Running Heading: Modulation of shoot branching by N supply

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Research Area:
Signaling and Response
Auxin and strigolactone signaling are required for modulation of Arabidopsis shoot branching by N supply

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Summary: Limited N supply suppresses shoot branching in Arabidopsis in a process that requires auxin and strigolactone signaling and involves an increase in auxin export from active shoot apices.
Financial support: This work was supported by the European Research Council grant No. 294514 EnCoDe (OL, MdJ, HM), the Gatsby Foundation (OL), the Swedish Governmental Agency for Innovation Systems (VINNOVA) and the Swedish Research Council (VR) (KL), a Burgess Studentship (GG), the UK Biotechnology and Biological Sciences Research Council (OL, VO and BW) and Norsk Hydro (OL, LW).

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Abstract

The degree of shoot branching is strongly affected by environmental conditions such as nutrient availability. Here we demonstrate that nitrate limitation reduces shoot branching in Arabidopsis thaliana both by delaying axillary bud activation, and by attenuating the basipetal sequence of bud activation that is triggered following floral transition. Ammonium supply has similar effects, suggesting that they are caused by plant N status, rather than direct nitrate signaling. We identify increased auxin export from active shoot apices, resulting in increased auxin in the polar auxin transport stream of the main stem, as a likely cause for the suppression of basal branches. Consistent with this idea, in the auxin response mutant, axr1, and the strigolactone biosynthesis mutant, max1, increased retention of basal branches on low N is associated with a failure to increase auxin in the main stem. The complex interactions between the hormones that regulate branching make it difficult to rule out other mechanisms of N action, such up-regulation of strigolactone synthesis. However, the proposed increase in auxin export form active buds can also explain how reduced shoot branching is achieved without compromising root growth, leading to the characteristic shift in relative biomass allocation to the root when N is limiting.
Plants continuously adjust their development to suit the environmental conditions in which they are growing. This spectacular developmental plasticity means that plants with identical genotypes can have very different morphologies. A good example of such developmental plasticity is the degree of shoot branching (Leyser, 2009). Shoot branches develop from axillary meristems laid down in the axils of the leaves produced by the primary shoot apical meristem. These axillary meristems can arrest after producing a few leaves to form a small dormant bud, or they can remain active or later reactivate, to produce a branch. The leaves produced on the branch also harbour axillary meristems, allowing higher order branching. Hence, depending on the activity of axillary meristems, the adult plant can range in form from a single unbranched stem to a highly ramified bush. Many environmental factors are integrated to regulate the dormancy-activity transitions of axillary meristems. Prominent among these is the supply of mineral nutrients such as inorganic phosphate and nitrate (Domagalska and Leyser, 2011; Brewer et al., 2013).

Limited nitrate (NO$_3^-$) availability can have profound effects on both root and shoot system architecture and biomass allocation, shifting the balance in favour of the root. In the root, low NO$_3^-$ can promote the elongation of both primary and lateral roots relative to shoot dry weight (Scheible et al., 1997; Zhang et al., 1999; Linkohr et al., 2002). Furthermore, a patch of high NO$_3^-$ can stimulate local proliferation of lateral roots into the patch and can suppress root growth outside the patch (Drew, 1975; Scheible et al., 1997; Zhang and Forde, 1998; Zhang et al., 1999; Linkohr et al., 2002). Some of these responses, such as proliferation of roots into a high NO$_3^-$ patch, are the result of direct local NO$_3^-$ signaling, but there is also clear evidence for systemic nitrogen (N) status effects. In particular, both the inhibitory effect on root growth by high NO$_3^-$ supply and the stimulatory effect of low NO$_3^-$ appear to be mediated by shoot N status (Scheible et al., 1997; Zhang et al., 1999). Thus variation in NO$_3^-$ availability triggers systemically co-ordinated changes in root and shoot system architecture.

There has been considerable progress in understanding how local NO$_3^-$-regulated changes in the root system are effected (reviewed in Bouguyon et al., 2012), but much less is known about how NO$_3^-$ availability regulates shoot branching, and how this is co-ordinated with the root. These are important questions because of the agricultural imperative to reduce fertiliser
inputs while maintaining yield. With respect to shoot branching, there is little physiological evidence for a direct role for mineral nutrients in bud activation, because although sustained growth of branches undoubtedly requires a nutrient supply, application of nutrients to dormant buds does not usually activate their growth (Cline, 1991). In contrast, there is ample evidence that hormones regulate bud activity and these same hormones are excellent candidates to act as systemic co-ordinators of nutrient signaling (Leyser, 2009). This suggests the hypothesis that NO$_3^-$-triggered changes in shoot system architecture are mediated by changes in hormone activity.

There is good evidence that NO$_3^-$ availability in the root system positively regulates cytokinin (CK) biosynthesis (Takei et al., 2002; Miyawaki et al., 2004; Takei et al., 2004; Wang et al., 2004) and this is required for at least some systemic responses to NO$_3^-$ availability (Ruffel et al., 2011). Two additional systemically mobile hormones have been strongly implicated in shoot branching control, namely auxin and strigolactones (SLs), both of which are generally considered to inhibit bud growth (reviewed in Dun et al., 2009; Domagalska and Leyser, 2011).

