The Compact Root Architecture1 Gene Regulates Lignification, Flavonoid Production, and Polar Auxin Transport in Medicago truncatula1[W]

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The root system architecture is crucial to adapt plant growth to changing soil environmental conditions and consequently to maintain crop yield. In addition to root branching through lateral roots, legumes can develop another organ, the nitrogen-fixing nodule, upon a symbiotic bacterial interaction. A mutant, cra1, showing compact root architecture was identified in the model legume Medicago truncatula. cra1 roots were short and thick due to defects in cell elongation, whereas densities of lateral roots and symbiotic nodules were similar to the wild type. Grafting experiments showed that a lengthened life cycle in cra1 was due to the smaller root system and not to the pleiotropic shoot phenotypes observed in the mutant. Analysis of the cra1 transcriptome at a similar early developmental stage revealed few significant changes, mainly related to cell wall metabolism. The most down-regulated gene in the cra1 mutant encodes a Caffeic Acid O-Methyl Transferase, an enzyme involved in lignin biosynthesis; accordingly, whole lignin content was decreased in cra1 roots. This correlated with differential accumulation of specific flavonoids and decreased polar auxin transport in cra1 mutants. Exogenous application of the isoflavone formononetin to wild-type plants mimicked the cra1 root phenotype, whereas decreasing flavonoid content through silencing chalcone synthases restored the polar auxin transport capacity of the cra1 mutant. The CRA1 gene, therefore, may control legume root growth through the regulation of lignin and flavonoid profiles, leading to changes in polar auxin transport.

Plant roots perform several crucial functions, including anchorage to the soil, water and nutrient uptake, as well as interactions with microorganisms of the rhizosphere. Postembryonic plasticity of the root system architecture, therefore, is essential to optimize the adaptation of plant growth to changing soil conditions (Malamy, 2005). As in soils biotic and abiotic constraints strongly affect crop yield, the regulation of root architecture represents a highly relevant agronomic trait. In legume plants, two types of below-ground organs can develop from roots depending on environmental conditions: lateral roots and symbiotic nitrogen-fixing nodules. Their development is regulated by water and nutrient availability as well as by the presence of symbiotic bacteria for nodulation (Sinorhizobium meliloti in the case of Medicago truncatula; Nibau et al., 2008; Gonzalez-Rizzo et al., 2009).

Whereas mechanisms underlying root growth and architecture are well studied in Arabidopsis (Arabidopsis thaliana); Benková and Hejátko, 2009; Fukaki and Tasaka, 2009), several specifics of legume roots suggest that regulatory mechanisms involved in determining root growth and architecture may differ (Gonzalez-Rizzo et al., 2009). Among them, the distinct cortical layers (outer and inner) present in legume roots show differential responses to rhizobial activation during nodule organogenesis. In addition, some phytohormonal regulations are different: for example, application of low concentrations of abscisic acid shows opposite regulation for lateral root formation when compared with Arabidopsis (Liang and Harris, 2005). Few mutants affected in both types of root lateral organogenesis have been described, such as M. truncatula root lateral organs deficient, affected in lateral root and nodule differentiation (Bright et al., 2005), or Lotus japonicus har1 (for hypernodulation and aberrant root), affected in the negative feedback regulation of lateral root and nodule numbers (Wopereis et al., 2000).

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Interestingly, the *M. truncatula* *sunn* (for super numeric nodules) mutant, affected in a CLAVATA-like receptor kinase similar to HARI, shows a hypernodulation phenotype but not increased lateral root numbers (Schnabel et al., 2005). Furthermore, *M. truncatula* roots in which the MCRE1 cytokinin receptor was down-regulated by RNA interference (RNAi) showed increased lateral root formation and decreased nodulation (Gonzalez-Rizzo et al., 2006), whereas perturbation of MtMIR166, an evolutionarily conserved microRNA, affects vascular bundle patterning and consequently initiation of both root lateral organs (Boualem et al., 2008). Certain nodulation mutants were reported to be also affected in root elongation. The *L. japonicus* brush mutant is primarily affected in root growth, showing distorted root apical meristem organization and reduced cell elongation, leading to a delayed shoot growth phenotype as shown by grafting experiments (Maekawa-Yoshikawa et al., 2009). Few nodules are formed, however, showing a wild-type structure. The *sickle* (*skl*) mutant affected in the Ethylene Insensitive2 ethylene signaling protein shows, in addition to a supernodulation phenotype, increased root elongation (Prayitno et al., 2006; Fenmetsa et al., 2008), and the *sunn*/har1 mutants show reduced root growth in addition to the nodulation phenotype (Wopereis et al., 2000; Schnabel et al., 2005). Interestingly, polar auxin transport (PAT) defects have been reported in *skl* and *sunn* mutant roots (Prayitno et al., 2006; van Noorden et al., 2006), suggesting a link with the well-documented effect of auxin and its polarized transport in the establishment of root meristems and lateral roots in Arabidopsis (Sabatini et al., 1999; Benková et al., 2003).

