Auxin and Strigolactone Signaling Are Required for Modulation of Arabidopsis Shoot Branching by Nitrogen Supply

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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 (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 nitrogen (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 more axillary growth1, 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 as up-regulation of strigolactone synthesis. However, the proposed increase in auxin export from 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 harbor 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 (NO₃⁻; Domagalska and Leyser, 2011; Brewer et al., 2013).

Limited NO₃⁻ availability can have profound effects on both root and shoot system architecture and biomass allocation, shifting the balance in favor of the root. In the root, low NO₃⁻ 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 NO₃⁻ 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 the proliferation of roots into a high-NO₃⁻ patch, are the result of direct local NO₃⁻ signaling, but there is also clear evidence for systemic nitogen (N) status effects. In particular, both the inhibitory effect on root growth by high NO₃⁻ supply and the stimulatory effect of low NO₃⁻ appear to be mediated by shoot N status (Scheible et al., 1997; Zhang et al., 1999). Thus, variation in NO₃⁻ availability triggers systemically coordinated changes in root and shoot system architecture.
There has been considerable progress in understanding how local \( \text{NO}_3^- \)-regulated changes in the root system are effected (for review, see Bouguyon et al., 2012), but much less is known about how \( \text{NO}_3^- \) availability regulates shoot branching and how this is coordinated with the root. These are important questions because of the agricultural imperative to reduce fertilizer 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 the 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 coordinators of nutrient signaling (Leyser, 2009).

This suggests the hypothesis that \( \text{NO}_3^- \) -triggered changes in shoot system architecture are mediated by changes in hormone activity.

There is good evidence that \( \text{NO}_3^- \) availability in the root system positively regulates cytokinin (CK) biosynthesis (Takei et al., 2002, 2004; Miyawaki et al., 2004; Wang et al., 2004) and that this is required for at least some systemic responses to \( \text{NO}_3^- \) 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 (for review, see 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 (Arabidopsis thaliana), this directional flow involves the PIN-FORMED1 (PIN1) protein, a member of a family of auxin efflux proteins that is localized 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 synthesized 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 may 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; Shinohara 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, 2012; López-Ráez et al., 2008; Umehara et al., 2010; Kohlen et al., 2011; Mayzlish-Gati 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 (Zea mays), 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-\( \text{NO}_3^- \) conditions, making use of auxin and SL mutants of Arabidopsis.

RESULTS

Low \( \text{NO}_3^- \) 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 shoot apical meristem. This means that in long-day conditions, there are usually no active branches produced in the vegetative phase. After the 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-borne cauline leaves and proceeds down into the rosette (Hempel and Feldman, 1994; Stirnberg et al., 1999; Grbić and Bleeker, 2000).
To investigate the effect of N supply on branching, we grew Arabidopsis plants with NO$_3^-$ supply ranging from 9 to 1.8 mM. Across this range, the mean total number of secondary shoots produced dropped from 4.25 on 9 mM NO$_3^-$ to 2.9 on 1.8 mM NO$_3^-$ (Fig. 1A). Measurement of free NO$_3^-$ in the leaves of 4-week-old plants revealed clear evidence of reduced NO$_3^-$ levels in the leaves of plants supplied with 2.25 mM NO$_3^-$ or less. Importantly, even plants grown on 1.8 mM NO$_3^-$ still showed substantial accumulation of free NO$_3^-$ during the vegetative phase, suggesting N limitation rather than N starvation (Fig. 1B). Based on these results, 9 and 1.8 mM NO$_3^-$ supply were selected as the standard N-sufficient and N-limited conditions for further analysis.

