A Role for Flavin Monooxygenase–Like Enzymes in Auxin Biosynthesis

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Although auxin is known to regulate many processes in plant development and has been studied for over a century, the mechanisms whereby plants produce it have remained elusive. Here we report the characterization of a dominant *Arabidopsis* mutant, *yucca*, which contains elevated levels of free auxin. *YUCCA* encodes a flavin monooxygenase–like enzyme and belongs to a family that includes at least nine other homologous *Arabidopsis* genes, a subset of which appears to have redundant functions. Results from tryptophan analog feeding experiments and biochemical assays indicate that YUCCA catalyzes hydroxylation of the amino group of tryptamine, a rate-limiting step in tryptophan-dependent auxin biosynthesis.

Auxin is an essential plant hormone that influences many aspects of plant growth and development, including cell division and elongation, differentiation, tropisms, apical dominance, senescence, abscission, and flowering (*1*). Although auxin has been studied for over 100 years, the mechanisms of its biosynthesis remain elusive. Multiple pathways have been proposed for the biosynthesis of indole-3-acetic acid (IAA) (the main auxin), including several tryptophan-dependent pathways and a tryptophan-independent one (*2*). Auxin biosynthesis can be catalyzed not only by endogenous plant enzymes, but also by enzymes encoded on plasmids of plant pathogens such as *Agrobacterium* (*2*). Whereas the bacterial pathways are well documented, knowledge of the endogenous plant pathways is scant. Rate-limiting steps have not been identified. Overexpression of the only enzyme conclusively shown to be involved in one of the tryptophan-dependent pathways does not cause any auxin-related phenotypes, although loss-of-function mutants are resistant to the effects of the auxin precursor indole-3-acetonitrile (*3–6*). Part of the difficulty in studying auxin biosynthesis has been the inability to generate auxin-deficient mutants, although genetic screens have identified several recessive *Arabidopsis* mutants with elevated levels of free auxin (*7–11*). However, it is not known whether elevated auxin levels in these mutants are caused by indirect effects on auxin biosynthesis or by defects in auxin conjugation. In addition, the mutants are either sterile or have intrinsic heterogeneity in phenotypes, which limits their utility for dissection of auxin biosynthetic pathways. Here we report a dominant and fertile mutant, *yucca*, which has an elevated level of endogenous auxin. We present evidence that YUCCA, a flavin monooxygenase (FMO)–like enzyme, catalyzes a key step in *Arabidopsis* tryptophan-dependent auxin biosynthesis.

Two independent *yucca* mutants were identified with long hypocotyls by activation tagging (*12*). The genomic DNA adjacent to the right border of the T-DNA insertions in the two alleles was cloned by plasmid rescue (Fig. 1A). Comparison of the rescued DNA insertions showed that the cauliflower mosaic virus 35*S* (CaMV 35*S*) enhancer arrays in the two mutants had inserted within a 3-kilobase (3-kb) region downstream of the coding region of the same gene (GenBank accession CAB79971). Northern blot analysis of total RNA from both alleles showed that the expression level of this gene was higher in *yucca* than in wild type. This indicated that the two mutants carried dominant alleles at