Auxin is mainly produced in the young expanding leaves at the shoot apex (Ljung et al., 2001). It is transported down the stem in the polar auxin transport stream (PATS) and inhibits bud growth. In *Arabidopsis*, this directional flow involves the PIN1 (PIN-FORMED1) protein, a member of a family of auxin efflux proteins that is localised to the basal membrane of cells of the PATS (Okada et al., 1991; Gälweiler et al., 1998; Wisniewska et al., 2006). Auxin moving in the PATS does not enter the bud in appreciable amounts, indicating an indirect mode of action (Brown et al., 1979; Everat-Bourbouloux and Bonnemain, 1980; Prasad et al., 1993; Booker et al., 2003). There is good evidence to suggest that auxin transport in the main stem inhibits bud outgrowth by preventing axillary buds from establishing their own PATS out into the main stem (Li and Bangerth, 1999; Bennett et al., 2006; Prusinkiewicz et al., 2009; Balla et al., 2011; Shinohara et al., 2013). In addition, auxin may act by regulating the production of one or more second messengers in the stem, which move up into the bud to regulate bud activity locally and directly (Snow, 1937). Both CK and SL are good candidate second messengers since the transcription of the biosynthetic genes for both these hormones is regulated by auxin (Nordström et al., 2004; Foo et al., 2005; Johnson et al., 2006; Hayward et al., 2009).
SLs are synthesised in both the root and shoot and are transported acropetally in the transpiration stream (Beveridge et al., 1996; Napoli, 1996; Booker et al., 2004; Kohlen et al., 2011). SLs may directly and locally inhibit bud activation, as mentioned above (Brewer et al., 2009; Dun et al., 2012, 2013), and/or they act systemically to influence bud growth by modulating PIN1 accumulation at the plasma membrane, and hence the establishment of canalization of auxin transport out of the bud (Bennett et al., 2006; Prusinkiewicz et al., 2009; Crawford et al., 2010; Shinozaka et al., 2013).

There is limited evidence about the roles of SLs and auxin in the control of shoot branching in response to N. SL exudation from the root can be up-regulated by N deficiency, and it has been demonstrated that SLs are required for the suppression of branching under phosphate deficiency, which also elevates SL biosynthesis and exudation from the root (Yoneyama et al., 2007a; Lopez-Raez et al., 2008; Umehara et al., 2010; Kohlen et al., 2011; Mayzlish-Gati et al., 2012; Yoneyama et al., 2012; Foo et al., 2013). In the case of auxin, increased auxin moving down the primary shoot would be predicted to suppress shoot branching, and there is some evidence to support such an increase from studies of root responses to N. For example in maize shoot-derived auxin appears to enhance root branching responses to N deprivation (Tian et al., 2008; Liu et al., 2010). Similarly, changes in shoot-to-root auxin transport have been implicated in the suppression of nodulation in response to N sufficiency, however there are apparently contradictory results as to whether nodule suppression results from high or low shoot-to-root auxin transport (van Noorden et al., 2006; Jin et al., 2012).

Here we present work aimed at testing the hypothesis that auxin and SLs are important mediators in shoot branching suppression under low NO₃⁻ conditions, making use of auxin and SL mutants of Arabidopsis.

Results

Low nitrate restricts shoot branching in Arabidopsis by early termination of the basipetal sequence of bud activation

In Arabidopsis, the primary shoot apical meristem remains indeterminate, producing leaves at its flanks with usually a single axillary meristem (AM) in each axil. During the vegetative phase the AMs are slow to develop and cannot be morphologically identified for many
plastochrons below the primary SAM. This means that in long-day conditions there are usually no active branches produced in the vegetative phase. After floral transition AMs arise rapidly in the axils of all leaves. These AMs activate to form lateral inflorescences in a basipetal sequence that usually includes all the inflorescence-born cauline leaves and proceeds down into the rosette (Hempel and Feldman, 1994; Stirnberg et al., 1999; Grbić and Bleecker, 2000).

To investigate the effect of N supply on branching, we grew Arabidopsis plants with NO$_3^-$ supply ranging from 9mM to 1.8mM. Across this range, the mean total number of secondary shoots produced dropped from 4.25 on 9mM NO$_3^-$ to 2.9 on 1.8mM NO$_3^-$ (Fig. 1A). Measurement of free nitrate in the leaves of 4-week-old plants revealed clear evidence of reduced NO$_3^-$ levels in the leaves of plants supplied with 2.25mM NO$_3^-$ or less. Importantly, even plants grown on 1.8mM NO$_3^-$ still showed substantial accumulation of free nitrate during the vegetative phase, suggesting N limitation rather than N starvation (Fig. 1B). Based on these results, 9mM and 1.8mM NO$_3^-$ supply were selected as the standard N-sufficient and N-limited conditions for further analysis.

To characterise in more detail the effect of low NO$_3^-$ on branching, the lengths of the three most apical branches were followed over time after anthesis of the first flower on the primary inflorescence. With 9mM NO$_3^-$ supply, these three branches activate near-simultaneously and rapidly elongate, reaching lengths in excess of 12cm over the time course of the experiment (Fig. 2A). On 1.8mM NO$_3^-$, bud activation and elongation is delayed, and the basipetal sequence of activation is much more obvious. By 6 days post anthesis, only the most apical bud has elongated significantly. By 11 days, the second bud is also actively growing, whereas there is still no significant elongation of the third bud (Fig. 2B). Even the first bud elongates more slowly than its counterpart on 9mM NO$_3^-$, reaching only approximately 4 cm by the end of the experiment. Thus reduced NO$_3^-$ availability results in delayed bud activation, reduced bud elongation, and early termination of the basipetal activation sequence, leading to reduced total branch number (Fig. 1A, Fig. 2A, B, and Fig. 4C).

**Branch suppression is not a direct effect of nitrate signaling**

There is good evidence that the NO$_3^-$ ion itself can act as a signal (Crawford, 1995; Stitt, 1999; Forde, 2002; Takei et al., 2002). To investigate whether NO$_3^-$ signals directly to
promote branching, plants were grown on limiting NO$_3^-$ supplemented with increasing amounts of ammonium as an N source. This approach was adopted because using ammonium as the sole N source was found to be toxic to Arabidopsis. Plants supplied with only 1mM NO$_3^-$ produced a mean of approximately one secondary shoot, while the number of branches was double for plants supplied with both 1mM NO$_3^-$ and 1mM NH$_4^+$. This mean increased slightly, but not significantly under 2mM NH$_4^+$ with 1 mM NO$_3^-$, and 3 mM NO$_3^-$ supply (Fig. 3A). Similar results were obtained when plants were grown under sterile conditions (Fig. S1), demonstrating that the effects are unlikely to be due to microbial conversion of NH$_4^+$ to NO$_3^-$. These findings suggest that branching is not directly triggered by NO$_3^-$ acting as a signal, but that the response relates to the overall N status of the plant. To investigate this idea further, the root/total mass ratio of these plants was examined, since low nutrient availability is well known to trigger redistribution of relative growth from shoots to roots. As expected, reduced branching is accompanied by increased proportional biomass allocation to roots, regardless of whether N was supplied as NO$_3^-$ alone or an NH$_4^+$ NO$_3^-$ mix (Fig. 3B).