To characterize 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 9 mM NO$_3^-$ supply, these three branches activated nearly simultaneously and elongated rapidly, reaching lengths in excess of 12 cm over the time course of the experiment (Fig. 2A). On 1.8 mM NO$_3^-$, bud activation and elongation were delayed, and the basipetal sequence of activation was much more obvious. By 6 DPA, only the most apical bud had elongated significantly. By 11 d, the second bud was also actively growing, whereas there was still no significant elongation of the third bud (Fig. 2B). Even the first bud elongated more slowly than its counterpart on 9 mM NO$_3^-$, reaching only approximately 4 cm by the end of the experiment. Thus, reduced NO$_3^-$ availability resulted in delayed bud activation, reduced bud elongation, and early termination of the basipetal activation sequence, leading to reduced total branch number (Figs. 1A, and 2).

**Branch Suppression Is Not a Direct Effect of NO$_3^-$ 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 (NH$_4^+$) as an N source. This approach was adopted because using NH$_4^+$ as the sole N source was found to be toxic to Arabidopsis. Plants supplied with only 1 mM NO$_3^-$ produced a mean of approximately one secondary shoot, while the number of branches was double for plants supplied with both 1 mM NO$_3^-$ and 1 mM NH$_4^+$. This mean increased slightly, but not significantly, under 2 mM 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 (Supplemental 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 the 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 as an NH$_4^+$ + NO$_3^-$ mix (Fig. 3B).

**Auxin and SL Are Both Involved in the 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 is required. The response of branching to N limitation in wild-type plants was compared with those of the auxin-resistant mutant axrT-3 (Lincoln et al., 1990) and the SL biosynthesis
mutant more axillary growth1-1 (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 signaling mutant max2 (Supplemental Fig. 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 (Supplemental Fig. 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 SL signaling.

It was apparent from these experiments that N deprivation also results in reduced stature (Fig. 4, B and 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 ability of the max mutants to suppress branching on low N suggests that either their buds are nearly 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 NO$_3^-$ 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

![Figure 2. Effects of NO$_3^-$ supply on the timing of bud activation.](image)

![Figure 3. Comparison of the effects of NH$_4^+$ and NO$_3^-$ as N sources on shoot branching and root fraction.](image)
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 NO$_3^-$ availability on RGI, we grew wild-type and SL-resistant mutant (max2) plants on 9 or 1.8 mM NO$_3^-$, excised a two-node segment from their bolting stems, and measured RGI under high and low NO$_3^-$, respectively, with or without basal addition of the synthetic SL, GR24. As reported previously, 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-N$_3^-$-grown plants; however, the wild-type RGI under both these treatments were nearly maximal. In these experiments, the wild-type RGI in N-sufficient conditions was, on average, 0.8, which is higher than that observed typically (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 on 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, 2012, 2013; Jamil et al., 2011; Foo 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 publicly available expression data for these genes (ArrayExpress; Rustici et al., 2013). To date, four such genes are known: MAX1, MAX3, MAX4, and DWARF27. Among six experiments examining the effects of NO$_3^-$ supply or resupply after NO$_3^-$ deprivation (Wang et al., 2003; Scheible et al., 2004; Gifford et al., 2008, 2013; Patterson et al., 2010; Krapp et al., 2011), we found no evidence of a consistent effect of N supply on the transcription of any of these genes (Supplemental Table S1). The transcript abundance of core genes in the SL signaling pathway, DWARF14 and MAX2, was also not affected (Supplemental Table S1).

**Figure 4.** Responses of auxin signaling and SL biosynthesis mutants to NO$_3^-$ supply. A and B, 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 9 or 1.8 mM NO$_3^-$ . Data are means ± s.e of 15 to 16 plants. Asterisks indicate statistically significant differences between the two NO$_3^-$ treatments (P < 0.05) as assessed using a Mann-Whitney U test (A) and a Student’s t test (B). C, Images of representative plants of each genotype grown on 9 mM (high [H]) or 1.8 mM (low [L]) NO$_3^-$.

