The Arabidopsis *TUMOR PRONE5* Gene Encodes an Acetylornithine Aminotransferase Required for Arginine Biosynthesis and Root Meristem Maintenance in Blue Light[1][C][W][OA]

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Arginine is an essential amino acid necessary for protein synthesis and is also a nitrogen storage compound. The genes encoding the enzymes of arginine biosynthesis in plants are not well characterized and have mainly been predicted from homologies to bacterial and fungal genes. We report the cloning and characterization of the *TUMOR PRONE5* (*TUP5*) gene of Arabidopsis (*Arabidopsis thaliana*) encoding an acetylornithine aminotransferase (ACOAT), catalyzing the fourth step of arginine biosynthesis. The free arginine content was strongly reduced in the chemically induced recessive mutant *tup5-1*, root growth was restored by supplementation with arginine and its metabolic precursors, and a yeast (*Saccharomyces cerevisiae*) ACOAT mutant was complemented by *TUP5*. Two null alleles of *TUP5* caused a reduced viability of gametes and embryo lethality, possibly caused by insufficient Arg supply from maternal tissue. *TUP5* expression is positively regulated by light, and a TUP5-green fluorescent protein was localized in chloroplasts. *tup5-1* has a unique light-dependent short root phenotype. Roots of light-grown *tup5-1* seedlings switch from indeterminate growth to determinate growth with arresting cell production and an exhausted root apical meristem. The inhibitory activity was specific for blue light, and the inhibiting light was perceived by the root. Thus, *tup5-1* reveals a novel role of amino acids and blue light in regulating root meristem function.

Arg is one of the 20 standard amino acids necessary for the biosynthesis of peptides and proteins. Therefore, it is vital for the functioning of the cell. Arg is a basic amino acid with a very low carbon:nitrogen ratio (6:4), which makes it ideal for nitrogen storage (Llácer et al., 2008). Arg can represent a significant part of the stored nitrogen in protein or as free amino acid in seeds, bulbs, or other parts of plants (Micallef and Shelp, 1989). Arg can account for 11% of the nitrogen pool in Arabidopsis (*Arabidopsis thaliana*) seeds (VanEtten et al., 1963; Zonia et al., 1995). Besides this, Arg is a precursor of compounds acting in developmental processes. Together with Orn, it is used for the production of polyamines (Slocum, 2005). Furthermore, it is a source of nitric oxide, which plays an important role in germination, defense responses, hormonal signaling, root growth, and flowering (Crawford, 2006; Grün et al., 2006). Taken together, Arg and its metabolism are of central importance in plant biology, but the genes and enzymes of Arg biosynthesis are only known partially.

Arg biosynthesis has been well studied in bacteria and fungi (Cunin et al., 1986; Davis, 1986). The enzymes of plant Arg biosynthesis have partly been characterized biochemically (Shargool et al., 1988), but little is known about the genes encoding these enzymes (Slocum, 2005). First, Orn is synthesized from Glu in bacteria, fungi, and plants in a highly conserved Orn pathway not found in most animals. Arg is then synthesized from Orn by the enzymes of the urea cycle in the Arg pathway (Slocum, 2005). Orn biosynthesis (see the overview in Supplemental Fig. S2) starts with Glu, which, in a first step, is acetylated by the *N*-acetyl-Glu synthase or by N^2-acetyl-Orn:Glu acetyltransferase to form *N*-acetyl-Glu, which is then phosphorylated in a second step by *N*-acetyl-Glu kinase to produce *N*-acetyl-Glu-5-P. The next step consists of the formation of *N*-acetyl-Glu-5-semialdehyde catalyzed by *N*-acetyl-Glu-5-P reductase. In the fourth step, an amino group is transferred from a Glu molecule to *N*-acetyl-Glu-5-semialdehyde by N^2-acetyl-Orn aminotransferase (ACOAT), yielding N^2-acetyl-Orn. Subsequently, the acetyl residue is either removed by N^2-acetyl-Orn:Glu acetyltransferase in the cyclic pathway found in nonenteric bacteria, fungi, and plants (Shargool et al., 1988) or by

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a putative N^2-acetyl-Orn deacetylase in the linear pathway found in enteric bacteria to obtain Orn (Slocum, 2005). In the sixth step, Orn transcarbamylase (OTC) catalyzes the transfer of a carbamoyl residue to Orn, yielding citrulline (Cit). Argininosuccinate is then formed from Cit and Asp by argininosuccinate synthase. In the last step, fumarate is cleaved from argininosuccinate by argininosuccinate lyase, yielding Arg (Slocum, 2005).

Only four Arabidopsis genes that encode three enzymatic steps of Arg biosynthesis have been studied by analyzing mutants or by testing their function biochemically. *N*-acetyl-Glu kinase activity was shown for At3g57560 (Chen et al., 2006). *ven3* and *ven6* carry mutations in the large and small subunits of the carbamoyl phosphate synthetase, respectively (Mollá-Morales et al., 2011). Carbamoyl phosphate and Orn are substrates of OTC that produce Cit (Slocum et al., 2000). A transfer DNA (T-DNA) insertion in the *OTC* gene caused an increased sensitivity to Orn (Quesada et al., 1999).

Not much is known about the putative functions of amino acids beyond their role as building blocks of proteins and storage molecules, and only a few reports indicate a role for them in plant growth and development. One such example is the characterization of a Trp biosynthesis mutant displaying a severe growth defect under high light by Last et al. (1991); another one is the description of a Lys biosynthesis mutant showing altered leaf morphology and mild dwarfism by Song et al. (2004). Amino acid metabolism might also influence root meristem maintenance, as well as root growth and development, as was indicated by mutants of Asp and His biosynthesis (Miesak and Coruzzi, 2002; Mo et al., 2006). It is well documented that endogenous factors controlling root growth, such as phytohormones, are often regulated by exogenous signals, for example, by water and nutrient conditions (López-Bucio et al., 2003; Monshausen and Gilroy, 2009), as well as light (Canamero et al., 2006; Tong et al., 2008). There is evidence that roots contain different photoreceptors, that they are able to perceive light of different wavelengths, and that this regulates root functions (Sakamoto and Briggs, 2002; Canamero et al., 2006; Galen et al., 2007; Tong et al., 2008; Costigan et al., 2011; Dyachok et al., 2011, and references therein). However, a connection between light perception and the control of root development involving amino acid homeostasis as an influencing factor has not yet been reported.

In this article, we describe the characterization of the mutant *tumor prone5-1* (*tup5-1*), which was initially identified in a screen for mutants responding to low concentrations of auxin and cytokinin by callus formation (Riefler, 2001). We found that *tup5-1* carries a mutation in the Arabidopsis *ACOAT* gene. The free Arg content was consistently reduced in *tup5-1* compared with the wild type, and root growth was restored in the mutant by supplementation with Arg. An interesting feature of the *tup5-1* mutant was a blue light-dependent defect in root meristem maintenance, the inhibiting light being perceived by the root. Our results showed that *TUP5* has a crucial role in plant development and that it connects primary metabolism, plant development, and blue light signaling.

RESULTS

The Mutant *tup5-1*

Initially, *tup5-1* was found in a screen for mutants that react with higher sensitivity than the wild type to the phytohormones auxin and cytokinin by the formation of tumorous tissue. The recessive ethyl methanesulfonate mutant *tup5-1* formed calli on hypocotyl and root explants when grown on medium containing low concentrations of the auxin α-naphthylacetic acid and the cytokinin isopentenyl adenine, which were not effective in the wild type (Fig. 1A; Riefler, 2001). Mutant seedlings had a very short root (no longer than 1–2 mm) when grown in vitro in light, and growth-arrested root primordia formed callus-like structures at the hypocotyl/root junction (Fig. 1, B and C; Riefler, 2001; Frémont, 2004). The shoot of in vitro-grown *tup5-1* plants was also impaired, probably due to the root growth arrest (Fig. 1B). In contrast, the shoots of soil-grown *tup5-1* mutants were morphologically similar to the wild type, flowered only approximately 2 d later and formed a normal root system (Fig. 1, D and E). This result indicated a potential role of light in inducing root growth inhibition in the *tup5-1* mutants, which will be explored further below.