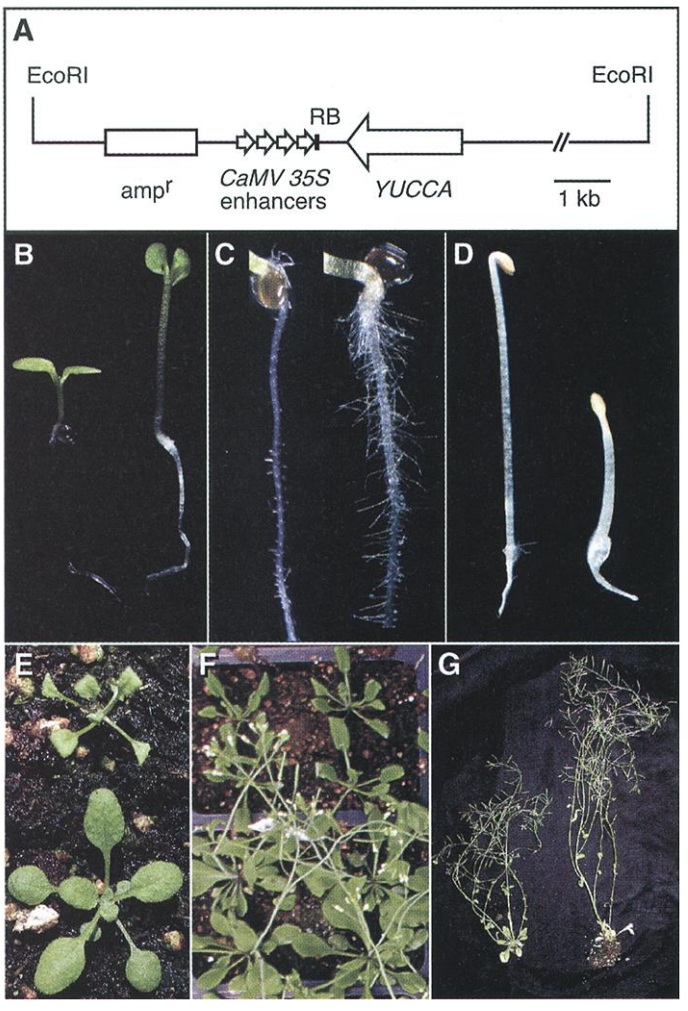

Fig. 1. Identification of YUCCA. (**A**) Rescued 10-kb Eco RI plasmid from *yucca-2D* mutant, which was used for recapitulation of phenotype. amp^r, bacterial ampicillin resistance; RB, right T-DNA border. (**B**) Wild-type (left) and *yucca* seedlings grown on 0.5X MS medium in white light. (**C**) Roots of wild-type (left) and *yucca* grown on a vertical plate in white light. (**D**) Dark-grown wild-type (left) and *yucca* seedlings. (**E**) Mature *yucca* (top) and wild type (bottom) grown in soil in the greenhouse. (**F**) Delayed development of *yucca* (top). (**G**) Increased apical dominance of *yucca* (right).

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the same locus. Furthermore, transforming wild-type *Arabidopsis* either with genomic DNA of this gene placed next to a tetramer of 35*S* enhancers or with a cDNA of this gene under the control of the complete 35*S* promoter recapitulated the *yucca* phenotype. Together, these experiments identified this gene as the *YUCCA* gene. Because the original *yucca* alleles were poorly fertile, recapitulation lines with weaker phenotypes were used for further studies. For simplicity, we refer to the recapitulation lines as *yucca* mutants as well.

In addition to long hypocotyls, *yucca* plants had phenotypes characteristic of elevated auxin levels during all stages of development. In the aerial part, *yucca* seedlings had epinastic cotyledons and elongated petioles when grown in white light (Fig. 1B) similar to known auxin overproduction mutants such as *sur1* (*7*, *9*), *sur2* (*8*), and transgenic plants that overexpress the *iaaM* auxin synthetic gene from *Agrobacterium* (*13*). Roots of *yucca* were shorter, but the root hairs were much longer and more plentiful than those of wild-type seedlings (Fig. 1C). Dark-grown *yucca* seedlings had short hypocotyls and lacked an apical hook (Fig. 1D). Mature leaves of *yucca* were narrower than those of wild type and were epinastic with long blades and petioles (Fig. 1, E and F). Adult *yucca* plants had increased apical dominance (Figs. 1G and 2G). All these traits are expected from elevated free auxin levels (*13*, *14*). In addition, the mature *yucca* leaves curled downward and had a semi-erect growth habit, resembling the commonly known yucca plant (*Agave* sp.) (Fig. 1, E and F). This gives rise to the mutant's name.