**Auxin and Strigolatone are both involved in branching response to N**

Since NO$_3^-$ is apparently not directly involved in the shoot branching response to N nutrition, we tested whether auxin or SL are required. The response of branching to N limitation in wild-type plants was compared with those of the auxin resistant mutant *axr1-3* (Lincoln et al., 1990), and the SL biosynthesis mutant *max1-1* (Stirnberg et al., 2002; Booker et al., 2005). Both mutants responded to N limitation by reducing their branching, although both mutants still produced more secondary shoots than wild-type plants under the same N-limiting conditions (Fig. 4A). Similar results were obtained for the SL-biosynthesis mutants *max3* and *max4*, and for the SL signalling mutant, *max2* (Figure S2). In the *axr1-3 max1-1* double mutant, there was no significant difference in branch numbers on high and low NO$_3^-$ in this experiment (Fig. 4A). There was some variability between experiments in the extent of the response in the double mutant (Figure S3), nonetheless these results suggest that the ability to reduce branching in response to low NO$_3^-$ is at least partially dependent on auxin and strigolactone signaling.
It was apparent from these experiments that N deprivation also results in reduced stature (Fig. 4B, C). Interestingly, this effect was abolished in the axr1 mutants, but was unaffected by mutation in max1, suggesting that it is auxin-dependent, but SL-independent.

**N deprivation and SL treatment have similar effects on bud growth**

The reduced of the max mutants to suppress branching on low N suggest that either their buds are near-constitutively active, or that bud suppression on low N is partly mediated by dynamic changes in SL levels. Direct assay of SLs, especially in Arabidopsis shoots, is currently extremely challenging. Therefore, we assessed whether low NO₃⁻ has SL-like effects on the shoot, using a well-established two-bud assay (Ongaro et al., 2008). In this assay, stem segments carrying two nodes are excised from plants and supplied with nutrient solution basally, which can be adjusted to include different hormones or nutrients, as required. The growth of the two buds is monitored over time and the relative growth index (RGI) of the two buds can be determined. RGI is the length of the longest bud divided by the total length of both buds. Usually, either one bud grows vigorously and the other does not, giving an RGI close to 1; or both buds grow equally well, giving an RGI close to 0.5. The mean RGI thus captures the relative frequency of these outcomes. In this assay, SLs have been shown to shift the RGI toward 1 (Crawford et al., 2010). To assess the effect of NO₃⁻ availability on RGI, we grew wild-type and SL-resistant mutant (max2) plants on 9mM or 1.8mM NO₃⁻, excised a two-node segment from their bolting stems and measured RGIs under high and low NO₃⁻ respectively, with or without basal addition of the synthetic SL, GR24. As previously reported, the RGI of wild-type explants under N-sufficient conditions was significantly higher than that of max2 mutants, and GR24 treatment increased the wild-type RGI further, but had no effect on that of max2 (Fig. 5A). The effect of low N supply resembled GR24 treatment. Low N significantly increased the RGI of wild-type explants, but had no significant effect on the RGI of max2 mutants (Fig. 5A). GR24 had no additional effect when supplied to low NO₃⁻-grown plants, however, the wild-type RGIs under both these treatments were near-maximal. In these experiments, the wild-type RGI in N-sufficient conditions was on average 0.8, which is higher than that typically observed (Ongaro et al., 2008; Crawford et al., 2010). This may be because to allow full control of N supply the plants were grown on a sand and terragreen mix, rather than compost.
This result is consistent with the hypothesis that shoot branching suppression on low NO$_3^-$ is partly attributable to increased SL production, as suggested by the observation that low NO$_3^-$ increases SL levels in root exudates from some species (Yoneyama et al., 2007a; Jamil et al., 2011; Yoneyama et al., 2012; Foo et al., 2013; Yoneyama et al., 2013). However, it is equally consistent with other hypotheses, such as increased auxin production on low NO$_3^-$.

To assess further whether low NO$_3^-$ increases SL levels, and in particular whether this is due to increased transcript abundance for SL biosynthetic genes, we examined publically available expression data for these genes (ArrayExpress, Rustici et al., 2013). To date 4 such genes are known- MAX1, MAX3, MAX4 and D27. Among 6 experiments examining the effects of NO$_3^-$ supply or re-supply after NO$_3^-$ deprivation (Wang et al., 2003; Scheible et al., 2004; Gifford et al., 2008; Patterson et al., 2010; Krapp et al., 2011; Gifford et al., 2013 ), we found no evidence of a consistent effect of N supply on the transcription of any of these genes (Table S1). The transcript abundance of core genes in the strigolactone signaling pathway, D14 and MAX2, was also not affected (Table S1).