TUP5 Encodes a Putative Acetyl-Orn Aminotransferase

The *tup5-1* mutation was located on the lower arm of chromosome I close to the telomere by marker-assisted gene mapping (Fig. 2A). No recombinants were found to further reduce the mapping interval of 88 kb containing 29 predicted genes. Therefore, 4- to 22-kb large subclones containing one to five predicted genes were generated from the two BAC clones T21F11 and F23A5 covering the *tup5-1* mapping interval. These subclones were then transformed into *tup5-1* plants to screen for root phenotype complementation. Three complementing BAC subclones contained only one predicted gene, At1g80600, in their common region. The *tup5-1* allele showed as a single point mutation, a transition from G to A in the third exon of At1g80600 at base number 1735 of the unspliced sequence, leading to an altered amino acid sequence of the predicted protein (G424R; Fig. 2, A and B). Transformation of *tup5-1* plants with the complementary DNA (cDNA) of *TUP5* under control of the 35S promoter complemented the root phenotype (Fig. 2C), demonstrating that the mutation in At1g80600 caused the *tup5-1* phenotype.

Protein sequence analysis identified *TUP5* as a putative ACOAT (EC 2.6.1.11.). The protein is predicted to have a length of 457 amino acids and a M_r of 48.8. The enzyme catalyzes the transfer of an amino group

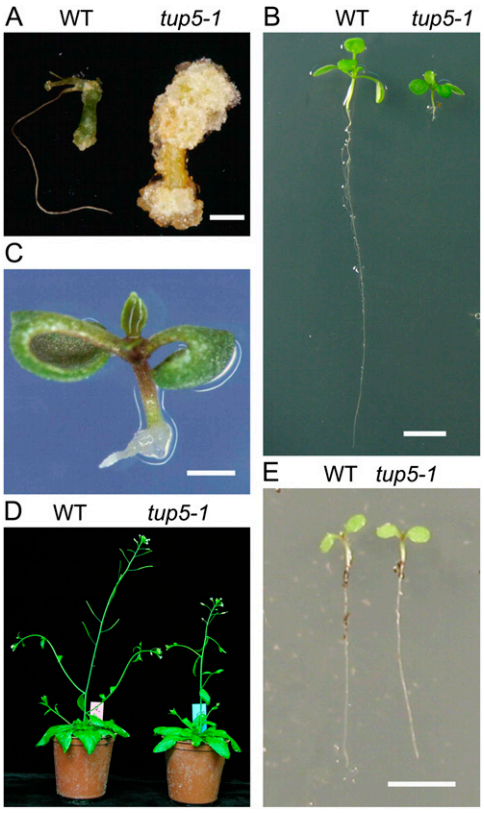

Figure 1. Root growth is arrested in *tup5-1* when grown in vitro, but *tup5-1* develops normally on soil. A, Hypocotyl explants of *tup5-1* formed callus tissue when cultivated on medium containing low concentrations of α-naphthylacetic acid and isopentenyl Ade, while wild-type explants did not (explants after 6 weeks on medium). B, Root phenotype of 12-d-old wild-type and *tup5-1* plants cultivated in vitro under standard light conditions. C, *tup5-1* seedling (10 d old) grown in vitro displayed a short primary root and formed adventitious root primordia in a tumorous fashion. D, Shoot phenotype of soil-grown *tup5-1* plants compared with the wild type. E, Roots of 5-d-old *tup5-1* seedlings developed normally in soil. Bars = 0.2 mm (A), 5 mm (B) and (E), 1 mm (C). WT, Wild type.

from L-Glu to acetyl-L-Glu 5-semialdehyde, yielding α-ketoglutarate and acetyl-Orn (www.expasy.org/enzyme/2.6.1.11; Fig. 3A), using pyridoxal 5-P as a cofactor (Albrecht and Vogel, 1964). The synthesis of acetyl-Orn is the fourth step (from Glu) in de novo Arg biosynthesis (Slocum, 2005; Fig. 3B). Proteins with high sequence similarity to TUP5 are encoded by bacteria, fungi, and plants. A sequence alignment with different known and putative ACOATs showed that the amino acid mutated in *tup5-1* (G424) was conserved in all enzymes from bacteria to higher plants, indicating that it is functionally relevant (Supplemental Fig. S1A). However, in silico analyses using different structure prediction programs have not been informative about the possible impact of the mutation on protein structure and catalytic activity (data not shown). The phylogenetic relationships of the seven aligned proteins correspond to the relationships between the source organisms. TUP5 of Arabidopsis is closely related to the *Populus trichocarpa* protein and farthest related to the bacterial and fungal ACOAT (Supplemental Fig. S1B).

The Root Phenotype of *tup5-1* Can Be Complemented by Exogenous Supplementation of Arg

tup5-1 is probably impaired in the biosynthesis of the amino acids that are produced downstream of the ACOAT enzymatic reaction in the Arg biosynthetic pathway (Fig. 3B; Slocum, 2005). Consistent with this assumption, it has been possible to complement the short root phenotype of *tup5-1* by supplementation of the medium with acetyl-Orn, L-Orn, L-Cit, and L-Arg (Fig. 3, B and C; data not shown). A number of other metabolites linked to Arg biosynthesis, including different polyamines and the nitric oxide donor sodium nitroprusside, were not effective in restoring the *tup5-1* root phenotype, indicating that the mutant phenotype is specifically linked to Arg biosynthesis (Supplemental Fig. S2).

Arabidopsis *TUP5* Rescues an ACOAT-Deficient Yeast Mutant

To test whether TUP5 has the predicted function as an ACOAT, we examined its ability to restore Arg autotrophy in the ACOAT-deficient yeast (*Saccharomyces cerevisiae*) mutant strain Y37711. For this purpose, the genomic Arabidopsis *TUP5* sequence and the *TUP5* cDNA were cloned downstream of the strong constitutive promoter of the *translation elongation factor1α* (*TEF*) gene in yeast vector p423TEF (Mumberg et al., 1995). The yeast mutant transformed with the empty vector p423TEF or the same vector containing the genomic *TUP5* sequence were unable to grow on medium lacking Arg (Fig. 3D). In contrast, the yeast mutant Y37711 expressing the *TUP5* cDNA became Arg autotrophic (Fig. 3D), thus confirming the function of TUP5 as an ACOAT.

tup5-1 Is Arg Deficient and Shows a General Deregulation of Amino Acid Metabolism

We measured the content of Arg and other amino acids and compared it with the wild-type content to analyze the consequences of the apparent block in Arg biosynthesis in the *tup5-1* mutant. The content of free Arg in *tup5-1* seedlings grown in vitro under long-day conditions was lowered to 31% of that of the wild type (Fig. 4A). Additional measurements in seedlings of different age (7, 11, and 17 d old) consistently showed a reduction of Arg content between 61% and 85% in *tup5-1* (data not shown). In contrast, no difference in Arg content was found between *tup5-1* and the wild type grown in darkness. Dark-grown seedlings of both genotypes showed a strong decrease of their Arg content (Fig. 4A).

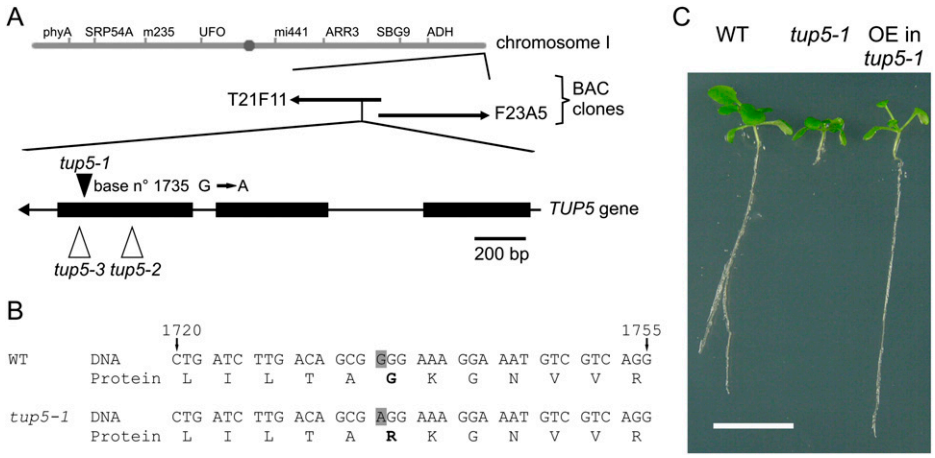

Figure 2. Identification of the *tup5-1* mutation and complementation of the phenotype. A, Genetic map position of the *TUP5* gene on chromosome I and scheme of the *TUP5* gene. The genomic sequence of *TUP5* is composed of three exons (indicated as black boxes) and two introns (shown as lines between exons). The locus of the *tup5-1* mutation is marked with a black arrowhead, and the positions of the two T-DNA insertions *tup5-2* and *tup5-3* are shown as white arrowheads. B, Nucleotide and amino acid sequences neighboring the *tup5-1* mutation. The *tup5-1* mutation (gray shade) is a G-to-A transition at base number 1735 of the genomic sequence leading to a Gly-to-Arg (G424R) exchange (marked in bold letters). C, Overexpression of *TUP5* complements the root phenotype of *tup5-1*. From left to right: wild-type, *tup5-1*, *P35S*:*TUP5* transgenic seedlings in *tup5-1* background grown in vitro (12 d old). Bar = 1 cm. WT, Wild type. [See online article for color version of this figure.]