We carried out several experiments to show directly that *yucca* plants contain elevated auxin levels. First, we used gas chromatography–mass spectrometry (GC-MS) to measure endogenous levels of free IAA (*15*). To obtain sufficient quantity of tissue for IAA measurements, we used a rather weak recapitulation line. This line contained about 50% more free IAA than wild-type plants, suggesting that strong *yucca* alleles contain even higher levels of free IAA (Fig. 2A).

Next, we used physiological and genetic experiments to test whether this modest increase in free IAA is physiologically important. It has been established previously that *Arabidopsis* explants cannot proliferate without addition of auxin to the medium. It has also been established that the ratio of auxin to cytokinin determines the relative growth of roots, shoots, and callus (*16*). In general, a high ratio of auxin to cytokinin leads to root growth, but a lower ratio induces callus and shoot growth (*16*). Extensive root growth was observed when *yucca* cotyledon explants were grown in Murashige-Skoog (MS) medium, whereas the wild-type explants did not proliferate at all (Fig. 2B). When cytokinin was added to the medium, the length and the abundance of the *yucca* explants' roots were both reduced, whereas the wild-type explants were left unchanged (Fig. 2C). As expected, an increase in cytokinin concentration led to the formation of callus, which produced shoots from which flowers were generated eventually (Fig. 2, C and D). The ability of *yucca* mutant tissue to proliferate and differentiate in an auxin-free medium indicates that *yucca* produces more auxin than the wild type. This conclusion is also supported by the observation that an auxin-reporter gene was much more active in *yucca* than in wild type (*17*) (Fig. 2, E and F).

If the *yucca* phenotype is caused by elevated levels of free auxin, then it should be possible to suppress the phenotype by reducing levels of free auxin. To test this hypothesis, we made use of the bacterial *iaaL* gene, which encodes an enzyme that conjugates free IAA to lysine. When *iaaL* is overexpressed in *Arabidopsis* plants, the pool of free IAA is reduced, which leads to decreased apical dominance (*18*). We found that overexpression of *iaaL* did indeed mask the *yucca* phenotype (Fig. 2G), consistent with the interpretation that elevated auxin levels were the cause of *yucca*'s phenotype.

The predicted YUCCA protein with 414 amino acid residues has similarity to FMOs from mammals (*19*, *20*) and contains conserved motifs for binding of flavin-adenine dinucleotide (FAD) and NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate) (Fig. 3A). BLAST searches indicated that there are two families of FMO-like proteins encoded in the *Arabidopsis* genome (Fig. 3B). The YUCCA family contains nine other proteins, which share 44 to 64% amino acid sequence identity with YUCCA. To test whether the YUCCA homologs have similar functions in vivo, we overexpressed several of them under the control of CaMV 35*S* enhancers. Overexpression of YUCCA2 (CAB41936), which has 53% amino acid sequence identity to YUCCA, caused *yucca*-like phenotypes (*21*). We also isolated two other *yucca*-like mutants with activation tagging. Both mutants have elevated expression of YUCCA3 (AAB80641), which has 51% amino acid sequence identity to YUCCA (*22*). The similar phenotypes of plants over-

