin exported by these buds into the stem can still reduce flux from more apical auxin sources (Prusinkiewicz et al., 2009). Indeed we have previously shown that when *Arabidopsis* bolting stem segments carrying two cauline leaves with their associated buds are excised, one bud often outgrows the other, and it can be either the basal bud or the apical bud that dominates (Ongaro et al., 2008). In similar two-node segments from *max* mutants, the two buds grow more evenly, consistent with reduced competition between the buds.

To determine whether GR24 is able to enhance competition between buds, we compared the growth of buds on one-node and two-node segments of wild-type, *max4* and *max2* plants, with or without basal 1 μM GR24. The results show that, again, isolated buds do not respond strongly to basal GR24 (Fig. 6A). After four days, the buds of all the genotypes had activated and begun to elongate. There were no significant effects of GR24 treatment for any genotype. By contrast, when two buds were present, there was a significant delay in the activation of both the apical and basal buds for wild type, and of the basal bud for *max4* mutants (Fig. 6A). However, both *max2* buds were unresponsive to GR24.

A second observation is that in the two-bud configuration, both buds activated more slowly than in the one-bud configuration, consistent with enhanced competition. To allow comparable stages of bud activation to be assessed, the mean bud lengths plotted in Fig. 6A were taken at day 4 for lone buds, but at day 6 for two-bud explants.

Furthermore, the graph in Fig. 6A does not capture a third feature. GR24 does not simply reduce elongation of both buds, but rather focuses growth into a single branch. This effect can be

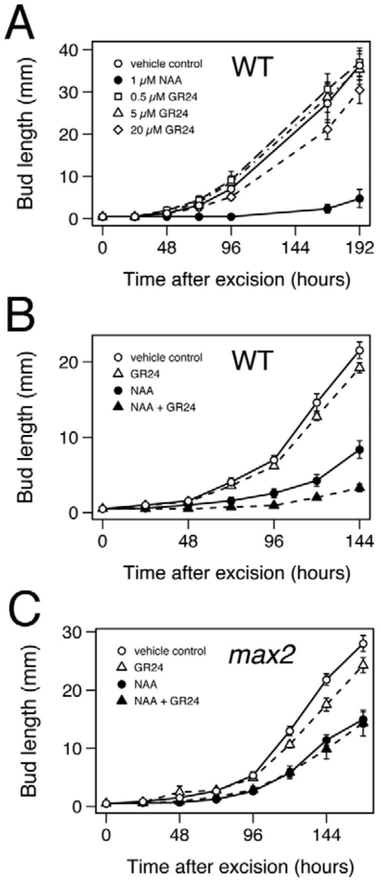

Fig. 5. Effect of GR24 on activation of buds on isolated nodal segments. Nodal segments bearing cauline buds less than 1 mm in length were excised from plants grown under sterile conditions in Weck jars on ATS medium. The segments were inserted between two agar slabs in a Petri dish. The indicated hormones were supplied to the basal or apical agar. (**A**) Dose response of buds to basally supplied GR24. Apical supply of 1 μM NAA was included as a positive control for bud inhibition. In comparison to the vehicle control, basal GR24 did not affect bud activation significantly ($P>0.05$), whereas apical NAA delayed bud activation significantly ($P<0.001$). (**B**) Effect of combined 5 μM basal GR24 and 0.5 μM apical NAA on bud activation. In the presence of apical NAA, basal GR24 delayed bud activation significantly ($P<0.05$). (**C**) Response of *max2* mutant buds to combined 5 μM basal GR24 and 0.5 μM apical NAA. Even in the presence of apical NAA, basal GR24 did not affect bud activation significantly ($P>0.05$). Means ± s.e.m. of 8-14 buds are shown. In each experiment, bud activation was assessed by comparing mean bud lengths at 96 hours using Student's *t*-test.