The distribution of free Arg between root and shoot was unequal. Wild-type roots contained about 50 times less Arg than wild-type shoots (61 ± 11 compared with 3084 ± 673 nmol g^{-1} fresh weight; Fig. 4B). This was the most extreme difference in shoot/root distribution of all amino acids analyzed (Supplemental Fig. S3). The Arg content of *tup5-1* shoots was approximately 39% of that of wild-type shoots (1214 ± 252 compared with 3084 ± 673 nmol g^{-1} fresh weight), which is similar to the difference found in whole seedlings.

We analyzed the content of 18 different amino acids to explore whether the disruption of Arg biosynthesis also affects the level of other amino acids. Out of these, the level of 13 amino acids was increased in 11-d-old seedlings (Fig. 4C). The strongest increases of up to approximately 800% and 400% compared with the wild type were found for Gly and Gln, respectively. Interestingly, only Asn and Gln were increased in *tup5-1* in 17-d-old seedlings, while the level of all other amino acids was comparable to the wild type, except for Arg, which was again reduced (Supplemental Fig. S4; data not shown).

Subcellular Localization of TUP5

The *TUP5* gene was fused at the 3′ end in frame to a *GFP* gene and positioned downstream of the 35S promoter to determine the subcellular localization of TUP5 (Karimi et al., 2005). The fusion gene complemented the *tup5-1* root phenotype, indicating its functionality (data not shown). The subcellular localization of the TUP5-GFP fusion protein was analyzed by confocal laser scanning microscopy in leaf protoplasts obtained from stably transformed lines in wild-type background. The TUP5-GFP signal colocalized with the autofluorescence of chloroplasts, indicating a chloroplastic localization (Fig. 5). This is in good agreement with the database predictions of TargetP (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al., 2000) and Aramemnon (http://aramemnon.botanik.uni-koeln.de/; Schwacke et al., 2003).

Expression of the *TUP5* Gene

The expression of *TUP5* was examined by quantitative real-time reverse transcription (RT)-PCR in different organs and under different light conditions. *TUP5* was expressed in all organs tested (root, rosette leaves, stem, flowers, and siliques). The highest expression was found in rosette leaves and the lowest in roots (Fig. 6A). The ubiquitous expression of *TUP5* is consistent with the results of microarray analyses found in databases such as the eFP browser and Genevestigator (Winter et al., 2007; Hruz et al., 2008).

The influence of white light on *TUP5* expression was then studied because *tup5-1* shows a light-dependent root phenotype. Seedlings were cultivated in standard conditions and then exposed to light or darkness for 24 h. The expression level of *TUP5* in light-exposed seedlings was 3 times higher than in dark-exposed seedlings (Fig. 6B).

Effects of Changes in *TUP5* Gene Dosage

We analyzed the consequences of lower and higher *TUP5* gene expression to gain a better understanding of the role of *TUP5*. Loss-of-function analysis was

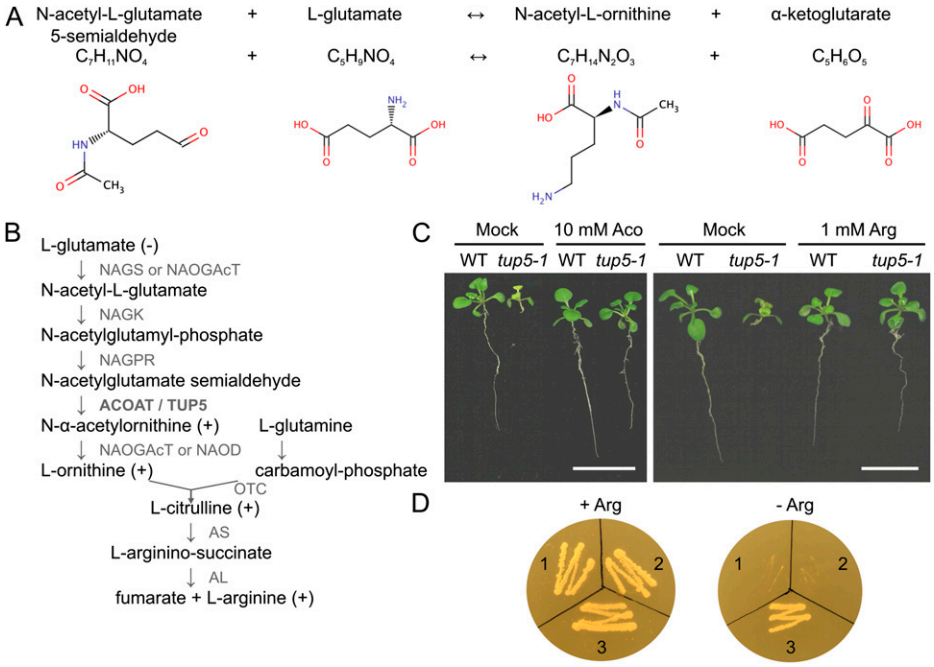

Figure 3. *TUP5* encodes an acetyl-Orn aminotransferase. A, Chemical reaction catalyzed by ACOAT (skeletal formula of compounds from the Human Metabolome Database; http://www.hmdb.ca). B, Arg biosynthesis pathway. *tup5-1* seedlings were supplemented with different compounds of the pathway to test for complementation of the short root phenotype. (−), the substance did not complement; (+), the substance complemented the root phenotype. The enzymes catalyzing the reaction steps of Arg biosynthesis are indicated in gray. NAGS, *N*-acetyl-Glu synthase; NAOGAcT, N^2-acetyl-Orn Glu acetyltransferase; NAGK, *N*-acetyl-Glu kinase; NAGPR, *N*-acetyl-Glu-5-P reductase; NAOD, N^2-acetyl-Orn deacetylase; AS, argininosuccinate synthase; AL, argininosuccinate lyase (modified from Slocum, 2005). C, Normal root development in *tup5-1* when supplemented with acetyl-Orn (Aco) or Arg (14-d-old seedlings in vitro). Bar = 1 cm. WT, Wild type. D, *TUP5* restores Arg autotrophy in the ACOAT-deficient yeast mutant Y37711. Left, transformed yeast on medium containing Arg; right, transformed yeast on medium lacking Arg. Y37711 transformed with 1, the empty yeast expression vector p423TEF; 2, vector containing the genomic Arabidopsis *TUP5* sequence; and 3, vector containing the Arabidopsis *TUP5* cDNA. [See online article for color version of this figure.]

carried out with two T-DNA insertion alleles of the SALK collection named *tup5-2* and *tup5-3* (Alonso et al., 2003). Sequencing confirmed the sites of both insertions within the third exon of the *TUP5* gene (Fig. 2A). F1 populations of heterozygous self-fertilized parents of both lines were screened by PCR for homozygous progeny, but no homozygous line was found among 118 F1 plants tested for *tup5-2* and 62 plants tested for *tup5-3*. This indicated that the homozygous state might be lethal for both alleles. Inspection of cleared siliques of heterozygous *tup5-2* and *tup5-3* plants revealed empty spaces between seeds, indicating premature termination of seed development (Fig. 7A). Microscopy examination showed that these spaces in the siliques contained aborted ovules or very small embryos (Supplemental Fig. S5). The percentage of abortion was about 42% ± 4% in *tup5-2* and 35% ± 7% in *tup5-3*, while in the wild type and *tup5-1*, only 1% to 2% of abortion was found (Supplemental Table S1).

In addition, a meiotic drift was found in the F1 generation of heterozygous self-fertilized *tup5-2* and *tup5-3* lines. According to Mendel's Laws, the ratio between wild-type and heterozygous F1 plants should be 1:2. Only 36% ± 10% of the plants in *tup5-2* and 43% ± 11% in *tup5-3* were heterozygous for mutated *tup5* allele (Supplemental Table S2). All other plants contained the *TUP5/TUP5* allele combination. Because of this strong underrepresentation of the T-DNA insertion alleles in the segregating progeny, the ratio between wild-type and heterozygous F1 plants was 1.7:1 in *tup5-2* progeny and 1.3:1 in *tup5-3* progeny.