Plant flavonoids function as pigments, antioxidants, and also auxin transport inhibitors (Winkel-Shirley, 2001). In Arabidopsis, a flavonoid-deficient mutant (*ht4*, for transparent testa4) defective in the first enzyme of the flavonoid biosynthesis pathway, chalcone synthase (CHS), shows increased auxin transport rates and is defective in auxin-regulated root phenotypes, such as lateral root formation and gravitropism response (Brown et al., 2001; Buer and Muday, 2004; Peer et al., 2004). However, it is possible that the effect of flavonoids in modulating auxin accumulation and subsequent development is indirect (Peer et al., 2004; Peer and Murphy, 2007). In addition, flavonoids are used as defense compounds in response to pathogens, and in the case of the legume symbiosis, a double action has been revealed: first, specific flavonoids from root exudates activate the synthesis of bacterial signals necessary to allow bacterial infection (so-called Nod factors; Cooper, 2007); and second, silencing CHS revealed that endogenous flavonoids are involved in the inhibition of PAT at the onset of nodule organogenesis (Wasson et al., 2006; Zhang et al., 2009). Specific flavonoids were shown to inhibit PAT, such as dihydroxyflavone, the flavanone liquiritigenin, and the flavonols quercetin and kaempferol in several plants, including *M. truncatula* roots (Stenlid, 1976; Jacobs and Rubery, 1988; Brown et al., 2001; Taylor and Grotewold, 2005; Zhang et al., 2009).

Recently, it was shown that inhibition of lignin biosynthesis led to an activation of the flavonoid pathway, as both types of compounds are derived from a common intermediate metabolite of the phe-nylpropanoid pathway (*p*-coumaroyl CoA; Besseau et al., 2007; Supplemental Fig. S1). Angiosperm lignins are constituted of three types of units (syringyl [S], guaiacyl [G], and hydroxyphenyl [H]) and are responsible for plant cell wall rigidification and the control of water diffusion between cells. Mutants or silenced lines affecting lignin metabolism were obtained in several plants, such as Arabidopsis, maize (*Zea mays*), poplar (*Populus* spp.), tobacco (*Nicotiana tabacum*), and alfalfa (*Medicago sativa*), and often show an altered growth correlated with vascular bundle defects (Anterola and Lewis, 2002; Boerjan et al., 2003; Hoffmann et al., 2004; Abdulrazzak et al., 2006; Besseau et al., 2007). Interestingly, in a few cases, reduced lignin content was not associated with growth defects, indicating that lignin per se may not be responsible for this phenotype (Lee et al., 1997; Hu et al., 1999; Boerjan et al., 2003). In addition, plants negatively affected in lignin biosynthesis frequently show an accumulation of anthocyanins (Boerjan et al., 2003), and Arabidopsis plants defective in hydroxycinnamoyl transferase activity, involved in the biosynthesis of S and G lignin units, accumulated flavonoids, including flavonols and anthocyanins (Besseau et al., 2007).

In the model legume *M. truncatula*, we identified the compact root architecture1 (*cra1*) mutant, characterized by short and thick roots likely resulting from the deregulation of root cell elongation. Other defects in leaf size, trichome density, lengthened life cycle, as well as partial sterility were observed in this mutant. To gain insight into the mechanisms affected in *cra1* roots, we analyzed its transcriptome and show that cell wall metabolism genes were down-regulated, including a Caffeic Acid O-Methyl Transferase (COMT) involved in lignin synthesis. Accordingly, defective lignification was detected in mutant roots, together with an accumulation of the flavonoid formononetin and an inhibition of PAT capacity. Treatment of wild-type *M. truncatula* roots with formononetin reduced PAT capacity and mimicked the mutant root phenotype. In addition, decreasing flavonoid content in *cra1* using a RNAi CHS construct restored wild-type PAT capacity. Overall, these results indicate that CRA1 regulates lignin and flavonoid contents and, consequently, affects PAT and root growth.

RESULTS

**Root Architecture and Shoot Phenotypes of the *cra1* Mutant**

The *cra1* mutant was isolated from a *M. truncatula* T-DNA mutant collection (Scholte et al., 2002). The most striking defect observed in *cra1* corresponds to...
reduced root growth, which can be observed both on young in vitro-grown seedlings and at later developmental stages in primary and lateral roots of greenhouse-grown plants (Fig. 1, A–C). In order to characterize defects occurring in the mutant, we analyzed other root parameters. _cra1_ has an increased root diameter (Fig. 2, A and B) but has normal ability to initiate lateral organs, either symbiotic nodules or lateral roots (Fig. 2C). This result indicates that the root architecture phenotype of _cra1_ likely corresponds mainly to a root growth defect. Accordingly, cell size in the root differentiated zone was strongly perturbed even though the number of cells files was conserved (Fig. 2A). Measurements of cell size (monitored on outer cortical layers of the root differentiated zone) revealed that cell diameter (i.e. radial length) was increased (Fig. 2D, left), whereas longitudinal length was reduced (Fig. 2D, right) in _cra1_ mutants. These analyses suggest that the main defect leading to decreased root growth and increased root diameter in _cra1_ resides in abnormal cell elongation.

