Running head:

A post apical dominance mechanism

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Research area:

Development and hormone action
Title:

AtMYB2 regulates whole plant senescence by inhibiting cytokinin-mediated branching at late stages of development in Arabidopsis

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Financial sources:

DOE DE-FG02-02ER15341, NSF MCB-0445596, and BARD IS-3645-04 (S.G.).

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ABSTRACT

Whole plant senescence of monocarpic plants consists of three major processes: arrest of shoot apical meristem, organ senescence, and permanent suppression of axillary buds. At early stages of development, axillary buds are inhibited by shoot apex-produced auxin, a mechanism known as apical dominance. How the buds are suppressed as an essential part of whole plant senescence, especially when the shoot apexes are senescent, is not clear. Here we report an $AtMYB2$-regulated post apical dominance mechanism by which Arabidopsis inhibits outgrowth of axillary buds as part of the whole plant senescence program. $AtMYB2$ is expressed in the compressed basal internode region of Arabidopsis at late stages of development to suppress production of cytokinins, the group of hormones that are required for axillary bud outgrowth. $atmyb2$ T-DNA insertion lines have enhanced expression of cytokinin-synthesizing isopentenyltransferases genes, contain higher levels of cytokinins, and display a bushy phenotype at late stages of development. And as a result of continuous generation of new shoots, $atmyb2$ plants have prolonged life span. $AtMYB2$ promoter-directed cytokinin oxidase 1 gene in the T-DNA insertion lines reduces the endogenous cytokinin levels and restores the bushy phenotype to wild type.
INTRODUCTION

Plants, like many other organisms, exhibit various life history patterns and possess a broad spectrum of longevity, ranging from a few weeks to several thousand years. Annuals (e.g., *Arabidopsis*), biennials (e.g., wheat), and some perennials (e.g., bamboo) possess a monocarpic lifestyle, which is characterized by only a single reproductive event in the life cycle. After flowering (and setting seeds or fruits), the whole plant will senesce and die (Gan, 2003). The monocarpic senescence includes three coordinated processes: (a) senescence of somatic organs and tissues such as leaves, (b) arrest of shoot apical meristems (SAM), and (c) suppression of axillary buds to prevent formation of new shoots/branches. The longevity of an Arabidopsis plant could be prolonged by mutations leading to either long lived leaves, prolonged SAM activity, or continued generation of new shoots as a result of axillary bud outgrowth (Nooden and Penney, 2001; Gan, 2007).

Axillary buds are groups of meristematic cells located in leaf axils. Outgrowth of the buds leads to branching. The axillary buds are generally suppressed by shoot apex-produced auxin, a mechanism known as apical dominance (Thimann and Skoog, 1933; Napoli et al., 1999). Removal of the shoot apex (i.e. decapitation) alleviates this inhibition, allowing for the outgrowth of lateral buds. Since auxin appears not to enter axillary buds (Hall and Hillman, 1975; Morris, 1977), and exogenous auxin applied directly to buds does not inhibit their growth (Cline, 1996), it is believed that auxin is transported down the stem to inhibit axillary bud outgrowth indirectly (Leyser, 2009).

Another class of hormones that has an important role in regulating apical dominance and axillary bud outgrowth are cytokinins. Direct application of cytokinins to axillary buds could reverse the inhibitory effect of auxin (Sachs and Thimann, 1964). The expression of cytokinin
biosynthetic genes correlates with bud outgrowth (Ferguson and Beveridge, 2009). It has been shown that synthesis of cytokinins is regulated by auxin (Li et al., 1995; Nordstrom et al., 2004) and auxin negatively regulates local biosynthesis of cytokinins by controlling expression of isopentenyl transferase (IPT) genes (Tanaka et al., 2006). IPT catalyzes the first and rate-limiting step in the biosynthesis of cytokinins. This suggests that auxin could act by reducing the supply of cytokinins to axillary buds, thereby inhibiting their growth.