**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 min, independently of new transcription, and therefore is a primary SL response (Shinohara et al., 2013). To assess the effects of low NO$_3^-$ on auxin transport through the stem, radiolabeled 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 demonstrated...
plants grown on 9 or 1.8 mM NO$_3^-$ from 4-week-old plants harboring a PIN1:GFP transgene and accumulated PIN1 at the rootward plasma membrane of xylem parenchyma cells of basal inflorescence stems. Stems were collected for fluorescence of PIN1:GFP at the rootward plasma membrane of xylem parenchyma cells from either the apical or the basal 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. 6A, 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 reported previously (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 nonsignificant increase in auxin in the basal stem segments of low-NO$_3^-$-grown plants compared with those grown on high NO$_3^-$ (Fig. 6A, 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 (Figs. 2B and 4C).

As reported previously, both axr1 mutants and max1 mutants have more auxin in the PATS than the 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 that from both single mutants. In basal stem segments, max1 exports the most auxin, with a substantial increase compared with the apical segments. The mutant backgrounds involving axr1 show little increase in auxin levels over those exported from apical segments, as reported previously (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.

Reduced N Availability Increases Auxin Levels in the Main Stem PATS

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 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-h 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 each active apex was greater than that exported from apical segments, as reported previously (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 nonsignificant increase in auxin in the basal stem segments of low-NO$_3^-$-grown plants compared with those grown on high NO$_3^-$ (Fig. 6A, 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 (Figs. 2B and 4C).

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SL De
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ence Alters Resource Allocation

To examine the wider consequences of SL de
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ence on low-NO$_3$-induced changes in resource allocation, we investigated the effect of N deprivation on the 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 were reduced under limiting NO$_3$ supply, but root biomass was relatively protected (Fig. 7C). For the shoot, max1 had slightly higher biomass than the wild type under both N-suf
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ent conditions, although this was not statistically significant on high NO$_3$ (Fig. 7A).

For the root, while max1 mutants had slightly higher mean biomass on high NO$_3$, they had slightly lower mean biomass on low NO$_3$ (Fig. 7B). Thus, while both genotypes showed a shift toward an increased proportion of biomass in the root on low NO$_3$, in the max1 mutant this shift was 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 postflowering 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 was obtained when an alternative N source was used (Fig. 3; Supplemental 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 favored over shoot growth; but

Figure 6. Effects of NO$_3$ supply on the amount of auxin in the PATS. Amounts of auxin exported from apical (A) or basal (B) stem segments of wild-type (WT), axr1-3, max1-1, and axr1-3 max1-1 plants grown on 9 or 1.8 mM NO$_3$ are shown. The inset is an enlargement of the data for the wild-type stem segments. Eighteen-millimeter apical and basal segments were excised from the inflorescence stems of 6-week-old plants. Data are means ± se of two to four pools of four stems. Asterisks indicate statistically significant differences between the two NO$_3$ treatments ($P < 0.05$, by Student’s t test). Different letters indicate statistically significant differences between the genotypes ($P < 0.05$, by ANOVA).