**Auxin transport in the main stem is unaffected by N availability**

To probe further the possibility of changes in SL levels on low N we assessed auxin transport changes. A signature of GR24 treatment and increased endogenous SL synthesis is reduced polar auxin transport in the main stem (Crawford et al., 2010; Shinohara et al., 2013). This effect is associated with a rapid SL-induced depletion in PIN1 accumulation on the plasma membrane of xylem parenchyma cells in the stem. Depletion of PIN1 by SL can occur within 10 minutes, independently of new transcription, and is therefore a primary SL response (Shinohara et al., 2013). To assess the effects of low NO$_3^-$ on auxin transport through the stem, radio-labelled auxin was applied to the apical end of stem segments excised from plants grown with low or high NO$_3^-$ supply, and the amount transported to the basal end was measured. As previously demonstrated, reduced SL levels in the max1 mutant resulted in increased auxin transport, but mutation in axr1 had no effect (Bennett et al., 2006; Prusinkiewicz et al., 2009; Crawford et al., 2010). NO$_3^-$ supply had no significant effect on auxin transport in any of the genotypes assessed (Fig. 5B), and accumulation of PIN1 on the plasma membrane of xylem parenchyma cells in the stem was also unaffected by NO$_3^-$ supply (Fig. 5C). These results argue against the hypothesis that reduced NO$_3^-$ supply increases endogenous SL levels, consistent with the lack of effect on transcript abundance of SL.
biosynthetic genes. More generally, the results suggest that changes in auxin transport are not involved the branching response to NO$_3^-$ supply.

**Reduced N availability increases auxin levels in the main stem polar auxin transport stream**

As mentioned above, an alternative hypothesis for branch inhibition by low NO$_3^-$ is that N limitation increases auxin levels in the polar transport stream in the shoot. To test this hypothesis, we measured the amount of auxin moving in the polar auxin transport stream (PATS) of stem segments of the various genotypes under investigation by collecting the auxin that emerged from the base of the stem segments over a 24 hour period and subjecting the exudate to mass spectrometric analysis. Auxin exudates from stem segments taken from either the apical part of the bolting stem, between the most apical cauline node and the first silique, or the basal part of the bolting stem, between the most basal cauline node and the rosette, were assayed. In wild-type plants grown on low NO$_3^-$, the amount of auxin exported from apical stem segments was double that of plants grown on high NO$_3^-$ (Fig. 6 inset). For basal stem segments from plants grown on high NO$_3^-$, the amount of auxin exported was greater than that exported from apical segments, as previously reported (Prusinkiewicz et al., 2009). This is likely due to the increased number of active shoots feeding auxin into the basal part of the stem (Prusinkiewicz et al., 2009). However, there was only a small non-significant increase in auxin in the basal stem segments of low NO$_3^-$-grown plants compared to those grown on high NO$_3^-$ (Fig. 6 inset), consistent with our observation that the buds of the more basal nodes on the bolting stem on low NO$_3^-$ remain dormant and thus do not export auxin (Fig. 2B and 4C).

As previously reported, both *axr1* mutants, and *max1* mutants have more auxin in the PATS than wild-type, although in this experiment not all these differences are statistically significant (Fig. 6) (Bennett et al., 2006; Prusinkiewicz et al., 2009). The amount of auxin exported from apical stem segments of the double mutant exceeds both single mutants. In basal stem segments, *max1* exports the most auxin, with a substantial increase compared to the apical segments. The mutant backgrounds involving *axr1* show little increase in auxin levels over those exported from apical segments, as previously reported (Prusinkiewicz et al., 2009).
In contrast to the wild-type, the amount of auxin exported from the apical stem segments of the various mutants was not significantly affected by NO$_3^-$ supply (Fig. 6). These results support the idea that reduced branching on low NO$_3^-$ is caused at least in part by increased auxin export from each active apex. This low NO$_3^-$-induced increase in auxin is dependent on auxin signaling and SLs.

**Strigolactone deficiency alters resource allocation**

To examine the wider consequences of SL deficiency on low NO$_3^-$-induced changes in resource allocation, we investigated the effect of N deprivation on relative allocation of biomass to the root and shoot in the *max1* mutant. The *axr1* mutation was not included in this work because of its highly pleiotropic effects, for example on fertility (Lincoln et al., 1990), which make the comparisons difficult to interpret. Both shoot (Fig. 7A) and root (Fig. 7B) biomass are reduced under limiting NO$_3^-$ supply, but root biomass is relatively protected (Fig. 7C). For the shoot, *max1* has slightly higher biomass than the wild-type under both N sufficient and N deficient conditions, although this is not statistically significant on high NO$_3^-$ (Fig. 7A). For the root, while *max1* mutants have slightly higher mean biomass on high NO$_3^-$, they have slightly lower mean biomass on low NO$_3^-$ (Fig. 7B). Thus while both genotypes show a shift toward an increased proportion of biomass in the root on low NO$_3^-$, in the *max1* mutant this shift is attenuated (Fig. 7C).

**Discussion**

Our results clearly show that low NO$_3^-$ availability suppresses shoot branching in Arabidopsis. This is achieved by slowing and early termination of the post-flowering basipetal sequence of bud activation characteristic of Arabidopsis plants (Fig. 2). Consequently, when NO$_3^-$ is limiting, only the most apical cauline nodes produce actively elongating branches, whereas all the cauline nodes, as well as some upper rosette nodes carry active branches when NO$_3^-$ is sufficient (Fig 4). Shoot NO$_3^-$ levels are lower in plants grown on low NO$_3^-$ (Fig. 1), but branch suppression appears to be a response to whole plant N status rather than a direct signaling effect of NO$_3^-$, because a similar response is obtained when an alternative N source was used (Fig. 3, Fig. S1).

**Systemic signaling and N status**
Both root-to-shoot and shoot-to-root signals are required to explain known responses to whole plant N status. This is perhaps seen most clearly in split root system experiments (Zhang et al., 1999; Zhang and Forde, 2000; Forde, 2002; Ruffel et al., 2011). When both parts of a divided root system have a low supply of N, root growth is favoured over shoot growth; but when one part of the root system is in low N and the other part in high N, root growth on the low N side is suppressed. This effect must be mediated via the shoot, since when the shoots are removed, the roots can still respond to local NO$_3^-$, but have lost the response to systemic N signaling (Ruffel et al., 2011).