Strigolactone has been recently identified as a novel branch-inhibiting hormone (Gomez-Roldan et al., 2008; Umehara et al., 2008). Plant mutants deficient in biosynthesis of or response to strigolactone show specific increase in bud outgrowth (reviewed in Beveridge and Kyozuka, 2010; Stirnberg et al., 2010). Auxin positively regulates expression of strigolactone biosynthetic genes (Sorefan et al., 2003; Arite et al., 2007), suggesting that strigolactone also functions downstream of auxin. While cytokinins can directly promote cell proliferation via upregulation of cell cycle components (Riou-Khamlichi et al., 1999; Rashotte et al., 2003), it is not clear how strigolactone regulates axillary bud outgrowth. It has been suggested that strigolactone may function by repressing the cytokinin pathway (Dun et al., 2009).

In some plants, apical dominance becomes weakened when the main shoot has grown beyond the suppression range of the apex, resulting in the outgrowth of the axillary buds to form bushy plants. In unbranched or less branched plants, the suppression of the axillary buds is believed to be “permanent” (Napoli et al., 1999; Beveridge, 2006). Our present study on AtMYB2, however, indicates that, in the less-branched monocarpic Arabidopsis, an AtMYB2-regulated post apical dominance mechanism functions at late stages of plant development to reduce cytokinin production and inhibit axillary bud outgrowth, which facilitates whole plant senescence.
RESULTS

AtMYB2 transcript accumulates in the compressed basal internodes at late stages of development and is not detectable in two T-DNA insertion lines.

AtMYB2 encodes a transcription factor containing R2R3 MYB domains. We previously found that this gene was highly represented in an Arabidopsis senescence cDNA library (Guo et al., 2004). RNA gel blot and RT-PCR analyses confirmed the high expression of this gene during leaf senescence (Fig. S1). Expression of AtMYB2 in the compressed basal internodes (rosette area) is also induced by age. Messenger RNA of this gene was detected in the compressed internodes region of 8- and 12-week-old plants but not in this region of 4-week-old plants (Fig. 1A). Consistent with the RT-PCR results, GUS staining was detected only at late stages of development in the compressed internodes region of transgenic plants harboring the AtMYB2 promoter (hereafter P_{AtMYB2})-GUS reporter construct (Fig. 1B).

The atmyb2 plants showed a bushy phenotype at late stages of development and have prolonged life span.

To investigate the role of AtMYB2 in plant senescence, we obtained two T-DNA insertion lines by screening the Wisconsin Arabidopsis (accession: Wassilewskija or Ws) T-DNA insertion collection (Sussman et al., 2000). In both lines the T-DNA was inserted in the 3rd exon of AtMYB2 (Fig. 1C) and resulted in no detectable AtMYB2 transcript (Fig. 1D, Fig. S2). The two lines were very similar to wild type (WT) at early stages of development phenotypically (Fig. 2A); the onset and progression of leaf senescence in these mutants were also the same as those in WT (Fig. 2C). Up to an age of 8 weeks the number of branches does not differ between mutant lines and wild type (Fig. 2B). But after about eight weeks of growth (a time when the main
meristem of WT was arrested) the axillary buds in the mutant plants started to grow out (Fig. 2E) while the axillary buds in WT remained suppressed (Fig. 2D). The suppression of these buds in WT was further confirmed by the fact that these buds expressed bud dormancy-associated genes AtDRM1 (At1g28330) and AtDRM1 homolog (At2g33830) (Tatematsu et al., 2005) (Fig. S3). By approximately 12 weeks of growth, the null plants became very bushy and continued to produce new branches (Fig. 2B, F). A 12-week-old null mutant plant had approximately 1150 branches (n=3) compared to 80 branches in WT (n=3).

We further studied the branching patterns in these plants. Branches generated directly from the primary inflorescence are designated the first order (1°) branches, those generated from the 1° branches are referred to as the second order (2°) branches, and so on. An atmyb2 null plant had not only more branch orders (up to 14 branch orders vs. 4 in WT) but also more branches at each order than WT. The typical branching patterns of 1° branches of atmyb2 and WT are shown in Fig. 2G. A null plant has 34 1° branches on average compared to 12 in WT. In addition to the bushy phenotype, the atmyb2 null plants have reduced seed set (Fig. S4). The reduced seed setting, the basis of which has not been further investigated in the current study, however, was not the cause for the bushy phenotype because manual removal of fruits from WT did not phenocopy atmyb2 (Fig. S5).

Because both T-DNA insertion lines, atmyb2-1 and atmyb2-2, displayed the same phenotype, only atmyb2-1 was used for further analyses.