At later developmental stages, other defects appear in _cra1_ mutants. One-month-old plants grown in a greenhouse develop smaller leaves, irregularly shaped, with a white refringent epidermis (Fig. 2E). Careful analysis of leaf epidermis revealed a high number of trichomes present in _cra1_ (Fig. 2E), and quantification using scanning electron microscopy confirmed the strong increase in trichome density when compared with wild-type plants (Fig. 2F). Upon flowering, reduced pod numbers and seed progeny were observed in _cra1_ plants, a phenotype that could be partially relieved by increasing relative humidity conditions, suggesting that fertilization was impaired. Interestingly, _cra1_ plants were not dwarf (Fig. 1, D and E) and even exhibited a delay in flowering of 3 to 4 months and, consequently, an extended life cycle.

Genetic analysis of these different phenotypes was studied by backcrossing a _cra1_ homozygous line. Root growth, leaf shape, trichome density, and flower (sterility) phenotypes showed a 3:1 segregation, as expected for recessive monogenic characters. In addition, analysis of 77 individuals in the backcrossed progeny showed no segregation of these different phenotypes, suggesting their linkage. To determine which of these defects was primarily responsible for the overall delayed development leading to an increased life cycle in _cra1_, we performed grafting experiments (Fig. 3). Analysis of root architecture and leaf phenotypes (shape and trichome density) in the different grafting combinations revealed that none of the aerial phenotypes was graft transmissible from roots (Fig. 3A). Quantification of graft dry weight indicates that the delayed _cra1_ shoot growth was primarily caused by the root phenotype (Fig. 3B).

These results, therefore, indicate that the _cra1_ mutation provokes pleiotropic growth alterations but that the root growth defect is primarily responsible for the delayed and lengthened life cycle.

The CRA1 Gene Controls Root Lignification Levels

Despite the _cra1_ mutant having been initially identified in a T-DNA insertion collection, cosegregation analyses revealed that the single T-DNA present in this line was not linked to the observed phenotypes. To gain insight into the molecular nature of the mutation responsible for the phenotypes, we used Mt16K+ microarrays (containing approximately 17,000 oligonucleotides; Henckel et al., 2009) to analyze transcrip-
tomes of 1-week-old wild-type and cra1 seedlings, a stage where both genotypes are phenotypically very similar, to avoid any growth or developmental bias. Statistical analysis revealed few consistent changes between wild-type and cra1 seedling transcriptomes at this stage (Supplemental Table S1): using stringent criteria (i.e. an adjusted $P < 0.005$ threshold), five genes were identified as differentially regulated and all showed down-regulated expression in cra1 compared with the wild type (Supplemental Table S2). In an attempt to perform transcript-based cloning (Mitra et al., 2004), we sequenced the cra1 most down-regulated transcripts showing a signal intensity in arrays similar to background, but no genetic lesion could be identified.

Analysis of functional categories down-regulated in cra1 seedlings (based on an adjusted $P < 0.05$ threshold) revealed enrichment for cell wall-related genes (Fig. 4A; functional categories defined based on MapMan BINs for M. truncatula; Tellstro¨m et al., 2007). Strikingly, one of the most down-regulated transcripts in cra1 encoded a COMT (EC 2.1.1.68; Mt16K+ identifier, MT007074; MtG8, TC100394; MtG9, TC130531; available from The Institute for Genomic Research database [http://compbio.dfci.harvard.edu/tgi/]), an enzyme involved in the biosynthesis of S lignin units (Supplemental Fig. S2; Supplemental Table S1; Besseau et al., 2007). The impact of the cra1 mutation on lignification was studied by thioacidolysis, which leads to H, G, and S thioethylated lignin-derived monomers from H, G, and S lignin units, respectively. Therefore, the total thioacidolysis yield provides an estimate of parent lignin structure content without any interference from nonlignin phenolics. Interestingly, a significantly lower thioacidolysis yield was observed in cra1 2-month-old mutant roots, which could not be

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**Figure 2.** cra1 root and leaf phenotypes. A, Transverse sections of wild-type (WT; 108-R4) and cra1 roots (at 1.5 cm distance from the root tip) from 2-week-old seedlings grown in pots containing perlite-sand, observed in bright field after toluidine blue staining (top row) or under UV light excitation (emission maximum at 365 nm) to visualize autofluorescence of tissues (bottom row). Bars = 100 μm. B, Quantification of wild-type (108-R4) and cra1 root diameter (at 1.5 cm distance from the root tip) from 4-week-old plants grown in the greenhouse. C, Quantification in wild-type (108-R4) and cra1 plants grown in the greenhouse of nodule density (nodule no. per centimeter of root at 3 weeks after inoculation with S. meliloti 1021 strain; left graph) and of lateral root density (lateral root no. per centimeter of root in plants grown for 4 weeks; right graph). D, Quantification of cortical cell size (at 1.5 cm distance from the root tip) in radial (left graph) and longitudinal (right graph) directions in 2-week-old seedlings grown in vitro. E, Representative images of wild-type (108-R4) and cra1 leaf phenotypes (top row; bars = 0.5 cm) and details of trichomes (bottom row; bars = 0.5 mm). F, Quantification of trichome density (per mm$^2$) in wild-type (108-R4) and cra1 leaves. In all cases, error bars represent confidence intervals ($α = 5%; n = 10$), and a Mann-Whitney test was used to determine significant differences (*** $α < 0.1%$).
Transport Ability Flavonoids Correlated with Decreased Polar Auxin Roots Show a Differential Accumulation of Flavonoids Correlated with Decreased Polar Auxin Transport Ability