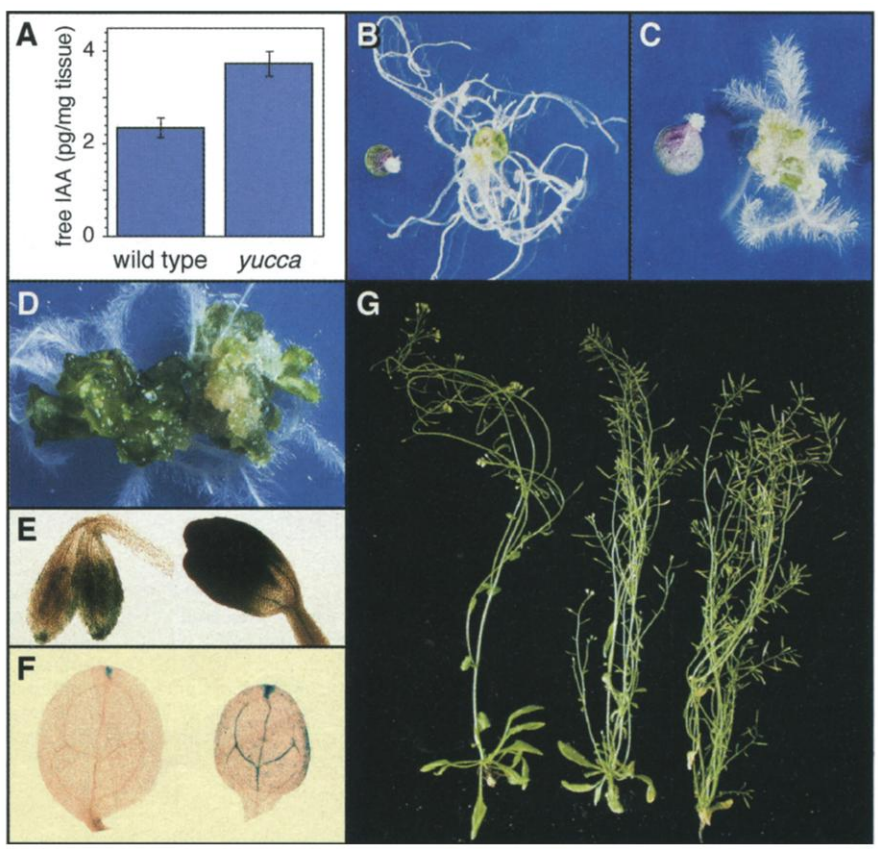


Fig 2. Evidence for elevated levels of endogenous auxin in *yucca*. (**A**) GC-MS analysis of free IAA levels (*15*). (**B**) Explants of wild-type (left) and *yucca* cotyledons grown on MS medium for 4 weeks. (**C**) Explants of wild-type (left) and *yucca* cotyledons grown on MS containing 6-(γ,γ-dimethylallylamino)-purine riboside (IPAR) (2 mg/l) for 4 weeks. (**D**) Explants of *yucca* cotyledons grown on MS containing IPAR (2 mg/l) for 7 weeks. (**E**) Histochemical staining of dark-grown DR5-GUS (left) and *yucca*/DR5-GUS seedlings. (**F**) Histochemical staining of light-grown DR5-GUS (left) and *yucca*/DR5-GUS leaves. (**G**) Suppression of the *yucca* adult phenotype by overexpression of the *iaaL* gene: *yucca* (left), *yucca*/*iaaL* (middle), and *iaaL* overexpression line (right).

expressing *YUCCA*, *YUCCA2*, and *YUCCA3* demonstrate that these genes are functional paralogs and that they likely have redundant functions. Functional redundancy may explain why neither single- nor double-knockout alleles of *YUCCA* and *YUCCA2* have obvious phenotypes (*23*). However, not all *YUCCA*-like genes have identical effects. *BAS3* (CAA22980), which has 50% and 63% amino acid sequence identity with YUCCA and YUCCA3, respectively, was identified in an activation-tagging screen for *phyB4* suppressors. Whereas dominant *yucca* mutants have long hypocotyls in the light, overexpression of *BAS3* shortened the long hypocotyls of *phyB* mutants (*24*). Thus, plant FMO-like enzymes have distinct in vivo functions, which cannot be predicted a priori from sequence analysis alone.