**Root-to-shoot signals under limiting N**

There are two candidates that can act as N-regulated root-to-shoot signals: cytokinin and strigolactone. Nitrate can up-regulate the synthesis of cytokinin in roots (Takei et al., 2001; Sakakibara et al., 2006), and this has been implicated in mediating root-to-shoot N signaling (Takei et al., 2002). In Arabidopsis, this response is apparently mediated by rapid up-regulation of the $IPT3$ cytokinin biosynthetic gene (Takei et al., 2004). This gene is up-regulated by nitrate in a cycloheximide-independent manner and its loss of function causes a severely attenuated CK synthesis response to nitrate addition. However, $IPT3$ transcription is unaffected by other N sources, such as NH$_4^+$ and thus while nitrate-induced CK synthesis may contribute to the branching phenotypes that we observe, it is unlikely to be the only cause.

Our results demonstrate that SL mutants are compromised in their ability to reduce branching when N is limiting. This result contrast with those of Zhu and Kranz (2012) who report that Arabidopsis SL biosynthetic mutants show the same low branch number as wild type when N is limiting. The reason for this difference is unclear. Zhu and Kranz (2012) used lower levels of N, but more rosette branches were retained, so clearly conditions were different. We consistently and robustly observe high branching on low N for all $max$ mutants compared to wild-type, indicating that SL is required for full branch suppression by low N. Furthermore, in a two-bud competition assay, the effects of low N are very similar to SL treatment (Fig. 5A). These results suggest that either there is near-constitutive bud activation in SL mutants, or that dynamic changes in SL levels are involved in mediating the N response. Under this latter hypothesis, low N response would trigger up-regulation of SL synthesis. There is
mounting evidence that the suppression of Arabidopsis and rice shoot branching by limiting phosphate is mediated in this way (Umehara et al., 2010; Kohlen et al., 2011). In wild-type plants, P limitation leads to dramatic up-regulation of SL synthesis (Yoneyama et al., 2007b; Lopez-Raez et al., 2008) and triggers a range of responses including suppression of shoot branching. Shoot branching in SL mutants is completely insensitive to P limitation and the SL pathway is required in roots to trigger a range of rapid local responses to P deprivation (Mayzlish-Gati et al., 2012). However, the evidence that N limitation has similar effects is weak. Although up-regulation of SL biosynthesis by N limitation has been reported, recent results suggest that this might be due to effects of N limitation on shoot P levels (Yoneyama et al., 2012), which then in turn affect root SL synthesis. Similarly, although the ability to reduce branching in response to low NO$_3^-$ seems to require SL, our results do not support increased SL being a major cause of reduced branching under N limitation. There is no evidence for transcriptional up-regulation of SL biosynthetic genes in response to low N supply, and plants grown with limited N supply do not show reduced auxin transport or reduced PIN1 accumulation, which are hallmarks of high SL (Bennett et al., 2006; Lazar and Goodman, 2006; Crawford et al., 2010; Shinohara et al., 2013).

**Shoot-derived signals under limiting N**

Although we cannot rule out a role for direct effects of N limitation on either CK or SL synthesis in roots in the suppression of shoot branching, our results suggest a substantial contribution from systemic shoot-based effects. This is consistent with previous evidence for shoot-driven hormonal responses to N (Chen et al., 1998; Walch-Liu et al., 2006; Tamaki and Mercier, 2007; Ruffel et al., 2011). Our data suggest that under N limitation each active shoot apex exports more auxin, such that the amount of auxin moving in the PATS in wild-type Arabidopsis apical stem segments doubles when N is limiting (Fig. 6A inset). This increase is consistent with the suppression of shoot branching concomitant with the relative protection of root biomass observed in response to low N (Fig. 3 and 7). Shoot-to-root auxin transport has previously been implicated in N response. For example, the stimulatory effects of low N on root growth in maize have been associated with increased shoot-derived auxin, and there is some evidence that nodulation depends both on high shoot-to-root auxin transport and N limitation (Carroll et al., 1985; van Noorden et al., 2006).
The *axr1* and *max1* mutants both have constitutively high auxin in the PATS that is unaffected by N supply (Fig. 6). In *axr1* this likely results from reduced feedback inhibition on auxin synthesis due to reduced auxin sensitivity (Romano et al., 1995; Prusinkiewicz et al., 2009). In *max1* mutants, it is also likely due to reduced feedback inhibition on auxin synthesis, but in this case caused by increased auxin transport away from the sites of synthesis (Bennett et al., 2006; Prusinkiewicz et al., 2009; Crawford et al., 2010; Shinohara et al., 2013). The additivity of this phenotype in the double mutant is consistent with different mechanisms operating in the two mutants. The inability of these mutants to change their auxin levels in response to low N could contribute to their reduced response to N deprivation (Fig. 4A).

The remaining response of each mutant could be mediated by auxin-independent mechanisms, for example reductions in CK mediated directly by N status, or in the case of *axr1*-3, increases in SL. In this context, it is interesting that the shoot elongation suppression observed on low N is MAX1-independent but AXR1-dependent (Fig. 4B). This suggests that the *max1* mutant retains some auxin-regulated changes induced by low N, despite constitutively high auxin levels in the polar auxin transport stream. One possibility is N-induced changes in AXR1-dependent auxin sensitivity, as suggested for roots (Vidal et al., 2009).

**Nutrient responses and auxin-mediated bud inhibition**

Taken together, our data suggest that in wild-type plants, low N results in increased auxin export from active shoot apices, which reduces shoot branching while maintaining strong root growth (Fig. 8). There is a substantial body of evidence that auxin in the main stem can inhibit bud activation by preventing auxin transport canalisation out of buds, thereby preventing their growth (Li and Bangerth, 1999; Bennett et al., 2006; Prusinkiewicz et al., 2009; Balla et al., 2011; Shinohara et al., 2013). A rapid primary response to SLs is to reduce PIN1 accumulation at the plasma membrane, making canalization harder to achieve, thus reducing the number of buds that can activate (Crawford et al., 2010; Shinohara et al., 2013). As a result, SL mutants have increased branching, increased auxin transport, and buds that are resistant to auxin-mediated growth inhibition. Therefore, an important role for SL in branch inhibition on low N is to clamp plasma membrane PIN1 at a constant level, thereby rendering buds sensitive to N-mediated increases in apical auxin. The reduced ability of *max1*
mutants to respond to N limitation is therefore likely due to their inability to increase bud auxin export on low N combined with the general difficulty in preventing the establishment of auxin transport canalisation out of buds in the absence of SL.