The connection recently established in Arabidopsis between decreased lignification and increased flavonoid accumulation (Besseau et al., 2007) and our transcriptome analysis revealing four up-regulated genes linked to flavonoid metabolism (Table I) pointed us to analyze flavonoid content in cra1 roots. Main flavonoid aglycones present in 3-month-old pot-grown roots of the wild-type genotype were identified as formononetin, medicarpin, and isoliquiritigenin. Both total and free (nonglycosylated) flavonoids were then analyzed, revealing that cra1 roots contained significantly more free formononetin than wild-type roots (Fig. 5A), despite no significant change in the total pool of formononetin (free and glycoside forms) being detected (Supplemental Fig. S4A). The concentration of free formononetin in wild-type young roots is estimated to be approximately 0.5 to 10 nmol g\(^{-1}\) fresh weight (depending on seedling age), which would result in low micromolar concentrations in the tissue (U. Mathesius, unpublished data). Medicarpin and isoliquiritigenin were not detectable as free isoforms in our conditions, but their total pools were significantly enhanced in cra1 (Supplemental Fig. S4, B and C).

As flavonoid accumulation may affect polar auxin transport, we attempted to correlate the observed changes in flavonoid patterns with potential defects in acropetal auxin transport in cra1 mutants. Interestingly, the most abundant nonglycosylated flavonoid present in cra1 roots, the isoflavone formononetin, was able to significantly inhibit acropetal PAT in M. truncatula roots (Fig. 5B), whereas isoliquiritigenin and medicarpin did not significantly affect PAT (Supplemental Fig. S4D). In addition, measurements of acropetal PAT in cra1 versus wild-type roots revealed a decreased ability of the mutant to transport auxin (Fig. 5C). To directly determine the contribution of flavonoid accumulation in PAT defects observed in cra1, an RNAi construct targeting the CHS gene family was used (previously shown to be efficient for silencing the flavonoid pathway in M. truncatula; Wasson et al., 2006). As expected, this construct provoked an increase of PAT in wild-type roots but also restored a wild-type PAT capacity in the cra1 mutant (Fig. 5D). While the increase in auxin transport in CHS-silenced wild-type roots was slightly higher than in the CHS-silenced cra1 mutant, this difference was not statistically significant (\(P > 0.05\)). These results support the hypothesis that inhibition of PAT in cra1 roots could be due to the increased accumulation of flavonoids, particularly formononetin. Furthermore, exogenously applied formononetin at concentrations (0.2, 2, and 10 \(\mu M\)) similar to those estimated to occur inside root tissues induced a slight but significant reduction of root length as well as an increased root diameter in wild-type seedlings (Fig. 5E; data not shown), which are reminiscent of the cra1 phenotype. However, we could not assess the potential complementation of cra1 growth phenotypes by CHS silencing, due to the heterogeneity of roots initiated on composite plants. Finally, the PAT defect observed in cra1 roots was correlated with decreased auxin sensitivity (Fig. 5F).

Altogether, our results show that the CRA1 gene regulates appropriate root lignification and flavonoid patterns, the latter likely consequently affecting PAT and root development.

DISCUSSION

The cra1 mutant described in this work revealed a correlation between reduced root growth, cell elongation defects, perturbations in lignin and flavonoid contents, and the ability to polarly transport auxin in M. truncatula. In Arabidopsis, a number of mutants affected in cell elongation were shown to be directly related to cell wall expansion, such as korrigan and procuste1 affecting cellulose synthesis (Nicol et al., 1998; Fagard et al., 2000; Sato et al., 2001). Even though observed in shoots (Fig. 4B; Supplemental Fig. S3). Even though the proportion of each lignin unit remained similar as in the wild type, abundance of all types of lignin units tested (S, G, or H) was decreased in the mutant (Fig. 4, B and C), indicating that the whole lignin pathway is down-regulated in cra1 roots.

**Figure 3.** The cra1 delayed life cycle is primarily due to its compact root architecture. Representative images (A) and quantification of plant dry weight (B) of various combinations of wild-type (WT; 108-R4) and cra1 grafts grown for 8 weeks are shown. The insets show details of representative leaves of the corresponding grafting combinations. Bar = 3 cm. Error bars represent confidence intervals (\(\alpha = 5\%\); \(n = 5\)), and a Kruskal-Wallis test was used to determine significant differences (\(*\alpha < 5\%\)) between the wild-type/wild-type control graft and other grafting combinations.
the genetic lesion associated with the cra1 mutation could not be identified in this study, transcriptome analysis at an early developmental stage revealed few highly significant changes, notably pointing to cell wall modifications in this mutant. One of the most down-regulated transcripts encoded a COMT involved in S lignin biosynthesis (Besseau et al., 2007), but the abundance of all types of lignin units was decreased in cra1 roots. This suggests that COMT per se is not primarily affected in the mutant, as confirmed by...
sequencing of the corresponding genomic locus. The whole lignin pathway, therefore, seems modified in cra1 roots, even though this effect is partial and leads to an approximately 30% reduction in lignin levels.