To test whether the elevated level of auxin in *yucca* is produced via a tryptophan-dependent auxin biosynthetic pathway or a tryptophan-independent pathway, we grew plants in media containing tryptophan analogs. Analogs such as 5-methyl-tryptophan (5-mT) are toxic to plants because they inhibit tryptophan biosynthesis and disrupt the functions of proteins in which they have been incorporated (*25*). Although 5-mT is ultimately toxic to both wild type and *yucca*, *yucca* plants are much more resistant to its effects (Fig. 4A). Although wild type cannot grow in MS medium containing 100 μM 5-mT, *yucca* not only survived but also gained additional phenotypes, including the growth of adventitious roots from the hypocotyl (Fig. 4A). The same root phenotype could also be produced by the addition of 5-methyl IAA to the medium. This suggests that 5-mT is rendered nontoxic by conversion of 5-mT to 5-methyl IAA and that the latter is an active auxin that induces adventitious root growth. These results indicate that auxin is produced in *yucca* via a tryptophan-dependent pathway.

To test whether the *YUCCA* pathway is generally used by other plants to synthesize auxin, *YUCCA* was overexpressed in tobacco (*26*) (Fig. 4B). The transgenic tobacco plants had a dramatic change in phenotype, including long, narrow, epinastic leaves, similar to the phenotypes observed in *Arabidopsis*.

There are several proposed auxin biosynthetic pathways that use tryptophan as the precursor (Fig. 5). Based on the well-characterized oxidation of heteroatom-containing compounds by mammalian FMOs (*19*, *20*, *27*, *28*), we identified the proposed auxin biosynthetic intermediate tryptamine as the most likely candidate as a YUCCA substrate. Indeed, when tryptamine was used in vitro as a substrate for recombinant YUCCA, a product was formed that had a mass-spectral fragmentation pattern consistent with that of N-hydroxyl tryptamine (Fig. 3C) (*29*).

These results lead us to propose that YUCCA catalyzes the N-oxygenation of tryptamine, and that this transformation is a rate-limiting step in auxin biosynthesis in many plants (Fig. 5). We propose furthermore that the limiting factor is the level of YUCCA and its paralogs, consistent with the observation that indole-3-acetaldoxime but not tryptamine has auxin activity when added to growth medium (*30*).

Although mammalian FMOs have been well characterized by biochemical means (*27*, *28*), their physiological functions remain unknown (*19*, *20*). That an *Arabidopsis* FMO-like enzyme is involved in tryptophan metabolism suggests that some mammalian FMOs may also have important physiological functions in tryptophan metabolism. Further genetic analysis of *Arabidopsis* FMO-like genes