In addition auxin, signaling through the AXR1-dependent pathway, can up-regulate the transcription of SL biosynthetic genes and down-regulate the transcription of CK biosynthetic genes (Nordström et al., 2004; Hayward et al., 2009). Both of these hormones have been proposed to act locally in buds to regulate bud outgrowth by influencing the transcription of genes of the TCP family that are known to be required to inhibit shoot branching (Doebley et al., 1997; Takeda et al., 2003; Kebrom et al., 2006; Aguilar-Martinez et al., 2007; Dun et al., 2012, 2013). This mechanism could contribute to branch reduction on low N. Furthermore increased strigolactone produced in this way is predicted to be important for clamping plasma membrane PIN1 levels despite auxin-induced increases in PIN1 transcription. However, the failure of the max1 and axr1 mutants to show an increase in auxin in the PATS on low N leaves these hypotheses untested.

Conclusions

Shoot branching is regulated by the actions and interactions of at least three systemically moving plant hormones- auxin, SL and CK. There is an increasing body of evidence that the environmental control of shoot branching is mediated at least in part by this hormonal network. Concordantly, our data suggest that the effects of N supply on branching depend at least in part on both SL and auxin signaling. Reduced N supply is associated with an increase in the amount of auxin moving in the PATS of apical stem segments (Fig. 6, Fig. 8), which is not observed in the axr1 auxin signaling mutant or the max1 SL synthesis mutant. This auxin increase can plausibly contribute to the changes in branching phenotype and root fraction we observe in response to N supply in the genoptyes tested. However, the complexity of the feedback interactions in the hormonal network make it difficult to rule out other mechanisms of N action, and indeed the remaining N responses in the mutants support the existence of such addition mechanisms.

Materials and Methods

Plant Material and Growth Conditions
Arabidopsis thaliana wild-type, accession Columbia, and isogenic mutants axr1-3 (Lincoln et al., 1990), max1-1 (Stirnberg et al., 2002), and max2-1 (Stirnberg et al., 2002) and transgenic plants harbouring the pPIN1::PIN1:GFP transgene (Benkova et al., 2003) were used. Plants were grown in greenhouse conditions with a temperature regime of between 15-24°C. To provide constant long-day conditions (16/8h light/dark), natural daylight was supplemented with artificial light to ~150µmol photons m⁻² s⁻¹.

Plants were grown on low nitrate substrates consisting of either 90% sand (Leighton Buzzard sand from WBB Minerals Ltd, Cheshire, UK) 10% soil, or 50% sand 50% Terragreen (Oil-Dri Ltd, Cambridgeshire, UK). These substrates were wetted with Arabidopsis thaliana salts (ATS) solution (Wilson et al., 1990). High nitrate treatments (9mM) were supplied as 5mM KNO₃ and 2mM Ca(NO₃)₂. For low nitrate treatments (1.8mM), the 5mM KNO₃ was replaced by 1mM KNO₃ and 4mM KCl, and 2mM Ca(NO₃)₂ was replaced by 0.4mM Ca(NO₃)₂ and 1.6mM CaCl₂. Nutrient solutions containing other concentrations of nitrate were adjusted accordingly. In the ATS solutions used to study the effect of ammonium and nitrate, the 5mM KNO₃ and 2mM Ca(NO₃)₂ were replaced by appropriate quantities of NH₄Cl, KCl, and CaCl₂. From two weeks after sowing, the plants were fed on a weekly basis, using 10ml nutrient solution per plant.

To obtain the root and shoot dry weight, plant tissues were dried at 70°C o/n, and subsequently re-dried and cooled on silica gel. The root fraction was calculated as the dry weight of the roots divided by the total dry weight of the plant.

Nitrate content analysis
Leaf tissues were collected 4 weeks post germination, just prior to bolting. The tissues were dried and ground to a homogenous fine powder. Nitrate was extracted from 5mg of powder with deionized water for 30 min at 80°C. The nitrate concentration was determined by the rapid colorimetric method described by Cataldo et al. (1975).

Auxin analysis
For the auxin export assay, 18mm segments were excised from the bolting stems of 6-week-old plants. The basal ends of these segments were incubated in 50µl 2.5mM diethylthiocarbamate for 24h. 15µl of 4 samples were pooled together, and 2-4 such pools per genotype were collected. The auxin was purified from the exudate and quantified by gas
chromatography-selected reaction monitoring-mass spectrometry as described in Prusinkiewicz et al. (2009).

The auxin transport assay was performed as described in Bennett et al. (2006) with minor modifications. 15mm basal stem segments were excised from the bolting stems of 6-week-old plants. The apical ends of these segments were incubated in 30µl of 0.5x ATS solution, containing 1µM 14C-labelled IAA (American Radiolabeled Chemicals, St Louis, MO, USA). After 24h of incubation under constant light conditions, the 5mm basal ends of the segments were cut and left in 80% (w/v) methanol for 48h to extract the radiolabel. The amount of radiolabel was measured by scintillation in the presence of MicroScint-20 (PerkinElmer, USA).