Few studies are available on the developmental consequences of lignin biosynthesis down-regulation. In tobacco, silencing of a Caffeic Acid-CoA O-Methyl Transferase enzyme involved in lignin biosynthesis induced phenotypes tightly related to cra1, including delayed overall growth, formation of narrower leaves, and defective flower development and fertilization (Pinçon et al., 2001). In addition, silencing of the coumaroyl ester-3’-hydroxylase CYP98A3 in Arabidopsis led to shortened and thicker hypocotyls and roots as well as flower male sterility, resulting in a lower number of viable seeds (Abdulrazzak et al., 2006). In contrast to cra1, however, these mutant plants showed smaller cells in leaf epidermis and remained dwarf despite a lengthened life cycle. In M. truncatula, we show that the decrease in lignin content observed in cra1 roots was correlated with a limitation of root growth due to defective cell elongation.

Changes in lignin content and/or structure using transgenic approaches could not be strictly correlated with the reported growth phenotypes, suggesting that lignin itself may not be responsible for these phenotypes (Boerjan et al., 2003). By combining lignin- and flavonoid-deficient Arabidopsis plants, it was elegantly shown that the flavonoid accumulation observed in lignin-deficient plants, likely through its effect on PAT, was responsible for the shoot growth phenotypes (Besseau et al., 2007). We also observed a flavonoid accumulation in cra1 lignin-deficient roots, which was correlated with up-regulation of four transcripts encoding crucial flavonoid biosynthesis enzymes. Coordinated up-regulation of these three enzymes is predicted to ultimately lead to an increase in isoflavone biosynthesis (Supplemental Fig. S1); strikingly, we observed an accumulation of the isoflavones medicarpin and formononetin. It is tempting, therefore, to speculate that cra1 root and cell elongation defects may be primarily due to changes in flavonoid accumulation. Indeed, reduction of flavonoid accumulation in this mutant by expressing a CHS RNAi construct restored a wild-type PAT, as observed previously in Arabidopsis plants defective for hydroxycinnamoyl transferase activity (Besseau et al., 2007). In the latter case, a reversion of shoot developmental phenotypes occurred, indicating a crucial role of flavonoid accumulation in the growth phenotype of lignin-defective plants. In Medicago, we showed that exogenous formononetin treatments mimicked the root growth phenotypes observed in cra1, indicating that changes in flavonoid accumulation patterns are likely to affect root length and width in this legume plant. However, as disruptions of the flavonoid pathway can have other effects on phenylpropanoid accumulation (Peer et al., 2004), on the availability of reactive oxygen species (Pourcel et al., 2007), or on the phosphorylation of proteins (Bernasconi, 1996), we cannot exclude that the phenotypes of cra1 are indirect consequences of flavonoid alterations.

Another phenotype found in cra1 leaves is increased trichome density, which agrees with observations made in Betula species, where concentrations of leaf surface flavonoids correlated positively with the density of glandular trichomes (Valkama et al., 2004). In addition, analysis of the alfalfa legume trichome transcriptome revealed that enzymes for all steps of flavonoid biosynthesis were expressed, as confirmed by histochetry and metabolite profiling, suggesting their involvement in trichome biology (Aziz et al., 2005). Finally, the Arabidopsis Transparent Testa Glabra locus regulates the formation of hairs and the production of anthocyanin pigments, further linking flavonoids and trichome development (Walker et al., 1999). In cra1, however, no visible alteration in anthocyanin accumulation could be detected in the shoot.

Despite cra1 roots showing a reduced PAT, we did not observe changes in their ability to form lateral roots and nodules. This is in contrast to Arabidopsis mutants defective in PAT, which often show lateral root phenotypes (Bennett et al., 1996; Benková et al., 2003; Swarup et al., 2008). The decreased lignin content and PAT observed in cra1 roots is modest (approximately 30% and 20% of wild-type contents, respectively), and we can speculate that higher reductions of lignin content are required to affect root lateral organogenesis, even though the pleiotropic plant growth reduction may bias the analysis. Interestingly,
M. truncatula CHS-silenced hairy roots showed a highly impaired nodulation (Wasson et al., 2006) but no defect in lateral root formation (Wasson et al., 2009). This is in contrast to the CHS-deficient Arabidopsis tt4, tt5, and tt6 mutants, respectively defective in CHS, chalcone isomerase, and flavonone 3-hydroxylase, enzymes involved in flavonoid biosynthesis, which all are affected in lateral root formation (Brown et al., 2001; Buer and Djordjevic, 2009). It is then possible that M. truncatula might have different requirements for auxin transport with respect to lateral root formation than Arabidopsis. For nodule development, an inhibition in auxin transport after inoculation appears to be an important step for organ initiation (Wasson et al., 2006), whereas shoot-to-root auxin transport is correlated with nodule numbers (van Noorden et al., 2006). Acropetal polar auxin transport capacity at the root tip, as measured in this study (Fig. 5), is not directly related to nodule numbers: indeed, CHS-silenced roots show increased auxin transport but are not able to nodulate, whereas roots of the supermodulating mutant sunn have wild-type root auxin transport rates (van Noorden et al., 2006; Wasson et al., 2006). The ethylene-insensitive mutant skl is supermodulating and also shows decreased auxin transport rates in the root tips (Prayitno et al., 2006).