Figure 7. Effects of NO$_3$ on resource allocation in SL-deficient mutants. Shoot dry weight (DW; A), root dry weight (B), and root fraction (C) of wild-type (WT) and max1 plants grown on 9 or 1.8 mM NO$_3$ are shown. Plants were grown for 7 weeks with four plants per pot. Data are means ± se of six pots. For both lines, the NO$_3$ treatment significantly affected the shoot dry weight, root dry weight, and root fraction, as indicated by the different letters ($P < 0.05$, by Student’s t test). Asterisks indicate statistically significant differences between the two genotypes ($P < 0.05$, by Student’s t test).
when one part of the root system is in low N and the other part is 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 \( \text{NO}_3^- \) but they 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: CK and SL. \( \text{NO}_3^- \) can up-regulate the synthesis of CK 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 \( \text{ATP/ADP ISOPENTENYL TRANSFERASE3} \) (\( \text{IPT3} \)) CK biosynthetic gene (Takei et al., 2004). This gene is up-regulated by \( \text{NO}_3^- \) in a cycloheximide-independent manner, and its loss of function causes a severely attenuated CK synthesis response to \( \text{NO}_3^- \) addition. However, \( \text{IPT3} \) transcription is unaffected by other N sources, such as \( \text{NH}_4^+ \); thus, while \( \text{NO}_3^- \)-induced CK synthesis may contribute to the branching phenotypes that we observed, 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 is in contrast with those of Zhu and Kranz (2012), who report that Arabidopsis SL biosynthetic mutants show the same low branch number as the 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 observed high branching on low N for all \( \text{max} \) mutants compared with the 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 were very similar to SL treatment (Fig. 5A). These results suggest either that there is nearly constitutive bud activation in SL mutants or that dynamic changes in SL levels are involved in mediating the N response. Under this latter hypothesis, a low-N response would trigger the up-regulation of SL synthesis. There is mounting evidence that the suppression of Arabidopsis and rice (\( \text{Oryza sativa} \)) shoot branching by limiting phosphate is mediated in this way (Umehara et al., 2010; Kohlen et al., 2011). In wild-type plants, phosphate limitation leads to dramatic up-regulation of SL synthesis (Yoneyama et al., 2007b; López-Ráez et al., 2008) and triggers a range of responses, including the suppression of shoot branching. Shoot branching in SL mutants is completely insensitive to phosphorus limitation, and the SL pathway is required in roots to trigger a range of rapid local responses to phosphorus deprivation (Mayzlish-Gati et al., 2012). However, the evidence that N limitation has similar effects is weak. Although the up-regulation of SL biosynthesis by N limitation has been reported, recent results suggest that this might be due to the effects of N limitation on shoot phosphorus levels (Yoneyama et al., 2012), which in turn affect root SL synthesis. Similarly, although the ability to reduce branching in response to low \( \text{NO}_3^- \) 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 the 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; Shinozara 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; Crawford et al., 2010; 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 (Figs. 3 and 7). Shoot-to-root auxin transport has previously been implicated in the 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 it is 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, such as 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 PATS. One possibility is N-induced changes in AXR1-dependent auxin sensitivity, as suggested for roots (Vidal et al., 2010).

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 canalization 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, therefore, is 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 canalization 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 TEOSINTE BRANCHED-CYCLOIDEA-PCF family that are known to be required to inhibit shoot branching (Doebley et al., 1997; Takeda et al., 2003; Kebrom et al., 2006; Aguilar-Martínez et al., 2007; Dun et al., 2012, 2013). This mechanism could contribute to branch reduction on low N. Furthermore, increased SL 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.

CONCLUSION

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 (Figs. 6 and 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 observed in response to N supply in the genotypes 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 additional mechanisms.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) wild type, accession Columbia, isogenic mutants axr1-3 (Lincoln et al., 1990), max1-1 (Stirmberg et al., 2002), and max2-1 (Stirmberg et al., 2002), and transgenic plants harboring the pPIN1::PIN1-GFP transgene (Benková et al., 2003) were used. Plants were grown in greenhouse conditions with a temperature regime of between 15°C and 24°C. To provide
constant long-day conditions (16/8 h of light/dark), natural daylight was supplemented with artificial light to approximately 150 μmol photons m⁻² s⁻¹. Plants were grown on low-No3⁻ substrates consisting of either 90% sand (Leighton Buzzard sand from WBB Minerals) and 10% (v/v) soil or 50% sand and 50% (v/v) Terragreen (Oil-Dri). These substrates were wetted with Arabidopsis salts (ATS) solution (Wilson et al., 1990). High-No3⁻ treatments (9 mM) were supplied as 5 mM KNO3 and 2 mM Ca(NO3)2. For low-No3⁻ treatments (1.8 mM), the 5 mM KNO3 was replaced by 1 mM KNO3 and 4 mM KCl, and 2 mM Ca(NO3)2 was replaced by 0.4 mM Ca(NO3)₂ and 1.6 mM CaCl2. Nutrient solutions containing other concentrations of NO3⁻ were adjusted accordingly. In the ATS solutions used to study the effect of NH4⁺ and NO3⁻, the 5 mM KNO3 and 2 mM Ca(NO3)2 were replaced by appropriate quantities of NH4Cl, KCl, and CaCl2. From 2 weeks after sowing, the plants were fed on a weekly basis, using 10 mL of nutrient solution per plant.