**AtMYB2 restored atmyb2 to wild type.**

To confirm that the knockout of AtMYB2 was responsible for the bushy phenotype described above, we performed a complementation experiment by introducing the intact ATMYB2 (including its promoter) into the null plants. The complementation lines were resistant to both
gentamycin (rendered by the complementation construct) and kanamycin (rendered by the original T-DNA construct), and displayed a WT phenotype (Fig. 3A).

**The atmyb2 null mutant had elevated levels of cytokinins.**

We hypothesized that the branchy phenotype in *atmyb2* was caused by elevated levels of cytokinins in *atmyb2*. We determined the endogenous levels of cytokinins in compressed internodes of 8-week-old *atmyb2* and WT plants, respectively, using an indirect competitive ELISA (Setter et al., 2001) on the compressed internodes tissues of 8-week-old plants. Anti-ZR (*trans*-zeatin riboside) monoclonal antibody and anti-IPA (isopentenyladenosine) monoclonal antibody were used to quantify all the ZR-type and IPA-type cytokinins. The levels of both total ZR-type and IPA-type cytokinins extracted from *atmyb2* were more than 90% higher than those in WT (Fig. 3B).

**P*AtMYB2* directed expression of a cytokinin oxidase restored atmyb2 to wild type.**

To further investigate whether the increased level of cytokinins caused the bushy phenotype in the *atmyb2* null plants, we used the *AtMYB2* promoter (hereafter *P*<sub>AtMYB2</sub>) to direct *AtCKX1* (encoding an *Arabidopsis* cytokinin oxidase that destroys cytokinins (Schmulling et al., 2003)) in the *atmyb2* plants. The *P*<sub>AtMYB2</sub>*-AtCKX1* transgene reduced the higher levels of cytokinins in *atmyb2* to levels that were even lower than those in WT (Fig. 3B), and restored the *atmyb2* bushy plants to WT (Fig. 3A). The *P*<sub>AtMYB2</sub>*-AtCKX1* transgenic plants (*atmyb2* null background) had almost the same number of branches as WT. These data strongly suggest that the increased cytokinin levels are responsible for the bushy phenotype of the *atmyb2* plants.
The overaccumulation of cytokinins in the atmyb2 plants likely resulted from enhanced local biosynthesis in the stem.

The high levels of cytokinins in the compressed internodes region of atmyb2 could result from (a) increased transport from roots (cytokinins are generally believed to be synthesized mainly in roots (Sakakibara, 2005)); or (b) locally enhanced cytokinin biosynthesis. To distinguish these possibilities, we performed reciprocal grafting experiment between the atmyb2 null plants and WT. As shown in Figure 4, the graft with WT as stock (bottom) and atmyb2 as scion (shoot) displayed the bushy phenotype, whereas the graft with atmyb2 stock developed as WT. Thus, the root system was not responsible for the elevated levels of cytokinins in the compressed basal internodes.

We further performed RT-PCR analysis of the expression patterns of isopentenyltransferase genes (IPTs) in the compressed internode tissues of 8 week-old atmyb2 and WT plants. IPT catalyzes the first step in cytokinin biosynthesis (Gan and Amasino, 1995). There exist 9 IPTs (Miyawaki et al., 2004) in the Arabidopsis genome. As shown in Figure 5, IPT1, 4, 5, 6 and 8 were up-regulated in atmyb2 relative to WT, suggesting that locally enhanced cytokinin biosynthesis is responsible for the elevated cytokinin levels, and that AtMYB2 is likely a negative regulator of some IPTs.
DISCUSSION

Our data reported here reveal a new mechanism of AtMYB2 controlling branching at late stages of development in Arabidopsis (Fig. 6). AtMYB2 is expressed at late developmental stages in the compressed basal internodes to suppress cytokinin biosynthesis and prevent axillary buds from outgrowth. In atmyb2 null plants, the suppression is removed, resulting in elevated levels of cytokinins that promote the outgrowth of the axillary buds, leading to a bushy phenotype. The expression of AtCKX1 under the direction of PA MYB2 destroys cytokinins in the mutant plants and restores the bushy phenotype to WT. We propose that as part of the whole plant senescence program, AtMYB2 functions to suppress axillary bud outgrowth at late developmental stages when the auxin-mediated apical dominance mechanism is no longer active.