Figure 5. cra1 roots show changes in root flavonoid patterns correlated with a decreased polar auxin transport capacity and auxin sensitivity. A, Quantification of nonconjugated formononetin in wild-type (WT; 108-R4) and cra1 3-month-old roots (n = 3 replicates with five plants each). B, Effect of formononetin (2 μM) on polar auxin transport in wild-type (108-R4) 4-d-old roots (n > 15). C, Quantification of polar auxin transport in root apices of wild-type (108-R4) and cra1 5-d-old seedlings (n > 23). D, Impact of reducing flavonoid content using a CHS RNAi construct in wild-type (108-R4) and cra1 roots on polar auxin transport (n > 15). E, Effect of formononetin (2 μM) on wild-type (108-R4) root length and width (at 15 mm from the tip) after 10 d (n > 20). Results for treatments with 0.2 and 10 μM formononetin were similar to the ones shown here. F, Root growth of 4-d-old wild-type (108-R4) and cra1 seedlings after transfer onto auxin (IAA; 10^{-7} or 10^{-8} M) for 10 d. Length of root segments grown on auxin was measured (n > 20) and normalized for each genotype to the nontreated condition. In A to C, E, and F, a Mann-Whitney test was used to determine significant differences (*p < 0.05, **p < 0.01); in D, a Kruskal-Wallis test was used to determine significant differences relative to the wild-type control (*p < 0.05). In all cases, error bars represent confidence intervals (p < 5%).
leading to the observed phenotypes. This study also underlines that strategies to improve digestibility (notably for forage legumes such as *Medicago* spp.) or industrial uses of plant biomass through changes in lignin content and/or structure also have to consider the deleterious developmental side effects induced by lignin manipulation.

**MATERIALS AND METHODS**

**Plant Material, Grafting, and Generation of Composite Plants**

*Medicago truncatula* wild-type 108-R4 genotype, described by Trinh et al. (1998), and the ctn1 mutant (originally called Ms6 in the T-DNA mutant collection; Scholte et al., 2002) were used in this study. Seeds were scarified on sandpaper, sterilized in 6% (w/v) hypochlorite for 10 min, and thoroughly rinsed in sterile water. Seeds were stratified at 4°C for 5 days and then germinated at 25°C in the dark on inverteat water agar plates. Seedlings were grown in vitro in a growth chamber at 25°C, with a 16-h light period at 150 μE light intensity on Fähræus without nitrogen or “i” medium (Gonzalez-Rizzo et al., 2006) depending on experiments. Alternatively, plants were grown in a greenhouse 

(25°C, 16-h light period, 60%–70% humidity) in pots containing a perite: sand mixture (4:1) watered every 2 days with Fähræus or “i” medium depending on experiments. Nodulation was done in the greenhouse using the Sinorhizobium meliloti 1021 strain. For flavonoid treatment, seedlings were grown on nitrogen-free Fähræus medium for 8 days or without formononetin at 0.2, 0.2, or 10 μM. The root tip was marked on day 1 at the back of the plate. Root length was measured at 1, 4, 8, and 10 d after transfer to Fähræus medium. Root width was measured in 10-d-old plants at 1.5 cm from the root tip with a light microscope using a graticule. For auxin sensitivity assays, roots were in vitro grown onto growth papers (Mega International), placed on “i” medium for 4 d, and then transferred on indole acetic acid (IAA; Sigma-Aldrich) at 10^-5 or 10^-8 M. The position of root tips was marked at the time of transfer, and root growth from this point was measured after 10 d using ImageJ software (available at http://rsweb.nih.gov/ij/). Three independent biological replicates were performed for each experiment.

Graftings were performed as described in the “cuttings and grafts” chapter of the Medicago handbook (http://www.noble.org/medicagohandbook/). Grafts were initially generated in vitro, and after 3 weeks they were transferred into the greenhouse in pots containing a perlite:sand mixture (4:1) and watered every 2 d with Faăræus or “i” medium depending on experiments. Nodulation was done in the greenhouse using the Sinorhizobium meliloti 1021 strain. Flavonoid treatment, seedlings were grown on nitrogen-free Fähræus medium for 8 d or without formononetin at 0.2, 0.2, or 10 μM. The root tip was marked on day 1 at the back of the plate. Root length was measured at 1, 4, 8, and 10 d after transfer to Fähræus medium. Root width was measured in 10-d-old plants at 1.5 cm from the root tip with a light microscope using a graticule. For auxin sensitivity assays, roots were in vitro grown onto growth papers (Mega International), placed on “i” medium for 4 d, and then transferred on indole acetic acid (IAA; Sigma-Aldrich) at 10^-5 or 10^-8 M. The position of root tips was marked at the time of transfer, and root growth from this point was measured after 10 d using ImageJ software (available at http://rsweb.nih.gov/ij/). Three independent biological replicates were performed for each experiment.