Reciprocal crosses of *tup5-2* and *tup5-3* heterozygous plants with the wild type were carried out to find out whether embryo development, pollen, and/or ovule fertility were impaired. PCR analysis of F1 progenies showed that the T-DNA insertion allele was transmitted by both pollen and ovule. However, the transmission frequency in both cases was reduced: Instead of 50%, only approximately 24% to 42% (20 heterozygous plants of 82 plants [24%] in the case of wild type × *tup5-3*, 10 of 24 [42%] for wild type × *tup5-2*, and 18 of 48 plants [37%] in *tup5-3* × wild type) of the F1 generation carried the T-DNA insertion alleles, indicating that the *tup5-2* and *tup5-3* alleles reduce male and female gametophytic fitness leading to meiotic drift. In addition, the low T-DNA allele frequency in the self-fertilized *tup5-2* and *tup5-3* lines and in the reciprocal

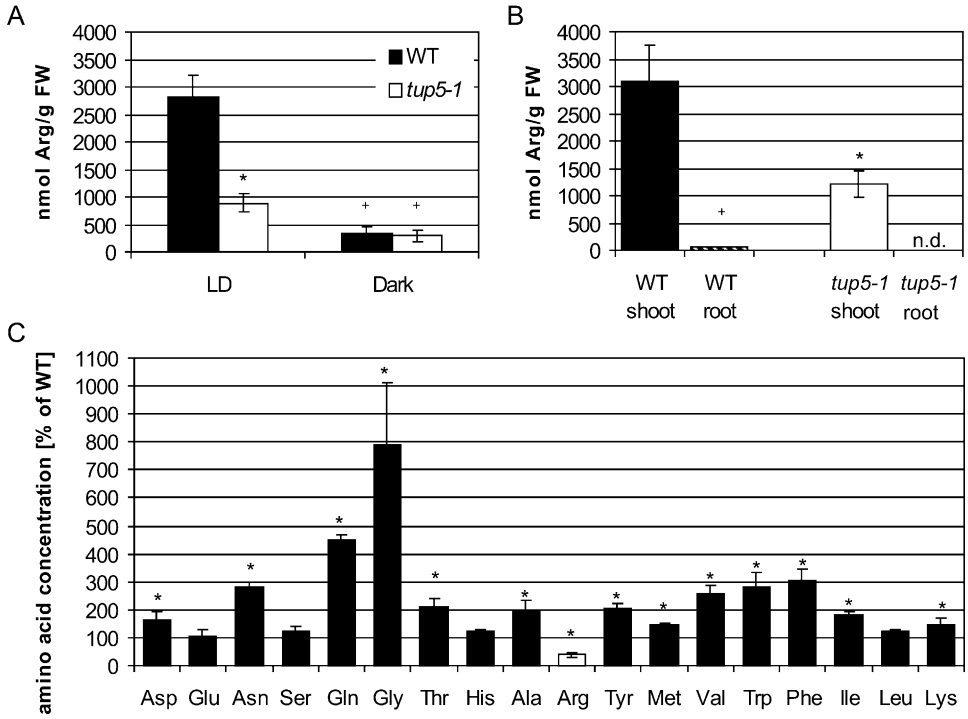

Figure 4. *tup5-1* plants have a reduced Arg content and a generally deregulated amino acid metabolism. A, Free Arg content of wild-type and *tup5-1* seedlings grown in long days (LD) or in darkness. $n = 6$. B, Free Arg content in wild-type shoots, wild-type roots, and *tup5-1* shoots grown in the light. Wild-type shoot, $n = 4$; wild-type root, $n = 3$; *tup5-1* shoot, $n = 4$. C, Free amino acid content of *tup5-1* shoots compared with wild-type shoots. The concentration of each amino acid in *tup5-1* is shown in percentage referring to the values measured in the wild type set as 100%. Arg content is shown as a white column. $n = 4$. Seedlings were 7 d old and grown in one-half-strength MS liquid culture (A) and 11 d old and grown on vertical plates of MS medium (B and C). Each sample was a pool of at least 30 seedlings. Data are shown as mean (±SD). Significant differences (calculated with Student's *t* test) between wild-type and *tup5-1* samples are marked with asterisks ($*P < 0.05$), while significant differences between standard light and other light conditions or tissues within a genotype are marked with a plus sign ($+P < 0.05$). FW, Fresh weight; WT, wild type; n.d., not determined.

crosses suggests an impairment of embryo development even in a heterozygous state. Taken together, null mutation of the *TUP5* gene impairs gametophytic fitness and is lethal at early stages of embryo development.

Plants overexpressing *TUP5* were produced by transformation of the wild type with the *35S*:*TUP5* gene used previously for complementation to test the consequences of increased *TUP5* expression (Fig. 2C). *TUP5* overexpression in the wild type caused two visible morphological changes: interveinal chlorosis in cauline leaves and the formation of wrinkled, bumpy siliques (Fig. 7, B and C). Interestingly, the Arg level was not increased above the wild type in cultivated *35S*:*TUP5* lines in vitro (Fig. 7D).

Influence of Light Conditions on *tup5-1* Root Meristem Development

As described above, the root phenotype of *tup5-1* mutants differed when plants were grown in vitro or on soil (Fig. 1, B, C, and E). The roots of *tup5-1* seedlings grown on Murashige and Skoog (MS) medium in short-day conditions grew longer than the roots of *tup5-1* seedlings cultivated under long-day conditions (data not shown). This indicated that day length, and therefore light, influenced root growth and not a component of the growth medium. The roots of *tup5-1* seedlings of different ages cultivated in darkness consistently immediately stopped growth when they were shifted into light.

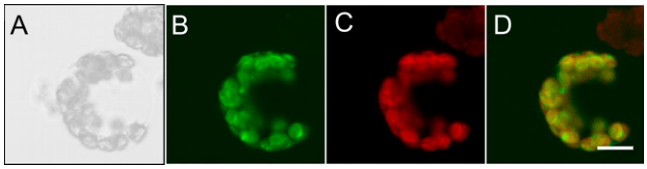

Figure 5. TUP5 is localized in chloroplasts. A, Protoplast of B, C, and D shown in bright field. B, Fluorescence signal of TUP5-GFP. C, Autofluorescence signal of the chloroplasts. D, Merged image of B and C showing an overlap (yellow) of TUP5-GFP and chloroplast signals. Bar = 10 μm. Leaf protoplasts were isolated from stably transformed Arabidopsis plants. Images were taken with a confocal laser scanning microscope.

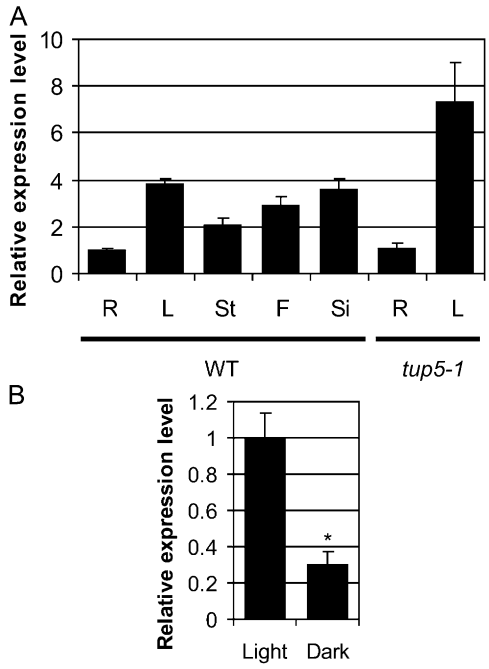

Figure 6. *TUP5* transcript level in different organs and its dependency on light conditions. A, *TUP5* and *tup5-1* expression in different plant organs in wild-type and *tup5-1* mutant plants. The transcript level was measured by quantitative real-time RT-PCR in roots (R) and rosette leaves (L) of 32-d-old plants and in stems (St), flowers (F), and siliques (Si) of 35-d-old plants grown in soil. Wild-type (WT) and *tup5-1* root and rosette leaves were also analyzed. The differences were not significant, as calculated with Student's *t* test. B, Effect of light on *TUP5* expression in Arabidopsis wild-type seedlings. Seedlings were grown in long-day conditions for 4 d and subsequently exposed for 24 h to continuous light or darkness and then harvested. Transcript levels are given as relative values compared with the wild-type root tissue (A) or light-treated wild-type seedlings (B). Relative expression levels were normalized using At *UBC10* (At5g53300) and At3g25800 as reference genes. Significance was calculated with Student's *t* test ($*P < 0.01$).

Microscopy analysis of the root tips of light-grown *tup5-1* plants revealed that the meristem had lost its organized structure, the cells had differentiated, and starch granules were absent in the columella cells (Fig. 8A). In contrast, dark-grown *tup5-1* seedlings had a root meristem similar to that of the wild type (Fig. 8A).