Recently, much progress has been made in unraveling regulation of axillary buds outgrowth at early stages of development in various plant species. Many plant mutants have been isolated to have impaired apical dominance and increased axillary bud outgrowth. This includes mutants with defects in auxin signal transduction pathway such as axr1 in Arabidopsis (Lincoln et al., 1990), cytokinin-overproducing mutants such as ampl (Chaudhury et al., 1993; Nogue et al., 2000), supershoot (sp) (Tantikanjana et al., 2001) and hoc (Catterou et al., 2002) in Arabidopsis. There are also mutants defective in biosynthesis or response to the branch-inhibiting hormone strigolactone, including the max mutants in Arabidopsis (Stirnberg et al., 2002; Booker et al., 2004; Booker et al., 2005; Stirnberg et al., 2007), the dad mutants in petunia (Snowden et al., 2005; Simons et al., 2007), the rms mutants in pea (Sorefan et al., 2003), and the dwarf mutants in rice (Arite et al., 2007; Arite et al., 2009).

Unlike atmyb2 plants, which do not show a massive outgrowth of axillary buds until at a very late stage of development, all of the above mentioned branching mutants start exhibiting a
bushy phenotype at vegetative growth stages or early reproductive stages. One important hypothesis of the apical dominance mechanism is that auxin (IAA) is produced at the growing shoot apex. It has been demonstrated that IAA is produced in tissues with rapid cell division and/or cell expansion such as apical meristems, young leaves, young stem tissues, developing fruits and seeds (Marumo et al., 1968; White et al., 1975). In this study, the massive outgrowth of axillary buds in atmyb2 plants was not observed until the plants were 8-week-old, a stage when the SAM was arrested, all leaves had started senescing, and majority of the siliques were mature (Fig. 2C). It is unlikely that the shoot apex at this stage is still actively producing IAA. Then, how the axillary buds are continuously suppressed without the auxin-mediated apical dominance? This study revealed a new mechanism that is designated “AtMYB2-mediated post apical dominance mechanism”.

Although distinct from the traditional auxin-mediated apical dominance, the AtMYB2-mediated branching control mechanism appears to have crosstalk with at least one class of the branching-regulating hormones: several cytokinin biosynthetic IPT genes were upregulated in the atmyb2-1 mutant (Fig. 5). It is interesting that auxin also regulates branching via suppressing biosynthesis of cytokinins. In addition to polar auxin transport which is responsible for apical dominance and many other important development processes, it has become clear that highly localized de novo auxin biosynthesis is also important in regulating auxin functions (Zhao, 2010). We therefore can not exclude the possibility of crosstalk between the “AtMYB2-mediated post apical dominance mechanism” and auxin signaling as the result of local biosynthesis. Further investigation on how AtMYB2 regulates transcription of IPTs, and how the AtMYB2-cytokinin pathway is related to local synthesis of auxin and the third branching regulating hormone strigolactone, will be necessary to unravel the mechanisms of AtMYB2-mediated branching control and whole plant senescence.
The AtMYB2-mediated branching regulation is of evolutionary significance. AtMYB2 has been shown to be induced by stresses such as drought and salinity (Urao et al., 1993; Abe et al., 1997), and plants under such stresses are often unbranched or much less branched (Frederick et al., 2001; Doust, 2007). As an evolutionary adaptation, plants under unfavorable environmental conditions minimize vegetative growth (e.g. branching) and finish their life cycle by producing seeds earlier with the limited resources. A change in phenotype in response to environmental factors such as this represents an example of phenotypic plasticity, which is controlled by gene expression (Sultan, 2000). The transgenic plants overexpressing AtMYB2 showed a phenotype of early flowering, less branching, and small stature (Fig. S6), as if the plants were stressed, suggesting that AtMYB2 might be a regulator of plant phenotypic plasticity under stressed conditions. Since stresses induce senescence (Guo and Gan, 2005), it will be interesting to find out whether under stressed conditions, AtMYB2 functions directly in response to stress or functions after senescence is induced. Functions of AtMYB2 might be different in different developmental processes and in response to different environmental stimuli. Although originally identified as a leaf senescence associated gene (Fig. S1), knocking out of AtMYB2 does not seem to affect the progression of leaf senescence (Fig 2C), suggesting the involvement of other factors which may function redundantly with AtMYB2 in regulating leaf senescence.
MATERIALS AND METHODS