Graftings were performed as described in the “cuttings and grafts” chapter of the Medicago handbook (http://www.noble.org/medicagohandbook/). Grafts were initially generated in vitro, and after 3 weeks they were transferred into the greenhouse in pots containing a perlite:sand mixture (4:1) for an additional 5 weeks. The root system and aerial parts were then separated individually, dried at 60°C for 48 h, and used to determine dry weight.

For composite plants, a CHS RNAI construct, which silences all known copies of CHS in *M. truncatula* and leads to the formation of transgenic roots with almost undetectable levels of flavonoids (Wasson et al., 2006), was introduced into the Agrobacterium *rhzogenes* A4TC24 strain and used for *M. truncatula* 108-R4 and ctn1 root transformation following the protocol of Boisson-Dernier et al. (2001). The empty vector served as a negative control.

Two weeks after inoculation with *A. rhizogenes*, composite plants were transferred to nitrogen-free Fähræus medium for an additional 3 weeks in a growth chamber at 25°C with a 16-h day at approximately 100 μE and 60% relative humidity. Roots were screened for the presence (empty vector control) or absence (CHS RNAI-transformed roots) of flavonoids by viewing roots with a fluorescent microscope (Leica DMLB) using a UV light excitation filter (excitation maximum at 365 nm, 425-nm long-pass filter) as described (Wasson et al., 2006). Flavonoid-deficient roots (and flavonoid-containing roots as controls) were selected and excised for auxin transport assays as described below.

**Microscopy**

Roots were cut into 5-mm segments and immediately embedded in 3% DNA-grade agarose (Progen Biosciences). They were then cross-sectioned at 150 μm thickness on a vibratome (1000plus; Vibratome Company). Sections were transferred to glass slides, kept under a coverslip in distilled water, and viewed immediately with an epifluorescence microscope (Leica DMLB) using a UV light excitation filter (excitation maximum at 365 nm, 425-nm long-pass filter). Root and cell length measurements were done using ImageJ software. Toluidine blue staining was done by incubating sections for 5 min in 0.5% toluidine blue (pH 4.4), washing sections with distilled water, and viewing under bright-field illumination. Images were taken with a mounted CCD camera (RT Slider; Diagnostic Instruments).

Scanning electron microscopy was performed on Hitachi S-3000N equipment directly on fresh leaves and roots.

**Hybridization and Statistical Analysis of Mt16K+ Microarrays**

Two independent biological replicates of wild-type (108-R4) and ctn 1 d-old seedlings grown in vitro (“i” medium) were performed. Two micrograms of total RNAs from each sample was isolated with Trizol reagent (Invitrogen), treated with DNase (RQ1 RNase-free DNase; Promega), and purified using the RNeasy Mini Kit (Qiagen) following the manufacturer’s instructions. RNA quality was controlled using a Bioanalyzer 2100 (Agilent), and RNA was quantified using a NanoDrop 1000 (LabTech) spectrophotometer. Purified RNAs were used to synthesize Cy5/Cy3-labeled cDNAs using the Amino Allyl MessageAmp rRNA Kit (Ambion) according to the manufacturer’s instructions. Cy5/Cy3-labeled cDNAs were hybridized with the 70-mer Mt16K+ oligonucleotide microarrays (http://www.ebi.ac.uk/arrayexpress; accession no. A-MEXP-138) for 16 h at 60°C.

Microarray slides were scanned with a Genepix two-laser scanner (Axon Instruments), and the resulting images were analyzed with GenePix 6.0 software (Molecular Devices). Data transformations and normalization, performed with the MAtrix R script (version 1.0; Marisa et al., 2007), consisted of a local background correction, omitting flagged spots, and successively an intensity-dependent print-tip Loess normalization and a scale between of array normalization (Yang et al., 2002). Differential analysis was based on an empirical Bayes moderated t test adjusted with the false discovery rate (Benjamini and Hochberg, 1995) multiple test correction. Differentially expressed genes were defined based on thresholds (Supplemental Table S1) on adjusted P values, fold change, and mean intensity of the two channels (A-mean > 7 on a log scale; threshold defined using negative controls available on the microarray).

Data were visualized using MapMan software (downloadable at http://gabi.rzpd.de/projects/MapMan/; Thimm et al., 2004; Tellström et al., 2007).

**Lignin Quantification**

Lignin content analysis was performed on air-dried defoliated stems or roots of 2-month-old plants by thioacidolysis as described by Hoffmann et al. (2004). Quantification of the main H, G, and S lignin-derived monomers, analyzed as their trimethylsilylated derivatives, was performed from specific ion chromatograms reconstructed at mass-to-charge ratio (m/z) of 239 for H monomers, m/z 269 for G monomers, and m/z 299 for S monomers after an appropriate calibration relative to the docosane internal standard.