Root growth of *tup5-1* was tested under various monochromatic light qualities to find out whether a specific part of the light spectrum causes this switch from an indeterminate growth to determinate growth. The inhibition of root growth in *tup5-1* was induced by blue light but not by red or far red light (Fig. 8B). The root growth inhibition caused by blue light was dose dependent (Fig. 8C). The root length was not significantly different between *tup5-1* and the wild type up to 0.2 μmol m^{-2} s^{-1}, while a blue light intensity of 0.6 μmol m^{-2} s^{-1} or more inhibited root elongation in *tup5-1*. To test whether a known light signaling pathway is involved in mediating this effect, we combined by crossing *tup5-1* with the following light receptor or light signaling mutants: the phytochrome apoprotein mutant *phyB-1* (*hy3-Bo64*; Reed et al., 1993; Rockwell et al., 2006), the phytochrome chromophore biosynthesis mutant *hy2-1* (Kohchi et al., 2001), the cryptochrome apoprotein mutant *cry1* (*hy4-1*; Ahmad and Cashmore, 1993), and the bZIP transcription factor mutant *hy5-1* (Oyama et al., 1997). Analysis of the double mutants in the F2 generation showed that none of these mutants suppressed the *tup5-1* root phenotype (data not shown). Therefore, the light receptors or light signaling factors analyzed are not epistatic to *tup5-1*.

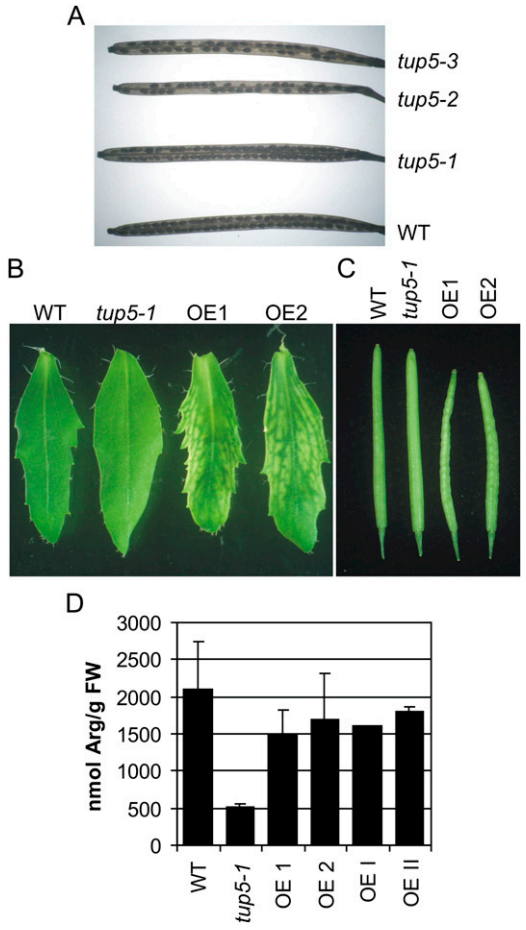

Figure 7. Developmental impairment caused by altered *TUP5* dosage. A, Empty seed positions in siliques of heterozygous *TUP5* T-DNA insertion lines compared with the wild type. Mature siliques were bleached with chloral hydrate. B, Cauline leaves of *TUP5* overexpressing transgenic lines show interveinal chlorosis. C, Siliques of *TUP5* overexpressing transgenic lines are shorter and wrinkled. D, Free Arg content of transgenic lines overexpressing *TUP5* in wild-type and *tup5-1* background compared with the wild type and *tup5-1*. FW, Fresh weight. The plants in B and C were cultivated on soil in a growth chamber under long-day conditions. Plants used for the analysis in D were cultivated in vitro for 17 d. OE1 and OE2, exemplary lines expressing *35S:TUP5* in the wild type; OEI and OEII, exemplary lines expressing *35S:TUP5* in *tup5-1* background. WT, Wild type.

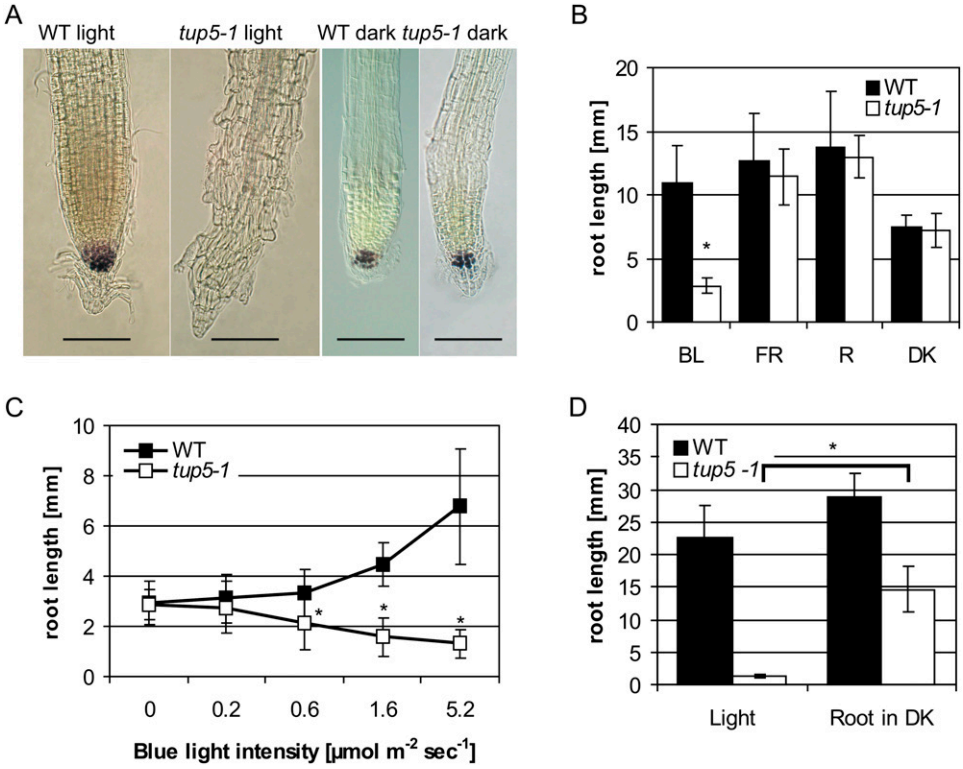

Figure 8. Influence of light on *tup5-1* root development. A, The root meristem of *tup5*-1 disappears in light-grown seedlings but not in dark-grown seedlings. Plants grown in light were 5 d old, and those grown in darkness were 6 d old. Starch granules were stained with Lugol solution. Bars = 100 μm. B, *tup5-1* root growth is specifically inhibited by blue light. BL, blue light (445 nm), approximately 2 μmol m^{-2} s^{-1}; FR, far-red light (724 nm), approximately 3.5 μmol m^{-2} s^{-1}; R, red light (660 nm), 3.5 μmol m^{-2} s^{-1}; DK, darkness. n = 18 to 26, except far-red light for the wild type, n = 5. Plants were treated with white light for 16 h to induce germination and then transferred to constant (24 h) monochromatic light exposure for 5 d. Student's t test was used to compare the wild type and *tup5-1* grown under the same light conditions; significance: $^*P < 0.001$. C, *tup5-1* root growth inhibition depends on the intensity of blue light irradiation. Seedlings were cultivated under various intensities of blue light (445 nm) for 6 d. $^*P < 0.05$ as calculated by Student's t test. D, The root growth inhibiting light is perceived in the root of *tup5-1*. Seedlings were cultivated for 28 d in vitro in light or with the roots kept in darkness. Mean ± SD of four to seven plants. Significance: $^*P < 0.001$. WT, Wild type; DK, darkness. [See online article for color version of this figure.]

Wild-type and *tup5-1* plants were grown in vitro in glass tubes with illumination of the whole plant or illumination of only the shoot to find out whether the perception of the inhibiting light takes place in the shoot or in the root. When grown in light, *tup5-1* exhibited its typical severe root growth defect (Fig. 8D). In contrast, when only the shoots were illuminated, the root length of *tup5-1* increased significantly. Although *tup5-1* roots did not reach the length of wild-type roots, which was probably due to the imperfect darkening of the roots in the experimental setup, it can be concluded that the root itself perceives the inhibiting light signal causing the *tup5-1* root phenotype.