**Plant materials and growth conditions.** The *Arabidopsis thaliana* T-DNA insertion lines and various transgenic lines were grown side by side with wild type for comparison. Seeds were sown on Petri dishes containing one-half strength of Murashige and Skoog salts, 0.8% (w/v) phytoagar, and appropriate antibiotics. Seeds were imbibed at 4°C overnight. Two-week-old seedlings were transplanted to 4” standard pots filled with Cornell mix soils (3 parts peat moss: 2 parts vermiculite: 1 part perlite). Plants were grown at 23°C with 60% relative humidity under constant light (150 µmol m\(^{-2}\) s\(^{-1}\) light from a mixture of fluorescent and incandescent bulbs). *A. thaliana* accession Wassilewskija (Ws) was used unless otherwise stated.

**Isolation of T-DNA insertion lines.** The alpha population of T-DNA insertion lines of the University of Wisconsin Arabidopsis Knockout Facility (Sussman et al., 2000) were screened using a pair of *AtMYB2*-specific primers, G468 and G484, and the T-DNA left border primer JL-202 (Table S1), according to the protocol available at [http://www.biotech.wisc.edu/Arabidopsis/](http://www.biotech.wisc.edu/Arabidopsis/). Two T-DNA insertion lines, designated as *atmyb2-1* and *atmyb2-2*, were obtained. Both lines displayed an identical phenotype, and *atmyb2-1* was used for further analyses unless otherwise indicated.

**Transgenic studies.** The genomic sequence of *AtMYB2* including a 2.5-kb promoter region, was PCR amplified with primers G910 (5’-ATCCTAGGATAGTACACGTT TTGGCATC-3’; the underlined section is an engineered Avr II site) and G911 (5’-AATTCGAAAACG TGACAAGGAA GAGAAT-3’) using *Pfu* DNA polymerase (Stratagene, La Jolla, CA). The 4.3-kb PCR product, upon an A-tailing procedure described by the manufacturer, was cloned into
pGEM-T (Promega, Madison, WI) to form pGL1805. The pGL1805 plasmid DNA was sequenced. pGL1805 was digested with Avr II and Pst I and the released \textit{AtMYB2} genomic fragment was cloned into the binary vector pPZP221 (Hajdukiewicz et al., 1994) at Xba I and Pst I sites, resulting in pGL1810. The pGL1810 was used for a complementation test involving the \textit{atmyb2-1} null plants.

The chimeric gene $P_{ATMYB2}$-$AtCKX1$, the Arabidopsis \textit{cytokinin oxidase 1} (\textit{AtCKX1}) directed by the \textit{AtMYB2} promoter, was constructed as follows. The coding region of \textit{AtCKX1} (\textit{At}2\textit{g}41510) was PCR amplified with primers G1401 (5’-ATGGA{T}C
\_CGCCTCTATTCTTTGC-3’; the underlined section is an engineered Bam HI site) and G1402 (5’-CGCCTAGGATTTTTATGGAGATCCT-3’; the underlined section is an engineered Avr II site) using the \textit{Pfu} DNA polymerase. The 2.4-kb PCR product was cloned into pGEM-T to form pGL1183. The promoter region of \textit{AtMYB2} was similarly cloned into pGL1187 by using primers G1469 (5’-ATCCTAGGTCCTATCCATCGGCTT-3’; the underlined section is an engineered Avr II site) and G1550 (5’-TAGGATCCTACTC TACCACACTTAC-3’; the underlined section is an engineered Bam HI site). Both pGL1183 and pGL1187 were sequenced. The \textit{AtCKX1} coding region was released from pGL1183 by Bam HI and Sph I, and was subsequently cloned into pGL1187 to form pGL1802. The $P_{ATMYB2}$-$AtCKX1$ fusion in pGL1802 was released upon Avr II digestion and subcloned into pGL1804, a binary vector containing the NOS terminator, to form pGL1808.