Microscopy
Wild-type plants, homozygous for the pPIN1::PIN1:GFP transgene (Benkova et al., 2003), were grown on high and low nitrate. Four weeks after germination, the basal segments (15mm) of the primary inflorescence stems were excised, longitudinally sectioned by hand with a razor blade and immersed in water as described in Shinohara et al. (2013). The sections were observed using a Zeiss LSM 780 confocal microscope. Images were acquired with excitation at 488nm and an emission spectrum from 493–550nm. The fluorescence intensity of PIN1-GFP at the plasma membrane of the xylem parenchyma cells was quantified using Zen 2010 Zeiss software. For each condition, regions of 4 to 11 xylem parenchyma cells were selected for analysis from each of 5 stem segments.

Two-bud assays
The two-bud assays were performed as described by Ongaro et al. (2008) with minor modifications. Wild-type and max2 mutant plants with bolting stems that carried two cauline nodes with associated buds were selected. The size of the buds was not bigger than 2.5mm. These two-node segments were excised and transferred to 1.5ml microcentrifuge tubes with the appropriate ATS solutions, with containing 1µM GR24 (LeadGen Labs, Orange, CT, USA), or 0.1% acetone as a carrier control. To reduce evaporation, tubes were placed in a tray with wet filter paper on the bottom and a propagator lid. The trays were transferred to a growth room, under the following conditions: 16 hours light, 8 hours dark, temperatures 19-22°C day, 18-20°C night, light intensity ~60-100µmol m⁻²s⁻¹. Bud lengths were measured daily for 7 days, and the ATS solutions in the 1.5ml microcentrifuge tubes were replenished
when necessary. The relative growth index (RGI) was calculated as the length of the longest bud divided by the total length of both buds.

**Jar assay**

To grow plants under sterile conditions, seeds were surface sterilized in 10% chlorine bleach and then washed with 70% ethanol (1x) and sterile distilled water (6x). After 48h of stratification at 4°C, seeds were sown into 500ml Weck jars (Weck, Germany) containing 50ml solid ATS medium (Wislon et al., 1990) with 0.8% agar, 1% sucrose, and depending on the treatment: 3mM NH$_4^+$, 3mM NO$_3^-$, 6mM NO$_3^-$, 3mM NH$_4^+$, 6mM NO$_3^-$, or 9mM NO$_3^-$. In each jar, 6 seeds were evenly spaced, and in total 10 jars per treatment were used. The jars were placed in a growth room under the following conditions: 16h light, 8 hours dark, temperature of 20°C, light intensity ~100-130 μmol m$^{-2}$s$^{-1}$. 5 Weeks after germination, branches of 10mm or more were counted.

**Acknowledgements**

We would like to thank Roger Granbom and Joe Johnson for excellent technical assistance.

**Literature cited**


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new linkages to glucose, trehalose-6-phosphate, iron, and sulfate metabolism. Plant Physiol 132: 556-567


Figure Legends

**Figure 1.** Effect of nitrate supply on shoot branching and leaf nitrate concentration. A, Mean number of secondary shoots of wild-type plants grown on nitrate concentrations ranging from 9mM to 1.8mM. The number of branches of 5mm or more was determined when the primary apex had ceased activity. Data are means ± SE of 8 plants. Different letters indicate statistically significant differences ($P<0.05$, Kruskal-Wallis H test). B, Leaf nitrate concentrations of wild-type plants grown on nitrate concentrations ranging from 9mM to 1.8mM. Nitrate measurements were conducted on leaves collected from 4-week-old plants, just prior to bolting. Data are means ± SE of 8 plants. Different letters indicate statistically significant differences ($P<0.05$, ANOVA).

**Figure 2.** Effect of nitrate supply on the timing of bud activation. A, Mean branch length from the three most apical nodes (N1, N2, N3) of wild-type plants grown on 9mM NO$_3^-$ at anthesis and 2, 6 and 11 days post-anthesis (DPA). N1 is the uppermost node. B, Mean branch length from the three most apical nodes of wild-type plants grown on 1.8mM NO$_3^-$ at anthesis and 2, 6 and 11 DPA. Data are means ± SE of 6-18 plants.

**Figure 3.** Comparison of the effect of ammonium and nitrate as N sources on shoot branching and root fraction. Mean number of secondary shoots (A), and root fraction (B) of wild-type plants grown with different N supplies. Plants were grown in 10% soil/90% sand supplemented with nutrient solutions containing 1mM NO$_3^-$, 1mM NH$_4^+$ + 1mM NO$_3^-$, 2mM NH$_4^+$ + 1mM NO$_3^-$, or 3mM NO$_3^-$. Plants were grown in 3 inch pots with 4 plants per pot and 8 pots per treatment. Data were collected at 6 weeks, when the first seed were ripe. For shoot number (A), branches of 5mm or more were counted. Data are means ± SE of 32 plants. Different letters indicate statistically significant differences ($P<0.05$, Kruskal-Wallis H test). The root fraction (B) was calculated per pot as the dry weight of the roots divided by the total dry weight of the four plants. Data are means ± SE of 8 replicates. Different letters indicate statistically significant differences ($P<0.05$, ANOVA).

**Figure 4.** Response of auxin-signaling and SL-biosynthesis mutants to nitrate supply. Mean number of secondary shoots (A) and primary shoot length (B) of 6 week-old wild-type plants (WT), auxin-resistant *axr1-3* mutants, SL-biosynthesis *max1-1* mutants, and *axr1-3 max1-1*
double mutants grown on either 9mM or 1.8mM nitrate. Data are means ± SE of 15-16 plants. * Indicate statistically significant differences between the two nitrate treatments \((P<0.05)\), for (A) assessed using a Mann-Whitney U test and for (B) using a Student’s T-test. C, Images of representative plants of each genotype grown on 9mM (H) or 1.8mM (L) nitrate.