To obtain the root and shoot dry weights, plant tissues were dried at 70°C overnight and subsequently redried 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.

NO3⁻ Content Analysis

Leaf tissues were collected 4 weeks after germination, just prior to bolting. The tissues were dried and ground to a homogenous powder. NO3⁻ was extracted from 5 mg of powder with deionized water for 30 min at 80°C. The NO3⁻ concentration was determined by the rapid colorimetric method described by Cataldo et al. (1975).

Auxin Analysis

For the auxin export assay, 18-mm segments were excised from the bolting stems of 6-week-old plants. The basal ends of these segments were incubated in 50 μl of 2.5 mM diethyldithiocarbamate for 24 h. Four samples of 15 microtubes (each) were pooled together, and two or four 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 by Prusinkiewicz et al. (2009). The auxin transport assay was performed as described by Bennett et al. (2006) with minor modifications. Fifteen-millimeter basal stem segments were excised from the bolting stems of 6-week-old plants. The apical ends of these segments were incubated in 30 μl of 0.5× ATS solution, containing 1 μCi 14C-labeled indole-3-acetic acid (American Radiolabeled Chemicals). After 24 h of incubation under constant light conditions, the 5-mm basal ends of the segments were cut and left in 80% (v/v) methanol for 48 h to extract the radiolabel. The amount of radiolabel was measured by scintillation in the presence of MicroScint-20 (PerkinElmer).

Microscopy

Wild-type plants, homozygous for the pPIN1:PIN1-GFP transgene (Benková et al., 2003), were grown on high and low NO3⁻. Four weeks after germination, the basal segments (15 mm) of the primary inflorescence stems were excised, longitudinally sectioned by hand with a razor blade, and immersed in water as described by Shinohara et al. (2013). The sections were immersed using a Zeiss ISM 780 confocal microscope. Images were acquired with excitation at 488 nm and an emission spectrum from 493 to 550 nm. The fluorescence intensity of PIN1-GFP at the plasma membrane of the xylem parenchyma cells was quantified using Zeiss Zen 2010 software. For each condition, regions of four to 11 xylem parenchyma cells were selected for analysis from each of five 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 2.5 mm. These two-node segments were excised and transferred to 1.5 mL microcentrifuge tubes with the appropriate ATS solutions containing 1 μCi GR24 (LeadGen Labs) or 0.1% (v/v) 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 h of light, 8 h of dark, temperatures of 19°C to 22°C day and 18°C to 20°C night, and light intensity of approximately 60 to 100 μmol m⁻² s⁻¹. Bud lengths were measured daily for 7 d, and the ATS solutions in the 1.5-mL microcentrifuge tubes were replenished when necessary. The 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% (v/v) chlorine bleach and then washed with 70% ethanol (v/v) and sterile distilled water (6×). After 48 h of stratification at 4°C, seeds were sown into 500-mL Weck jars containing 50 mL of solid ATS medium (Wilson et al., 1990) with 0.8% (w/v) agar, 1% (w/v) Suc, and, depending on the treatment, 3 mM NH₄⁺ + 3 mM NO₃⁻, 6 mM NO₃⁻, 3 mM NH₄⁺ + 6 mM NO₃⁻, or 9 mM NO₃⁻. In each jar, six 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: 16 h of light, 8 h of dark, temperature of 20°C, and light intensity of approximately 100 to 130 μmol m⁻² s⁻¹. Five weeks after germination, branches of 10 mm or more were counted.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Comparison of the effect of ammonium and nitrate as N sources on shoot branching under sterile conditions.

Supplemental Figure S2. Branching response of SL-biosynthesis and signaling mutants to nitrate supply.

Supplemental 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.

Supplemental Table S1. Transcriptomic changes of SL-biosynthetic and signaling genes in response to nitrate.

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LITERATURE CITED


Modulation of Shoot Branching by Nitrogen Supply