We studied the cell cycle marker gene *CYCB1;1pro*:*GUS-DBox* (Colón-Carmona et al., 1999) and QC25 (Sabatini et al., 1999), which specifically marks the quiescent center (QC), in the *tup5-1* background to follow more closely the light-dependent switch to determinate growth and the subsequent loss of the root meristem. The meristem of *tup5-1* roots grown in vitro in light showed a strongly reduced cell cycle-dependent GUS activity compared with the wild type. A weak signal was visible in only a few cells (Fig. 9A). The QC-dependent GUS activity was reduced in light-grown roots of *tup5-1*. When the meristematic structure was not yet completely lost, a light-blue color showed a decreased GUS activity in the QC cells (Fig. 9B). The shape of the QC cells was also changed in *tup5-1*: While the cells were flattened in the wild-type meristem, they stretched parallel to the root axis in *tup5-1* (Fig. 9B). When the loss of meristematic structure progressed, the GUS activity in the QC of *tup5-1* roots almost vanished (Fig. 9B). As *tup5-1* was found in a screen for callus formation on low concentrations of cytokinin and auxin, we also examined the hormonal status in the differentiated *tup5-1* root meristem using the cytokinin-sensitive marker *ARR5*:*GUS* (Sabatini et al., 1999; D'Agostino et al., 2000) and the auxin reporter *DR5*:*GUS* (Sabatini et al., 1999; D'Agostino et al., 2000). The GUS activity pattern in both cases was much weaker in *tup5-1* than in the wild type or had almost vanished in the mutant root (Fig. 9, C and D). Nevertheless, *DR5*:*GUS*

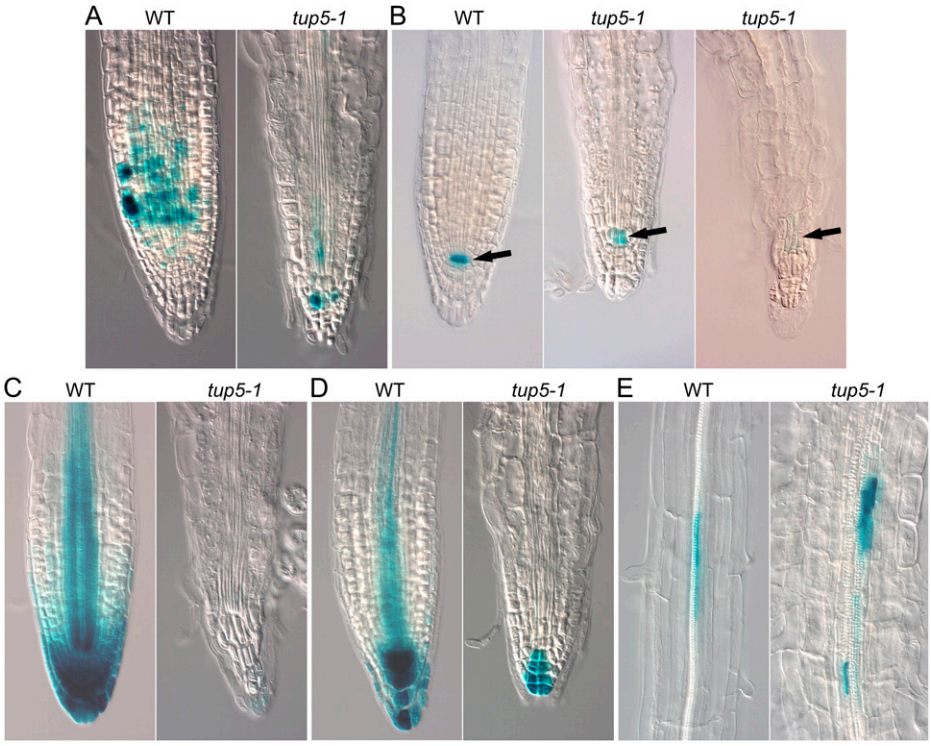

Figure 9. Expression of different marker genes is altered in the *tup5-1* root. A, Cell cycle-dependent GUS staining with the marker *CYCB1;1pro*::*GUS-DBox* is reduced in the primary root tip of *tup5-1*. B, GUS activity of the marker *QC25* in the QC of the root apical meristem in *tup5-1* primary roots decreases during meristem differentiation. Arrows point to the stained QC. C, Staining with the cytokinin-sensitive marker *ARR5*::*GUS* is strongly reduced in the primary root tip of *tup5-1*. D, Auxin-responsive *DR5*::*GUS* expression is decreased in the primary root tip of *tup5-1*. E, Auxin-responsive *DR5*::*GUS* expression indicating early events during the formation of lateral roots in the pericycle of the primary root is also found in *tup5-1*. All seedlings were 5 d old and grown in vitro. Photos were taken at a 200-fold magnification. WT, Wild type.

expression marking in pericycle cells at the beginning of lateral root primordia formation (Benková et al., 2003) was also observed in the short growth-arrested *tup5-1* primary roots (Fig. 9E).

DISCUSSION

TUP5 Encodes a Plastid-Localized Acetyl-Orn Aminotransferase

We showed that the phenotype of *tup5-1* is due to a point mutation in a gene encoding the Arabidopsis ACOAT required for Arg biosynthesis. The function of TUP5 as ACOAT is supported by the findings that *tup5-1* is Arg deficient and that the root phenotype could be complemented by supplementation with acetyl-Orn, Orn, Cit, and Arg, which are all synthesized downstream of ACOAT, while other metabolites did not complement. Additionally, a yeast ACOAT mutant was complemented by heterologous expression of the Arabidopsis *TUP5* gene. A *TUP5-GFP* fusion protein has been detected in chloroplasts. This is consistent with the database prediction of a plastidic localization of TUP5 and biochemical experiments showing acetyl-Orn aminotransferase activity in the plastid fraction of a soybean (*Glycine max*) suspension culture (Jain et al., 1987).

Mutation of *TUP5* Causes a Reduction of Arg Content and Has Pleiotropic Effects on Amino Acid Homeostasis

The Arg content of light-grown *tup5-1* plants was strongly reduced compared with the wild type. Interestingly, the Arg content was reduced in both the wild type and *tup5-1* to a comparable level in darkness. Arg in the wild type showed the most extreme difference in shoot/root distribution of all amino acids analyzed. The root contained 50 times less Arg than the shoot, indicating a fine-tuned and sensitive metabolic control system of Arg content in the root. It could be that further reduction of this already low Arg level causes a slowdown of root growth and, eventually, its complete arrest. Overexpression of *TUP5* in *tup5-1* restored the Arg content to the wild-type level, but overexpression in the wild type did not cause accumulation of higher Arg concentrations, indicating that other biosynthetic steps are rate limiting.

The content of most other amino acids was increased in *tup5-1* mutants, although to a different extent at different developmental stages. The increased content of Asn and Gln, two amino acids that play a major role in nitrogen storage and transport (Lam et al., 1995), may partially compensate for the absence of Arg as a nitrogen sink. Accumulation of surplus amino groups in these storage pools in the *tup5-1* mutant might also be caused by a reduced protein biosynthesis capacity due to the lack of Arg as a rate-limiting compound. The dynamic changes in the concentrations of numerous other amino acids support the notion of cross-pathway regulation of different amino acid biosynthesis pathways in plants. This mechanism has been shown to exist in yeast (Hinnebusch, 1992) and may also cause a general deregulation of free amino acid content in plants if one amino acid is not produced sufficiently (Guyer et al., 1995; Noutoshi et al., 2005). Indeed, a general amino

acid metabolic deregulation with a global increase of free amino acid content was also found in other mutants, for example, in *albino and pale green10*, which is disrupted in His biosynthesis (Noutoshi et al., 2005), *Phe insensitive growth1-1* (Voll et al., 2004), and *Gln dumper1* (Pilot et al., 2004). Alternatively, the increased free amino acid content in *tup5-1* might be due to protein degradation caused by deleterious effects due to the lack of Arg (Noctor et al., 2002). Together, the strong effect of a single partial loss-of-function mutation in the Arg biosynthesis pathway on amino acid homeostasis underpins that a tight regulatory network controls a delicate balance that is not yet fully understood.

TUP5 Is Required for Gametogenesis and Seed Development

Analysis of two *tup5* functional null alleles revealed the absolute requirement of Arg synthesis for proper male and female gametogenesis, as well as early seed development. Meiotic drift, as indicated by reduced transmission frequencies of the *tup5-2* and *tup5-3* insertion alleles in reciprocal crosses with the wild type, is likely due to a dysfunction of ovules and pollen grains carrying this genotype. The reduced fitness of gametes carrying a *TUP5* null allele indicates that they are not exclusively cross-fed from surrounding tissues but need a certain degree of Arg autotrophy (Muralla et al., 2011). However, survival of some gametes also shows that maternal tissue ensures at least limited Arg supply to ovules and pollen grains.