To make the $P_{ATMYB2}$-$GUS$ reporter construct, the \textit{AtMYB2} promoter was amplified using primers G618 (5’-GACTAGTTTTTTATATTATTATCCCAAC-3’) and G619 (5’-TCCATGGCTTTATAAGAAACTTTAAATGA-3’) with engineered Spe I and Nco I sites, respectively. The PCR product was cloned into pSG506 at Spe I and Nco I to generate pGL1131.
The $\text{P}_{\text{ATMYB2}}$-GUS-MAS terminator chimeric gene was released from pGL1131 with SpeI and BamHI and cloned into binary vector PZP221 by the XbaI and BamHI sites to create pGL1138.

To overexpress $\text{AtMYB2}$, the $\text{AtMYB2}$ coding sequence (1070 bp) was amplified with a pair of primers, G853 (5’-AACCTTCAATCTAATCCACAAAAC C-3’, the underlined section is an engineered Hind III site) and G854 (5’-CTGCAG G GGATTAAAACAAGAGAGGA-3’, the underlined section is an engineered Pst I site) using the Pfu DNA polymerase. The PCR product was digested with Hind III and Pst I, and cloned into the binary vector pGL800 in the sense orientation to form pGL1149. The pGL800 contains the 35S promoter-msc-the RBS terminator.

The constructs pGL1810, pGL1808, pGL1138, and pGL1149 were transferred into $\text{Agrobacterium tumefaciens}$ (strain: ABI) using the freeze–thaw method as previously described (Adachi and Lieber, 2002). The Agrobacterium cells containing the respective construct were then used to transform heterozygous $\text{atmyb2}$ or WT plants via vacuum infiltration (Bechtold et al., 1993). The $\text{atmyb2}$ plants are resistant to kanamycin. Transgenic plants harboring pGL1810 or pGL1808 were selected on plates containing both 50 mg/L kanamycin and 80mg/L gentamycin and were allowed to self. Progenies homozygous for $\text{atmyb2}$ were selected using PCR for further analysis.

RT-PCR analyses. Total RNA extraction was performed as described (Guo and Gan, 2006). RT-PCR reactions were carried out with the Ambion RetroScript Kit (Ambion, Austin, TX) according to the manufacture’s instruction. The QuantumRNA™ Universal 18S Internal Standard Kit (Ambion, Austin, TX) was used for internal standard. Primers used for RT-PCR and/or for amplification of RNA gel blot probe are listed in Table S1.
Counting and pattern of branches. The numbers of branches were counted using two methods: whole plant approach and partial counting approach. In the whole plant approach, all branches of a 3-month-old plant were counted. In the partial counting approach, a 3-month-old plant was cut at 3 inches above soil and the number of branches at the cutting was counted. For the purpose of examining branching pattern, the 1°, 2°, …, and N° branch order and the number of branches of a given order branch were individually counted. A 1° branch is the one originated directly from the primary inflorescence, including those generated from the upper part of the main shoot and from the compressed internodes region at the rosette, and a 2° branch is the one from the 1° branch, and so on.

Fruit removal and grafting experiments. For fruit removal experiment, newly developed fruits were removed from plants daily. Grafting was carried out as described by Turnbull et al (Turnbull et al., 2002). Arabidopsis seedlings were kept under dim light for 4 days to produce elongated hypocotyls. The seedlings were cut with a sharp surgical blade and the stock and scion were inserted into a 5-mm long silicone tube with 0.3 mm inner diameter (VWR, West Chester, PA). The two cut ends were firmly appressed inside the tube. The grafted seedlings were kept in Petri dishes for two weeks (by that time two true leaves should have been produced if the grafting was successful), and were transplanted to soil. Adventitious roots from the scions (if any) were removed. The number of branches of individual grafts was assessed after an additional 10 weeks of growth.

Measurement of cytokinin levels. The compressed basal internode tissues for cytokinin analysis were harvested from eight-week-old Arabidopsis plants. Extraction, purification and quantification of \textit{trans}-zeatin ribosides (ZR) and isopentenyladenosine (IPA) families of
cytokinins were performed according to Setter et al. (Setter et al., 2001) (See Supplemental Information for details).

**Microscopic analysis.** The basal internode regions including axillary buds from Arabidopsis plants at different developmental stages were examined, and images were taken under a dissecting microscope (Leica S6E).
SUPPLEMENTAL MATERIALS

Table S1. Primers used in this study.