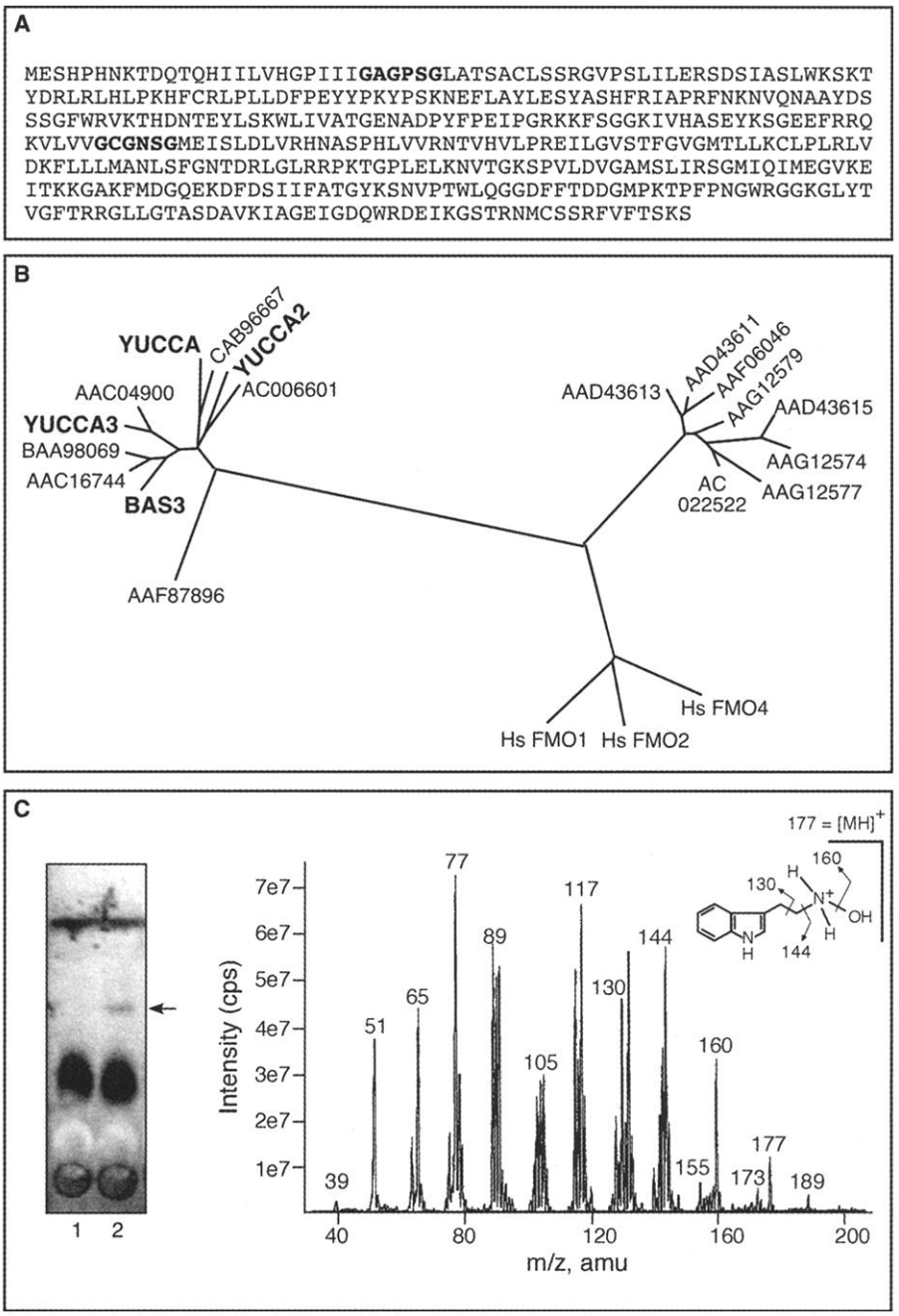
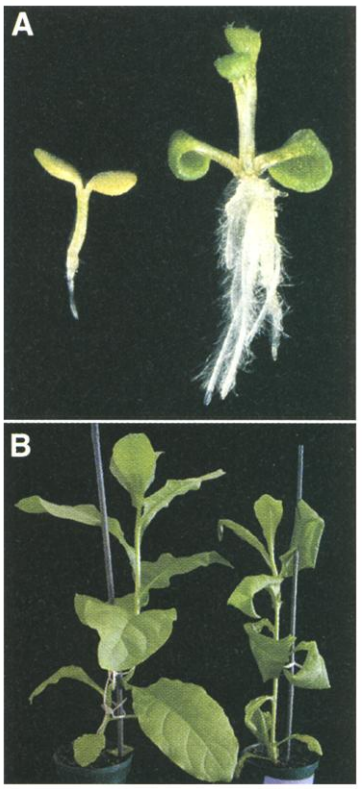


Fig. 3. YUCCA is a member of the FMO-like family. (**A**) Amino acid sequence of YUCCA. The putative FAD and NADPH binding motifs near the NH_2 terminus and in the middle of the protein, respectively, are indicated in bold. (**B**) Phylogenetic tree of YUCCA and related *Arabidopsis* genes along with human FMOs (Hs FMO1/2/4). GenBank accession numbers are shown. Proteins analyzed here are indicated in bold. (**C**) Tryptamine is a substrate for YUCCA in vitro. Reaction mixture with tryptamine as the substrate was separated on a TLC plate (*29*). The arrow points to the enzymatic product; 1 is the control and 2 is the reaction (*29*). The product was characterized by mass spectrometry (*29*). Assignments of the pseudo-molecular ion (MH^+) and major fragmentation ions are indicated.