Similarly, it has not been clear so far if Arabidopsis embryos need to be Arg autotrophic or whether their demand can be covered by maternal tissue. The knowledge of how amino acid supply of developing seeds takes place is still incomplete for Arabidopsis (Baud et al., 2008). All amino acids, but mainly Gln, Glu, Asp, Asn, Ser, and Ala, are transported from source tissues of the mother plant to the seed via the phloem (Sanders et al., 2009). The amino acids can be converted to other amino acids in the seed coat (maternal tissue) or in the embryo (Baud et al., 2008; Sanders et al., 2009). Early failure of seed development in *tup5* nulls indicated that these need their own Arg biosynthesis and are not exclusively nourished by the maternal tissue. It is not entirely clear from our analysis which tissue of the developing seed is primarily impaired by the lack of Arg and causes growth arrest, but the embryo is most likely affected. Different amino acid biosynthesis mutants showing embryo lethal phenotypes have been described, including mutants in His (Muralla et al., 2007), Lys (Song et al., 2004; Hudson et al., 2006), and Pro biosynthesis genes (Székely et al., 2008). Meinke (1991) and Bryant et al. (2011) also pointed out that embryonic development is often interrupted in mutants defective in essential housekeeping genes, including those of amino acid biosynthesis.

tup5-1 Mutants Show an Unusual Blue Light-Dependent Root Growth Inhibition

Probably the most intriguing aspect of the *tup5-1* mutant phenotype is the blue light-dependent switch from indeterminate growth of the root to a determinate growth phase. Importantly, the root growth arrest is not simply due to the arrest of cell cycling, but the apical root meristem is completely used up. This implies that central functions relevant for the maintenance of the QC and the surrounding initials are lost and, thus, critically depend on Arg in the presence of light.

We are not aware of any other mutant showing a blue light-dependent inhibition of root growth. This phenotype also distinguishes *tup5-1* from other Arabidopsis mutants impaired in amino acid biosynthesis or metabolism showing defects in root growth, such as the *hpa1* His biosynthesis mutant (Mo et al., 2006), the mutant Rm 57 disturbed in the Lys biosynthetic pathway (Sarrobert et al., 2000), and two mutants of the genes encoding Asp aminotransferase AspAT2, *aat2-2* and *aat2-T* (Schultz et al., 1998; Miesak and Coruzzi, 2002). The His biosynthesis mutant *hpa1* resembles *tup5-1* insofar as it has a short root when grown in vitro, but there is no obvious phenotype in the aerial part and *hpa1* mutants are fully fertile plants (Mo et al., 2006). Mutants for several Trp biosynthetic pathway genes are generally impaired in development under high-light but not under low-light conditions (Last et al., 1991; Radwanski et al., 1996). It has been suggested that the residual activity of the mutant enzyme might be able to cover the lower Trp demand of slow-growing plants in low light (Last et al., 1991). In contrast, the roots and shoots of soil-grown *tup5-1* plants grown under standard light develop normally, though high metabolic activity can be expected under these conditions. This argues for specifically light-induced changes in the root tissue.

One possibility is a light-dependent change in protein stability or catalytic activity of the mutated ACOAT ($TUP5^{G424R}$). The molecular environment of root plastids is presumably different from that of green tissue chloroplasts; therefore, some blue light-regulated proteins might interfere with TUP5 in the illuminated root, or proteins could be lacking due to the influence of light. It could also be hypothesized that shoot Arg production is sufficient even with the mutated protein and that the shoot exports Arg into the root when the latter is grown in dark, but not when grown in light. However, although significantly higher Arg concentrations are present in the shoot compared with the root (Fig. 4B), it is not clear yet whether Arg long-distance transport normally plays an important role in *Arabidopsis*. Glu, Gln, Asp, and Asn were found to be the predominant amino acids in phloem sap and xylem exudates and are therefore presumably the major nitrogen transport molecules (Lam et al., 1995). Other amino acids are assumed to be mainly synthesized from these transport amino acids at the site of requirement (Lea and Miflin, 1980).

The root growth arrest in *tup5-1* is specifically caused by blue light and very low fluence rates are sufficient to trigger the developmental switch. Light is encountered by roots on the soil surface where roots emerge from germinating seeds and in the upper soil layers. Light penetrates several millimeters through soil and also reaches plant roots through the vascular tissue (Tester and Morris, 1987; Kasperbauer and Hunt, 1988; Sun et al., 2003). The very low effective light fluence rates that act on *tup5-1* indicate a physiological relevance and suggest an as yet unknown connection between amino acid biosynthesis, light perception, and root development. Roots contain red and blue light photoreceptors that may perceive light directly (Sakamoto and Briggs, 2002; Galen et al., 2007; Costigan et al., 2011; Dyachok et al., 2011, and references therein) and also respond to UV-B (Tong et al., 2008). Our genetic analyses have largely excluded several known components from light signaling pathways (*CRY1*, *PHYB*, *HY2*, and *HY5*) to operate in the TUP5 pathway, but not all known light receptors that could have a function in light perception by roots have been tested. Other candidate proteins include cry2, though cryptochrome effect on root elongation was perceived in the shoot according to a study of Canamero et al. (2006). Phototropin phot1 was also found to have a role in blue light perception of the root where it mainly regulates directional growth (Galen et al., 2007; Kutschera and Briggs, 2012). phyA can absorb blue light and could thus also be a potential receptor in the signaling pathway regulating TUP5 (Lin, 2000). Intriguingly, recent work has demonstrated that phyA and phot1 interact functionally at the plasma membrane, which further increases the variety of light signaling pathways (Jaedicke et al., 2012). In addition, it cannot be ruled out that an unknown receptor is involved in the light regulation of TUP5.

Interestingly, three mutants linked to UV light perception and light stress have a light-dependent short root phenotype comparable to *tup5-1*. The *rus1* and *rus2* mutants have short roots under UV-B light and the inhibiting light is perceived by the root (Tong et al., 2008; Leasure et al., 2009). *pdx1-3* mutants missing a pyridoxal synthase necessary for the production of the TUP5 cofactor pyridoxal-5′-P show a light-dependent root growth arrest. However, it has not been tested whether the root perceives the inhibiting light and whether a specific wavelength is involved (Titiz et al., 2006; Havaux et al., 2009). Interestingly, a screen for second site suppressor mutants of the *rus* phenotype identified mutations in the *ASP AMINOTRANSFERASE2* gene, and adding pyridoxine (vitamin B_6) to the growth medium of *rus* mutants partly rescued root growth (Leasure et al., 2011). This illustrates that as yet unknown pathways connecting UV-B light sensitivity, vitamin B_6 homeostasis, and amino acid biosynthesis exist.

Our data show that for a complete understanding of root meristem function, essential metabolites, such as Arg, need to be considered in addition to plant hormones and transcription factors that are generally in the focus of root meristem research (Perilli et al., 2012). The relevance of additional poorly considered factors in meristem regulation is corroborated by the recent report that the metabolic cofactor thiamine has an important role during the proliferation of stem and initial cell populations in the shoot meristem (Woodward et al., 2010). The *tup5-1* mutant will be an important tool to investigate the mechanisms that link Arg to known regulators maintaining indeterminate root meristem development.

MATERIALS AND METHODS

Plant Material and Growth Conditions

tup5-1 was isolated from an ethyl methanesulfonate-mutagenized population of Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 *glabra* (Riefler, 2001). The T-DNA insertional mutants *tup5-2* (N878354) and *tup5-3* (N875828) and the light signaling pathway mutants *phyB-1* (N69; Reed et al., 1993; Rockwell et al., 2006), *hy5-1* (N71; Oyama et al., 1997), *cry1* (N70; Ahmad and Cashmore, 1993), and *hy2-1* (N68; Kohchi et al., 2001) were obtained from the Nottingham Arabidopsis Stock Center (http://arabidopsis.info/) and the Arabidopsis Biological Resource Center (http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm). *tup5-1* was combined by crossing with the marker gene lines *CYCB1;1pro:GUS-DBox*, QC25, *ARR5:GUS*, and *DR5:GUS* provided by Peter Doerner, Sabrina Sabatini, and Joe Kieber, respectively (Colón-Carmona et al., 1999; Sabatini et al., 1999; D'Agostino et al., 2000).

Unless stated otherwise, plants were grown at 22°C under long-day conditions (16-h light/8-h dark) on soil in a greenhouse (light intensity: 150–300 μmol m^{-2} s^{-1}; natural light supplemented with high-pressure sodium vapor lamps, type SON-T, 2,000K) or in vitro in a growth chamber (light intensity: 150 μmol m^{-2} s^{-1}). In vitro cultivation was on solid medium containing 1× MS salts (Duchefa Biochemie), 0.1 g L^{-1} myo-inositol, 0.5 g L^{-1} MES, 20 mg L^{-1} thiamine, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine, 1 mg L^{-1} biotin, 3% (w/v) Suc, and 0.9% (w/v) agar, pH 5.7, or in liquid medium containing 0.5× MS salts, 0.5 g L^{-1} MES, and 1% (w/v) Suc, pH 6 (modified from Murashige and Skoog, 1962).