Fig. S1. Expression of AtMYB2 during leaf senescence.

Fig. S2. Expression of AtMYB2 in WT and T-DNA insertion lines.

Fig. S3. Expression of bud dormancy-associated genes in WT and atmyb2-1.

Fig. S4. Inflorescences from 8-week-old plants of WT and atmyb2-1.

Fig. S5. Effect of fruit-removing on branching.

Fig. S6. Effect of overexpression of AtMYB2 on branching and other aspects of plant development.
ACKNOWLEDGEMENTS

We thank Tim Setter (Cornell) for help in cytokinin quantification, Richard Amasino (UW-Madison), June Nasrallah, and Steve Tanksley (Cornell) for critical reading of an early version of the manuscript.
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FIGURE LEGENDS

Fig. 1. Structure and expression of AtMYB2 in the compressed basal internode region in Arabidopsis. (A) RT-PCR analysis of AtMYB2 transcripts in the basal internode region of 8- and 12-week-old plants. (B) GUS staining in the compressed internode regions of 4- and 8-week-old transgenic plants harboring the P_atMYB2-GUS construct. (C) Structure of AtMYB2 and T-DNA insertion sites in two T-DNA lines, atmyb2-1 and atmyb2-2. (D) No detectable AtMYB2 transcripts in both T-DNA lines, suggesting both lines are null mutants (RT-PCR analysis of only atmyb2-1 line is shown).

Fig. 2. Phenotypic characterization of atmyb2-1 plants. Both atmyb2-1 and atmyb2-2 null lines display an identical phenotype and only atmyb2-1 is shown. (A) The atmyb2-1 null plants (on the left tray) and WT (on the right tray) at an early stage of development. Shown are approximately 4-week-old plants. (B) Number of branches counted from 3 inches above ground from WT (n=8, blue) and atmyb2-1 (n=9, fuchsia) plants at different development stages. Shown are averages with standard deviations. (C) WT (left) and atmyb2-1 plants that are approximately 8 weeks old. Rosette leaves of WT and the null mutant plant were all senescent. (D) Suppression of axillary buds of the compressed basal internodes region and of aerial stem (inset) of the WT plant shown in (C). (E) Outgrowth of axillary buds of the atmyb2-1 plant shown in (C). An axillary bud associated with a cauline leaf is shown in the inset. (F) Approximately 12-week-old WT (on the left) and the atmyb2-1 null plant (on the right). (G) Schematic diagrams of branching patterns of a typical first order (1°) branch from a WT and an atmyb2-1 plant. A 1° branch is generated directly from the primary florescence, and a 2° branch from the 1° branch, and so on.
**Fig. 3.** Restoration of the *atmyb2-1* plants to WT, morphologically and in terms of levels of endogenous cytokinins, by intact *AtMYB2* or by *P_{AtMYB2}-AtCKX1* (a chimeric gene in which the Arabidopsis cytokinin oxidase 1 gene is under the control of the *AtMYB2* promoter). (A) Phenotype. (B) Endogenous levels of the zeatin family (ZR) and isopentenyladenosines family (IPA) cytokinins in the basal internodes regions of approximately 8-week-old plants. The cytokinin levels were determined using ELISA. Data shown are averages with standard deviations from three biological replicates.

**Fig. 4.** Branching patterns in plants resulting from reciprocal grafts between *atmyb2-1* and WT plants. (A) Phenotype of various grafts. (B) Numbers of branches of the grafts. The grafts were cut at 3 inches above soil and the branches at the cut were counted. Four plants were counted for each type of grafting. Averages with standard deviations are shown.

**Fig. 5.** RT-RCR analysis of expression patterns of isopentenyltransferase-encoding genes (*IPT1*-9) in the basal internode regions of approximately 8-week-old WT and *atmyb2-1* plants.

**Fig. 6.** Model of *AtMYB2* action. At late stages of plant development, *AtMYB2* is expressed to inhibit the expression of the isopentenyl transferase genes to prevent cytokinin production. In *atmyb2* mutant plants, the inhibition is released, leading to the increased production of cytokinins, which promotes axillary bud outgrowth.
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