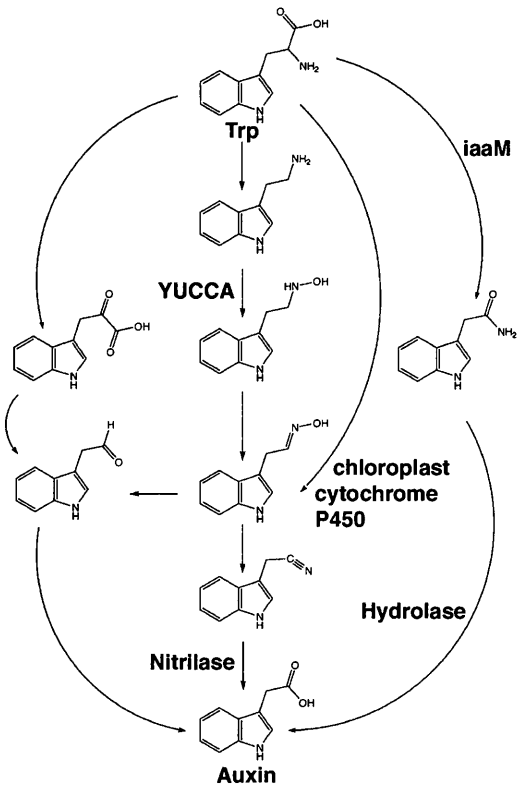


Fig. 4. (Left) YUCCA is involved in tryptophan-dependent auxin biosynthesis, and the YUCCA pathway is functional in other plants. **(A)** *yucca* is less sensitive to toxic tryptophan analogs. Wild-type (left) and *yucca* seedlings were grown on 0.5X MS medium containing 100-μM 5-mT for 10 days. **(B)** Comparison of wild-type (left) and transgenic tobacco plants overexpressing *YUCCA*. **Fig. 5. (Right)** YUCCA catalyzes a key step in auxin biosynthesis. Putative tryptophan-dependent auxin biosynthesis pathways and intermediates are shown (*2*). The indole-3-acetaldoxime intermediate was proposed recently (*25*).

may yield additional clues that can be used to elucidate the physiological roles of their mammalian counterparts.