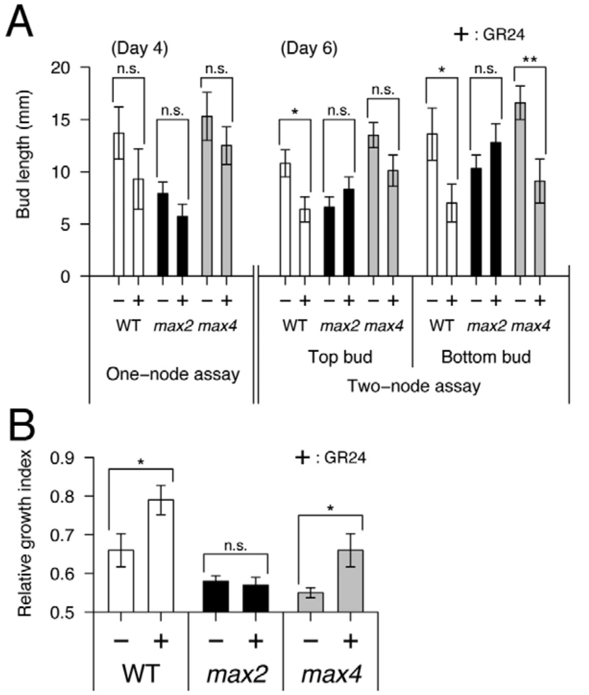

Fig. 6. Effect of GR24 on activation of buds on one- and two-node stem segments. Nodal segments bearing cauline buds less than 2 mm in length were excised from soil-grown plants. The basal stem was inserted into an Eppendorf tube containing ATS solution supplemented with 1 μM GR24 as indicated. (**A**) Branch length for lone buds 4 days post-excision with and without GR24, and for the top and bottom buds on two-node stem segments 6 days post-excision. (**B**) Relative growth index (RGI) at day 10 for branches of the two-node explants. The RGI is the proportion of branch length in the longest branch. Means ± s.e.m. of 10-17 plants are shown. Comparisons between the vehicle control group and the GR24 treated group were made using Student's *t*-test; n.s., not significant ($P>0.05$); *, $P<0.05$; **, $P<0.01$.

assessed using the relative growth index (RGI), which is the proportion of branch length in the longest branch (Ongaro et al., 2008). If both branches are the same length, the RGI will be 0.5. If one grows and the other remains dormant, the RGI will be 1. The RGIs at day 10 are plotted in Fig. 6B. As previously reported, the *max* mutants have lower RGIs than wild type (Ongaro et al., 2008). Either the top or bottom bud could dominate. In this experiment, for the wild-type sample the top bud was longer than the bottom bud in 7/16 cases. Basal GR24 significantly increased the RGI for both wild-type and *max4* mutants, but had no effect in *max2*. The treatment did not appear to affect which bud was favoured, with the top bud being longer than the bottom bud in 9/16 of the wild-type explants. These results are consistent with the idea that GR24 enhances competition between buds in a MAX2-dependent manner, further supporting the auxin transport canalisation dampening model for SL action.

DISCUSSION

MAX1 and strigolactone biosynthesis

A recent breakthrough in understanding shoot-branching control identified strigolactones, or their derivatives, as the upwardly mobile branch-inhibiting compound predicted by analysis of branching mutants of pea (*rms*), petunia (*dad*) and *Arabidopsis* (*max*) (Gomez-Roldan et al., 2008; Umehara et al., 2008). The involvement of strigolactones was hypothesised from evidence for their carotenoid origin (Matusova et al., 2005), coupled with observations that two of the branching loci encode carotenoid cleavage dioxygenases (CCD7/RMS5/DAD3/MAX3/D17 and CCD8/RMS1/DAD1/MAX4/D10) (Sorefan et al., 2003; Booker et al., 2004).

Little is known about strigolactone biosynthesis downstream of carotenoid cleavage (Humphrey and Beale, 2006; Rani et al., 2008). Grafting and expression analysis suggest that the *Arabidopsis* MAX1 P450 family member acts downstream of the CCDs after the formation of a mobile intermediate (Booker et al., 2005), and furthermore, cytochrome P450 enzymes have been postulated to be involved in SL biosynthesis (Matusova et al., 2005; Humphrey and Beale, 2006; Rani et al., 2008). Here we show that branching in *max1* mutants can be fully suppressed by GR24, suggesting that MAX1 is required for strigolactone synthesis.

Mechanism of strigolactone action

Two mechanisms have been proposed to explain the inhibitory effect of SLs on shoot branching. The first is that SLs act directly and locally in buds to inhibit their outgrowth (Brewer et al., 2009). The second is that SLs act systemically to dampen auxin transport canalisation, making it more difficult for buds to establish auxin export into the main stem in the presence of competing auxin in the PATS, thus preventing bud activation (Li and Bangerth, 1999; Bennett et al., 2006; Prusinkiewicz et al., 2009).

The results presented here strongly support the second hypothesis. Firstly, GR24 and endogenous SLs reduce auxin transport and PIN1 accumulation in isolated stem segments in a MAX2-dependent manner. The effects on transport are modest, consistent with a dampening effect rather than the strong inhibition typical of known pharmacological inhibitors of auxin transport. Such dampening can enhance competition between buds (Prusinkiewicz et al., 2009). Secondly, solitary buds receiving an ample supply of GR24 are not inhibited, but GR24 applied by the same method can enhance the inhibition of solitary buds by apical auxin, in a MAX2-dependent manner. Perhaps even more strikingly, GR24 can enhance the competition between buds on two-node stem segments, focusing growth into one of the two buds.

These observations are hard to reconcile with a model of direct local bud repression by SLs, but they are entirely consistent with the auxin transport canalisation model for bud regulation. Thus our results strongly support two hypotheses: that auxin in the main stem inhibits bud activity by preventing auxin transport canalisation out of the bud; and that SLs reduce bud activation by dampening auxin transport.