**Flavonoid Extraction and Quantification**

Three-month-old pot-grown roots were excised, weighed, and ground in liquid nitrogen. For each genotype, three batches of plants were extracted, containing root systems of five plants each. For every 100 mg fresh weight, 1 mL of 80% methanol was added and flavonoids were extracted overnight (approximately 16 h) at room temperature (20°C) in the dark on a rotating wheel. Tubes were centrifuged at 14,000 rpm on a bench centrifuge at room temperature for 30 min. The supernatant was taken into a new tube, separated into two equal samples, and evaporated in a Speedy-Vac. One-half of the sample was redissolved in 45% methanol and separated by HPLC. The other half of each sample was deacetylated by redissolving the pellets in 2 N HCl and heating to 80°C for 1 h. Flavonoid aglycones were extracted by shaking in an appropriate volume of ethyl acetate, and the ethyl acetate fraction was evaporated in a Speedy-Vac. The pellets were redissolved in 45% methanol at 1 mL^-1 fresh weight. Flavonoids were separated on a Shimadzu LC-10 VP series HPLC device, equipped with a diode array UV/visible light detector and a fluorescence detector, using an Altima C18 5 μm reverse-phase column (250 × 4 mm; Alltech Associates). Solvents were MilliQ water + 0.1% acetic acid (A) and acetonitrile + 0.1% acetic acid (B). Separation was over 70 min, as follows: 0 to 5 min, 100% A; 5 to 70 min, 0% to 80% B. Absorbance was recorded between 190 and 700 nm, and fluorescence emission was detected at 450 nm (excitation at 365 nm). To identify the separated flavonoid aglycones, a number of flavonoid standards were separated under the same conditions,
including 7,4-di-hydroxy-flavone, ononin, liquiritigenin, isoorientin (in-
dotine Chemicals), formononetin, kaempferol (Fluka Chemie), and medi-
carpin (Sequoia Research Products), as described by Wasson et al. (2006).

Auxin Transport Measurements

To compare wild-type (108-R4) and cra1 genotypes, 4- to 5-old seedlings
grown on Fähraeus medium under sterile conditions were used. Alternatively,
to measure auxin transport in roots of composite plants, transformed plantlets
were grown for a total of 5 weeks, the last 3 weeks on Fähraeus medium. To
test the effect of the flavonoids isoorientin, formononetin, and media-
carpin on auxin transport, wild-type seedlings were grown for 3 d on
Fähräeus medium and then transferred onto the same medium containing 2
μM of each flavonoid. Each flavonoid was dissolved in methanol at 1 mM and
then diluted 1:500 into warm Fähraeus medium. As a control, an equivalent
amount of methanol was used. Seedlings were left on these plates for 18 h
before measurement of acropetal (from the base to the root tip) auxin transport
as described below. During the 6-h period of auxin transport measurements,
roots remained on the flavonoid-containing medium.

A solution of [14H]IAA (7.5 μL of 1 mCi mL−1 [i.e. a specific activity of 23 Ci
mmol]−1: Amersham Biosciences) was diluted in 30 μL of ethanol and mixed
into 1.5 mL of melted and cooled 1% agarose medium, approximately pH
4.8, in a sterile petri dish. Once solid, the agar was cut into two 2-× 2-× 2-mm3
“donor blocks” containing approximately 250 nM auxin or 2 pmol per block,
similar to the amounts used in transport assays in Arabidopsis (Lewis and
Muday, 2009). Of these 2 pg, less than 10% is taken up by the root. Compared
with approximately 150 pg of auxin present in a 16-mm root segment of
M. truncatula seedlings (van Noorden et al., 2006), the added auxin constitutes
0.13% of the internal auxin. Plant roots of seedlings/composite plants were cut
16 mm/10 mm from the tip, respectively. The roots were then laid on plates
containing Fähräeus medium, with the basipetal (cut) end in contact with a
donor block and separated from the medium by a strip of Parafilm (to prevent
diffusion of the [14H]IAA through the agar). The plates were placed vertically
in a box and covered with aluminum foil. They were incubated at 20°C for 6 h.
For seedlings, three 4-mm segments were cut, after removal of the top 4 mm of
root in contact with the donor block, and each was placed into a scintillation
vial containing 4.5 mL of scintillation fluid (Perkin-Elmer). This allowed
monitoring the speed as well as the quantity of auxin transported. As we did
not detect any changes in the speed of auxin transport in any of the treatments,
but only of the quantity of auxin transported, we added the amounts of
radiolabeled auxin quantified in each of the segments to generate an overall
score. For auxin transport measurements in the shorter roots of composite
plants, only one 4-mm segment was collected, which was located between 4
and 8 mm away (acropetal) from the donor block. In all cases, vials were
shaken overnight and radioactivity was counted in a scintillation counter
(Beckman Coulter LS6500) over 3 min.

Supplemental Data

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

Supplemental Figure S1. Simplified diagram showing the relationship
between the flavonoid and lignin biosynthesis pathways.

Supplemental Figure S2. MapMan representation of the lignin metabolic
pathway.

Supplemental Figure S3. Quantification of lignin-derived H, G, and S
thioacidolysis monomers recovered from wild-type (108-R4) and cra1
shoots.

Supplemental Figure S4. Quantification of flavonoids in M. truncatula
roots, and effects of flavonoid aglycones on auxin transport.

Supplemental Table S1. Summary of differentially expressed genes in
cra1 versus wild-type (108-R4) 1-week-old in vitro-grown seedlings.

Supplemental Table S2. Detailed list of differentially regulated genes
between the wild type (108-R4) and cra1.

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