**Figure 5.** Effect of nitrate supply on strigolactone-related phenotypes. A, Relative growth index (RGI) of the buds from two-node explants of wild-type (WT) and the \(max2\) strigolactone signaling mutant grown on 9mM or 1.8mM nitrate, with or without 1\(\mu\)M GR24 treatment, as indicated. The data represents the RGI 7 days after excision, which and is calculated as the length of the longest branch divided by the total length of both branches. Data are means ± SE of 7-10 explants. Different letters indicate statistically significant differences \((P<0.05, \text{ANOVA})\). B, Amount of radiolabelled auxin transported basipetally through basal inflorescence stem segments of 36 day-old WT, \(axr1-3\), \(max1-1\) and \(axr1-3\ max1-1\) double mutant plants, grown on 9mM or 1.8mM nitrate. Data are means ± SE of 11-12 stem segments. There are no statistically significant differences between the two nitrate treatments \((P>0.05, \text{Student’s T-test})\). C, Mean fluorescence of PIN1:GFP at the rootward plasma membrane of xylem parenchyma cells of basal inflorescence stems. Stems were collected from 4 week-old plants harbouring a \(p\text{PIN1::PIN1::GFP}\) transgene, grown on 9mM (black bar) or 1.8mM (grey bar) nitrate. PIN1:GFP fluorescence at the plasma membrane was assessed using confocal microscopy. Data are means ± SE of 39-46 cells. There is no statistically significant difference between the nitrate treatments \((P>0.05, \text{Student’s T-test})\).

**Figure 6.** Effect of nitrate supply on the amount of auxin in the polar auxin transport stream. Amount of auxin exported from apical (A) or basal (B) stem segments of wild-type (WT), \(axr1-3\), \(max1-1\) and \(axr1-3\ max1-1\) double mutant plants grown on 9mM or 1.8mM nitrate. The inset is an enlargement of the data for WT stem segments. 18mm apical and basal segments were excised from the inflorescence stem of 6 week-old plants. Data are means ± SE of 2-4 pools of 4 stems. * Indicates statistically significant differences between the two nitrate treatments \((P<0.05, \text{Student’s t-test})\). Different letters indicate statistically significant differences between the genotypes \((P<0.05, \text{ANOVA})\).

**Figure 7.** Effect of nitrate on resource allocation in strigolactone deficient mutants. Shoot dry weight (A), root dry weight (B) and root fraction (C) of wild-type (WT) and \(max1-1\) mutant
plants grown on 9mM or 1.8mM nitrate. Plants were grown for 7 weeks with 4 plants per pot. Data are means ± SE of 6 pots. For both lines, the nitrate treatment significantly affected the shoot dry weight, root dry weight and root fraction, as indicated by the different letters ($P<0.05$, Student’s t-test). * Indicate statistically significant differences between the two genotypes ($P<0.05$, Student’s t-test).

**Figure 8.** Schematic representation of the hormonal pathways involved in modulation of Arabidopsis shoot branching in response to N supply. Our results suggest that N-limitation increases the amount of auxin exported from each active apex into the polar auxin transport stream (PATS) of the main stem. This prevents the activation of more basal branches, while maintaining the amount of auxin reaching the root. N limitation may also affect branching by increasing strigolactone synthesis and decreasing cytokinin synthesis in roots (dotted lines). Additional feedbacks in the system (not shown) are also likely to be important, for example up-regulation of SL synthesis by auxin is likely important in maintaining plasma membrane PIN1 at constant levels, contributing to auxin homeostasis in the system.

**Figure S1.** Comparison of the effect of ammonium and nitrate as N sources on shoot branching under sterile conditions. Mean number of secondary shoots of wild-type plants grown with different N supplies. Plants were grown in solid ATS medium containing 3mM NH$_4^+$ + 3mM NO$_3^-$, 6mM NO$_3^-$, 3mM NH$_4^+$ + 6mM NO$_3^-$, or 9mM NO$_3^-$. Plants were grown in sterile glass jars with 6 plants per jar and 10 jars per treatment. Branches of 10mm or more were counted at 5 weeks after germination. Data are means ± SE of 53-58 plants. The letters indicate statistically significant differences ($P<0.05$, Kruskal-Wallis H test).

**Figure S2.** Response of SL-biosynthesis and signaling mutants to nitrate supply. Mean number of secondary shoots of wild-type plants (WT), mutants in SL-biosynthesis *max1-1*, *max3-9*, *max4-1* and the SL-signaling mutant *max2-1* grown on either 9mM or 1.8mM nitrate. The number of branches was determined when the primary apex had ceased activity. Data are means ± SE of 14-15 plants. * Indicate statistically significant differences between the two nitrate treatments ($P<0.05$, Mann-Whitney U test).

**Figure S3.** Two repeats (A and B) of the experiment presented in Figure 4, showing the mean number of secondary shoots of auxin-signaling and SL-biosynthesis mutants in response to nitrate supply. The number of branches of 5mm or more were counted of 6 week-old wild-
type plants (WT), auxin-resistant *axr1-3* mutants, SL-biosynthesis *max1-1* mutants, and *axr1-3 max1-1* double mutants grown on either 9mM or 1.8mM nitrate. Data are means ± SE of 12-24 plants. * Indicate statistically significant differences between the two nitrate treatments (*P*<0.05, Mann-Whitney U test).

**Table S1.** Transcriptomic changes of SL-biosynthetic and signaling genes in response to nitrate. The expression of genes involved in strigolactone biosynthesis or strigolactone signaling in response to various nitrate supply regimes was compared to the responses of known nitrate-inducible genes. The Log2 fold change in expression of the genes was calculated from published microarray data obtained from 12d-old seedlings after 2h of nitrate induction (Gifford et al., 2008 and 2013), 9d-old seedlings after full nutrition or 2 days of nitrate deprivation (starv.) followed by 30min/3h readdition of nitrate (readd.) (Scheible et al., 2004), 10d-old seedlings after 20min of nitrate induction (Wang et al., 2003), adult plants at the stage of inflorescence emergence 1.5h or 8h after ammonium or nitrate addition (Patterson et al., 2010), and 5-week old plants after 2 or 10 days of nitrate starvation (Krapp et al., 2011).
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