Implications for shoot-branching control

The phenomenon of apical dominance has proved so mechanistically impenetrable that many authors prefer the less specific term ‘correlative inhibition’. The central paradox is the ability of auxin simultaneously to protect active buds from inhibition and keep inhibited buds dormant (Snow, 1937). Under the canalisation model for bud activity, this is no longer a paradox. All apices are equivalent and differ only in their auxin export status, with auxin export from active apices maintaining their activity and preventing activation of others.

There are many details of this model to be clarified. A central question is why auxin export is needed for bud activity. A contributory factor may be that polar auxin transport from bud to stem is required to establish vascular connectivity between them (Sachs, 1968). However, as previously active, vascularly connected buds can be re-inhibited, this cannot be the main reason. Instead, an attractive hypothesis is that auxin transport away from incipient leaf primordia at the meristem is necessary to allow phyllotactic patterning to proceed (Bayer et al., 2009). If this is the cause, it could explain why direct application of auxin to buds cannot trigger their ectopic activation, as flooding the apex with auxin is unlikely to promote auxin export from competing sources, i.e. the young leaves.

The auxin transport canalisation model for bud activation shifts focus away from a dominating primary apex inhibiting the branches below it, to a more dynamic integrated view (Sachs et al., 1993). All the meristems in a shoot are ultimately connected by their common auxin transport pathway through the main stem to the root. The positive feedback process of canalisation acts through this auxin transport network to balance bud activation across the shoot system.

The role of SL in this network in interesting. Instead of simply inhibiting any bud that it reaches, SLs set the global context in which buds compete for auxin export into the main stem. With low SL, many buds can activate, contributing auxin to the main stem. With high SL, the sources that activate first dominate the system, preventing activation of additional buds. As a root-derived signal transported upwards to the shoot, SLs can permeate the plant vascular system, reaching all the meristems in the shoot, and the tissue through which they must export auxin. Thus, factors such as nutrient availability in the root, which are known to affect SL synthesis (Yoneyama et al., 2007; López-Ráez et al., 2008), can globally set the level of competition between buds. Competition is resolved by local bud competitiveness, based on developmental and environmental factors such as shading.

In this context, is it is interesting to compare the behaviour of the two-branch *Arabidopsis* system with results we recently obtained for *Chrysanthemum* (Liang et al., 2010). Again, GR24 only inhibited buds in the presence of a competing auxin source, but in *Chrysanthemum* the top bud was always favoured over the bottom bud, whereas in *Arabidopsis* the choice is more random. This suggests different relative competitiveness of the top versus bottom buds in these two species. Further cross-species comparisons might provide explanations for these differences.

As described above, root-derived SLs provide a mechanism by which overall nutrient status can be globally reported, and integrated with local signals. It is likely that SLs also play a role in determining local bud competitiveness. SL biosynthesis mutant roots grafted to wild-type shoots have wild-type branching, demonstrating the activity of shoot-synthesised SLs (Beveridge et al., 1997; Morris et al., 2001; Napoli, 1996; Simons et al., 2007; Turnbull et al., 2002; Sorefan et al., 2003; Booker et al., 2005). This situation is reminiscent of cytokinin (Ck), where both root-derived Ck and local nodal synthesis are important (Li et al., 1995; Tanaka et al., 2006). The synthesis of both Ck and SL is regulated by auxin in the stem and root (Nordstrom et al., 2004; Li et al., 1995; Sorefan et al., 2003; Foo et al., 2005; Bainbridge et al., 2005; Johnson et al., 2006; Arite et al., 2007; Hayward et al., 2009). For example, following decapitation, auxin depletion in the stem leads to local downregulation of SL synthesis and upregulation of Ck synthesis. All these effects can contribute to local bud activation. Auxin export from activated buds will restore auxin levels in the stem, reducing stem sink strength for auxin, upregulating SL synthesis and downregulating Ck synthesis, re-equilibrating the system. In the case of SL there is an additional element in this loop. Buds activated in the presence of very low SL will export more auxin, as a result of higher PIN accumulation, and thus induce more SL biosynthetic gene transcription, than buds activated in the presence of higher SL levels.

Outlook

The discovery of SLs is a watershed in understanding shoot branching, but of course has much wider implications. SL pathway mutants have been recovered from wider screens for phenotypes including delayed senescence and defective light responses (Woo et al., 2001; Shen et al., 2007). SL action via auxin transport modulation could provide a unifying mechanism underlying these diverse roles. Our results will guide ongoing investigations of the molecular mechanism of SL action – in particular PIN protein accumulation.

In the context of shoot-branching control, the emerging picture is one of interlocking feedback loops that integrate systemic and local signals to control bud activity. This provides an excellent illustration of the prevalent shift in biological thinking away from simple pathways with upstream and downstream components to networked signals in which perturbations of the system have non-intuitive effects. Analysis of such systems is greatly facilitated by computational modelling, and shoot branching control is already proving a fertile ground for these approaches (Dun et al., 2009; Prusinkiewicz et al., 2009).

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Competing interests statement
The authors declare no competing financial interests.

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