Gene Mapping and BAC Subcloning

Homozygous *tup5-1* plants were crossed with plants of the ecotype Landsberg *erecta*. The mapping was done with approximately 2,300 homozygous mutant plants of the F2 generation, as described by Krupková et al. (2007). No more recombinants were found at a mapping interval of 88 kb. Therefore, DNA fragments of the two BAC clones T21F11 (Choi et al., 1995) and F23A5 (Mozo et al., 1998) covering the *tup5-1* mapping interval were used for complementation and gene identification.

Plasmid Construction for Plant Transformation

To generate subclones from the BAC clones T21F11 and F23A5, these were cut with appropriate restriction enzymes, separated by gel electrophoresis, the appropriate bands purified from the gel using QIAEX II gel extraction kit or QIAquick gel extraction kit (Qiagen), and cloned into vector pCB302 (Xiang et al., 1999). The resulting plasmids were used for transformation of *tup5-1*.

The *35S:TUP5* construct was produced using the *TUP5* cDNA clone U15579 provided by Arabidopsis Biological Resource Center (Yamada et al., 2003). The cDNA was recombined into the vector pB2GW7 (Karimi et al., 2005) with LR clonase (Invitrogen) according to the manufacturer's protocol.

To generate the *P35S:TUP5-GFP* gene fusion, the cDNA of *TUP5* was amplified from clone U15579 using the gene-specific primers 5′-ATGGCGTCTCTTAGCCAAATC-3′ (forward) and 5′-ATCAAGCGCAGTCAAATTTT-3′ (reverse; Yamada et al., 2003). The resulting PCR product was used as a template to add 12 bp of Gateway attachment sites at the 5′ ends with the primers 5′-AAAAAGCAGGCTATGGCGTCTCTTAGCCAAATC-3′ (forward) and 5′-AGAAAGCTGGGTCATCAAGCGCAGTCAAATTTT-3′ (reverse). Finally, the full-length attachment sites were added in a PCR

reaction with the primers 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′ (forward) and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′ (reverse). The resulting PCR product was inserted into the donor vector pDONR221 via recombination to create an entry clone for further use. The resulting entry clone was recombined with pB7FWG2 (Karimi et al., 2005), which inserts the *TUP5* gene between the 35S promoter and the *GFP* gene creating the *P35S:TUP5-GFP* fusion.

The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101:pMP90 (Koncz and Schell, 1986), which was used for Arabidopsis transformation (Clough and Bent, 1998). Transformants were selected on medium containing the appropriate antibiotics.

Heterologous Complementation with *TUP5* of a Yeast Mutant Defective in ACOAT

The yeast (*Saccharomyces cerevisiae*) strain with the accession number Y37711 (genotype: BY4743; Mat a/α; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; YOL140w::kanMX4/YOL140w::kanMX4) in which the ACOAT gene YOL140w (*ARG8*) is deleted was provided by Euroscarf. The genomic sequence and the cDNA of *TUP5* were amplified with the primers 5′-CGGGATCCATGGCGTCTCTTAGCCAAATC-3′ (forward) and 5′-ACGCGTCGACTCAATCAAGCGCAGTCAAAT-3′ (reverse), generating a *Bam*HI restriction site at the 5′ end and a *Sal*I restriction site at the 3′ end for plasmid construction. The PCR products were inserted into the yeast vector p423TEF by directional cloning using the *Bam*HI and *Sal*I sites (Mumberg et al., 1995). The resulting plasmid was transformed into Y37711 by the lithium acetate method (Gietz and Schiestl, 1995; Bürkle et al., 2005). Yeast transformants were first selected for His autotrophy and ampicillin resistance. Transformed yeast strains were grown on synthetic defined medium containing 20 mg Arg L^{-1} or no Arg to test Arg autotrophy.

Gene Expression Analysis

Total RNA was purified from Arabidopsis tissue by the TRIzol method, as described by Brenner et al. (2005). cDNA synthesis, quantitative real-time RT-PCR, and data analysis were performed as described by Werner et al. (2010) using 7500 FAST Software_v2.0.1 (Applied Biosystems). In addition to *UBC10*, we used At3g25800 as a reference gene for normalization (Czechowski et al., 2005). *TUP5* was amplified with the primers 5′-TCGGTGTGACTCCTGACAT-3′ (forward) and 5′-ACTGCACACAAGAGGGCT-3′ (reverse). The reference gene At3g25800 was amplified with the primers 5′-CCATTAGATCTTGTC-TCTCTGCT-3′ (forward) and 5′-GACAAAACCCGTACCGAG-3′ (reverse) for gene expression analyses.

Subcellular Localization of TUP5

One to three leaves from stably transformed *P35S:TUP5:GFP* transgenic Arabidopsis lines were treated according to the protoplast isolation protocol described by Damm and Willmitzer (1988). The protoplasts were analyzed for subcellular localization of the TUP5-GFP fusion protein with a Leica TCS SP2 confocal laser scanning microscope. An excitation wavelength of 488 nm and a filter of 510 to 550 nm were used for the analysis of GFP fluorescence signal and an excitation wavelength of 488 nm and a filter of 610 to 680 nm for the autofluorescence signal of chloroplasts.

GUS Staining and Light Microscopy

The plant material was treated following the GUS staining protocol described by Krupková et al. (2007). The tissue was cleared and mounted according to Malamy and Benfey (1997). Microscopy was carried out with an Axioskop 2 plus with AxioCam ICc3 (Zeiss).

Measurement of Free Amino Acids

Seedlings were cultivated in liquid one-half-strength MS medium for 7 d under long-day conditions at standard light intensity (150 μmol m^{-2} s^{-1}), at low light intensity (3 μmol m^{-2} s^{-1}), or in darkness. After harvest, seedlings (approximately 100 mg per sample) were washed once with 5 mL sterile *aqua bidest.*, frozen in liquid nitrogen, and stored at −80°C until extraction. Shoot and root samples were harvested from 11-d-old seedlings grown under long-day conditions on vertical plates containing 1× MS medium. The samples were extracted, derivatized, and analyzed as described by Rinder et al. (2008).

Sequence Comparison and Phylogenetic Analysis

The protein sequence of TUP5 was used for a search for homologous genes using BLAST search (Altschul et al., 1997). The amino acid sequences of six homologous proteins from different species were aligned with TUP5 using ClustalW 1.83 (http://www.ch.embnet.org/software/ClustalW.html; Larkin et al., 2007), and shading of conserved amino acids in the alignment was carried out with BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). The fraction of sequences that had to agree for shading was set at 0.9. The phylogenetic analyses were conducted using MEGA5 (Tamura et al., 2011). The phylogenetic tree was revised with CorelDRAW 12.

Prediction of Subcellular Localization

The prediction of subcellular localization was carried out using TargetP (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al., 2000) and Aramemnon (http://aramemnon.botanik.uni-koeln.de/; Schwacke et al., 2003).

Sequence data from this article can be found in the GenBank/EMBL database or the Arabidopsis Genome Initiative database under the following accession numbers: *TUP5/WIN1* (AT1G80600), *Populus trichocarpa* unknown gene ABK95025.1, rice (*Oryza sativa* ssp. *japonica* cultivar group; GI:51854368), putative ACOAT, *Physcomitrella patens* ssp. *patens* (GI:162664563), predicted protein ACOAT family, *Chlamydomonas reinhardtii* ACOAT (GI:159480034), yeast ACOAT (GI:151945496), and *Escherichia coli* ACOAT (GI:242378886).

Supplemental Data

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

Supplemental Figure S1. Amino acid alignment and phylogenetic analysis of known ACOATs and highly similar genes from different species.

Supplemental Figure S2. Metabolites of the Arg biosynthesis pathway and related compounds tested for a complementing effect on the *tup5-1* root phenotype.

Supplemental Figure S3. Free amino acid content differs between wild-type shoots and roots.

Supplemental Figure S4. Content of free Gln and Asn in *35S:TUP5* transgenic lines.

Supplemental Figure S5. Aborted seeds in siliques of heterozygous *tup5-2* plants.

Supplemental Table S1. Percentage of aborted seeds in siliques of wild-type, *tup5-1*, heterozygous *tup5-2*, and *tup5-3* plants.

Supplemental Table S2. Segregation analysis of heterozygous self-fertilized *tup5-2* and *tup5-3* F1 populations.

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