References and Notes

1. P. J. Davies, Ed., in *Plant Hormones: Physiology, Biochemistry and Molecular Biology*, (Kluwer Academic, Dordrecht, Netherlands, 1995), pp. 1–12.
2. B. Bartel, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 51 (1997).
3. ———, G. R. Fink, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6649 (1994).
4. D. Bartling, M. Seedorf, A. Mithofer, E. W. Weiler, *Eur. J. Biochem.* **205**, 417 (1992).
5. J. Normanly, P. Grisafi, G. R. Fink, B. Bartel, *Plant Cell* **9**, 1781 (1997).
6. R. C. Schmidt, A. Muller, R. Hain, D. Bartling, E. W. Weiler, *Plant J.* **9**, 683 (1996).
7. J. J. King, D. P. Stimart, R. H. Fisher, A. B. Bleecker, *Plant Cell* **7**, 2023 (1995).
8. M. Delarue, E. Prinsen, H. V. Onckelen, M. Caboche, C. Bellini, *Plant J.* **14**, 603 (1998).
9. W. Boerjan *et al.*, *Plant Cell* **7**, 1405 (1995).
10. A. Lehman, R. Black, J. R. Ecker, *Cell* **85**, 183 (1996).
11. J. L. Celenza Jr., P. L. Grisafi, G. R. Fink, *Genes Dev.* **9**, 2131 (1995).
12. D. Weigel *et al.*, *Plant Physiol.* **122**, 1003 (2000).
13. C. P. Romano, P. R. Robson, H. Smith, M. Estelle, H. Klee, *Plant Mol. Biol.* **27**, 1071 (1995).
14. R. J. Pitts, A. Cernac, M. Estelle, *Plant J.* **16**, 553 (1998).
15. Analysis of free auxin was done as described by K. Chen, A. N. Miller, G. W. Patterson, and J. D. Cohen [*Plant Physiol.* **86**, 822 (1988)]. Six-day-old *yucca* and wild-type seedlings grown in 0.5X MS medium at 22°C in white light were used for the analysis.
16. J. Mathur, C. Koncz, in *Arabidopsis Protocols*, J. M. Martinez-Zapater, J. Salinas, Eds. (Human Press, Totowa, NJ, 1998), vol. 82, pp. 31–34.
17. S. Sabatini *et al.*, *Cell* **99**, 463 (1999).
18. C. P. Romano, M. B. Hein, H. J. Klee, *Genes Dev.* **5**, 438 (1991).
19. D. M. Ziegler, *Drug Metab. Rev.* **19**, 1 (1988).
20. J. R. Cashman, *Chem. Res. Toxicol.* **8**, 165 (1995).
21. Y. Zhao, J. Chory, unpublished data.
22. Y. Zhao, S. Christensen, D. Weigel, J. Chory, unpublished data.
23. Y. Zhao, S. Christensen, J. Alonso, J. Ecker, J. Chory, unpublished data.
24. Y. Zhao, M. Neff, J. Chory, unpublished data.
25. A. K. Hull, R. Vij, J. L. Celenza, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 2379 (2000).
26. Transformation of tobacco was carried out as described by P. Gallois and P. Marinho [*Methods Mol. Biol.* **49**, 39 (1995).]
27. N. B. Beaty, D. P. Ballou, *J. Biol. Chem.* **256**, 4619 (1981).
28. ———, *J. Biol. Chem.* **256**, 4611 (1981).
29. Recombinant YUCCA was purified from *Escherichia coli* as a maltose binding protein (MBP) fusion according to the procedures for expressing human FMOs [A. Brunelle *et al.*, *Drug Metab. Dispos.* **25**, 1001 (1997)]. For activity assays, 350 μg of YUCCA-MBP was incubated with 2 mM tryptamine, 1 mM $NADP^+$, 1 mM glucose-6-phosphate, and 2.0 IU of glucose-6-phosphate dehydrogenase in 120-μL total volume at 37°C for 3 hours. The reactions were stopped by adding an equal volume of methanol. The control contained the same components, except methanol was added before the enzyme. The substrate and products were separated by thin-layer chromatography (TLC) with CH_2Cl_2/methanol/tetraethylammonium (TEA) (75:20:5). The product was eluted from the TLC plates, and Electrospray mass spectrometry was performed under positive mode to determine the molecular mass of the product.
30. Y. Zhao, J. R. Cashman, J. Chory, unpublished data.

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Transgenic Monkeys Produced by Retroviral Gene Transfer into Mature Oocytes

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Transgenic rhesus monkeys carrying the green fluorescent protein (GFP) gene were produced by injecting pseudotyped replication-defective retroviral vector into the perivitelline space of 224 mature rhesus oocytes, later fertilized by intracytoplasmic sperm injection. Of the three males born from 20 embryo transfers, one was transgenic when accessible tissues were assayed for transgene DNA and messenger RNA. All tissues that were studied from a fraternal set of twins, miscarried at 73 days, carried the transgene, as confirmed by Southern analyses, and the GFP transgene reporter was detected by both direct and indirect fluorescence imaging.

Although transgenic mice have been invaluable in accelerating the advancement of biomedical sciences (*1–5*), many differences between humans and rodents have limited their usefulness (*6–9*). The major obstacle in producing transgenic nonhuman primates has been the low efficiency of conventional gene transfer protocols. By adapting a pseu-