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Tryptophan auxotroph mutants suppress the *superroot2* phenotypes, modulating IAA biosynthesis in arabidopsis

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Plant phytohormone, Indole-3-acetic acid (IAA), is synthesized by tryptophan (trp) dependent and independent pathway. Here we report that tryptophan auxotroph mutants completely suppressed the abnormalities of auxin over production mutant, *superroot2*. SUR2 is considered to modulate Trp dependent pathway, resulting IAA accumulation in Arabidopsis. Tryptophan auxotroph mutants showed hyper-sensitivity to the auxin polar transport inhibitor, NPA, on the phenotype of reduced gravitropism. These results together with the results of histochemical analyses, tryptophan auxotroph mutants seem to have a complete defect in Trp dependent IAA biosynthesis pathway, and it is also suggested that the Trp dependent pathway is responsible for the normal root gravitropism.

Introduction

Auxin regulates many developmental processes in plant, and it has been studied for over a century. Multiple pathways have been proposed on biosynthesis of indole-3-acetic acid (IAA), including tryptophan (Trp) dependent and independent pathway.[7] Although bacterial pathways are well known, endogenous plant biosynthetic pathway is still scant. Overexpression of the *NIT1* and *AAO1*, which are suggested to be involved in Trp dependent IAA synthesis pathway, does not cause any auxin-related phenotypes, although *nit1* mutant showed resistibility to the effects of the putative auxin precursor indole-3-acetonitrile.[10] *superroot1, superroot2,* and *yucca* mutants is known to have increased IAA content showing long hypocotyl, epinastic cotyledone, and excess lateral root formation.[4,8,16] TAA1/TIR2 is recently isolated to be responsible for indole-3-pyruvic acid (IPA) branch of the auxin biosynthetic pathway.[12,15] However, it is not known the IAA deficient mutants. Analyses of the mutants with reduced IAA content would provide us a new insight to the mechanisms of IAA biosynthesis.

Biochemical analysis suggested that tryptophan auxotroph mutants, *trp2-1*, are defective in the Trp dependent IAA biosynthetic pathway.[9] Here we report that *trp2-1* and *trp3-1* are genetically defective on the Trp dependent IAA synthesis pathway, suppressing the *sur2* phenotypes. *trp2-1* and *trp3-1* also showed significant sensitivity to the auxin transport inhibitor, NPA. Although *trp2-1* and *trp3-1* showed reduced lateral root formation, its phenotype was suppressed by application of Trp, but not by the Indole. Furthermore, activities of the auxin responsible promoter, *DR5* and *AtAUX2-11,* were reduced in tryptophan auxotroph mutants. These results indicate that Trp dependent IAA biosynthesis in root is responsible for the normal root gravitoropism and lateral root formation.

Results

sur2 mutant showed increased IAA content by modulating the Trp dependent IAA biosynthesis pathway.[1,2] To examine whether Trp biosynthesis is responsible for phenotypes of the *sur2* mutant or not, we generated a series of double mutants of *sur2-1 trp2-1, sur2-1 trp2-8, sur2-1 trp3-1, sur2-1 trp3-100,* and *sur2-1 trp1-1. sur2-1* mutant produced long hypocotyl, epinastic cotyledone and excess lateral root formation from a hypocotyl (**Fig. 1A**), and the *sur2* phenotypes were completely suppressed by *trp2-1* mutation, which is known as a strong allele of the *trp2* mutant (**Fig. 1B and C**). In the case of the *sur2-1 trp2-8* double mutants, *trp2-8* mutation partially suppressed the *sur2-1* mutation. *sur2-1 trp2-8* double mutants produced long hypocotyl, whereas it did not show epinastic cotyledone and excess lateral root formation from a hypocotyl (**Fig. 1D and E**). This partial suppression by the *trp2-8* mutation is consistent to the fact that the *trp2-8* is a weak allele of the *trp2* mutant. Similarly, *trp3-1 and trp3-100* mutation completely or partially suppress the *sur2* phenotypes, respectively (**Fig. 1F–I**). Further, *trp1-1,* which is known as a strong allele of the *trp1* mutant, could also completely suppress the *sur2* phenotype (**Fig. 1J and K**).

To determine the IAA content of *sur2-1 trp2-1* double mutant, we measured it by mass spectrometry (GC-SIM-MS) analyses. As shown in **Figure 2**, the average level of three replicate measurements of free IAA in the double mutant of *sur2 trp2-1*

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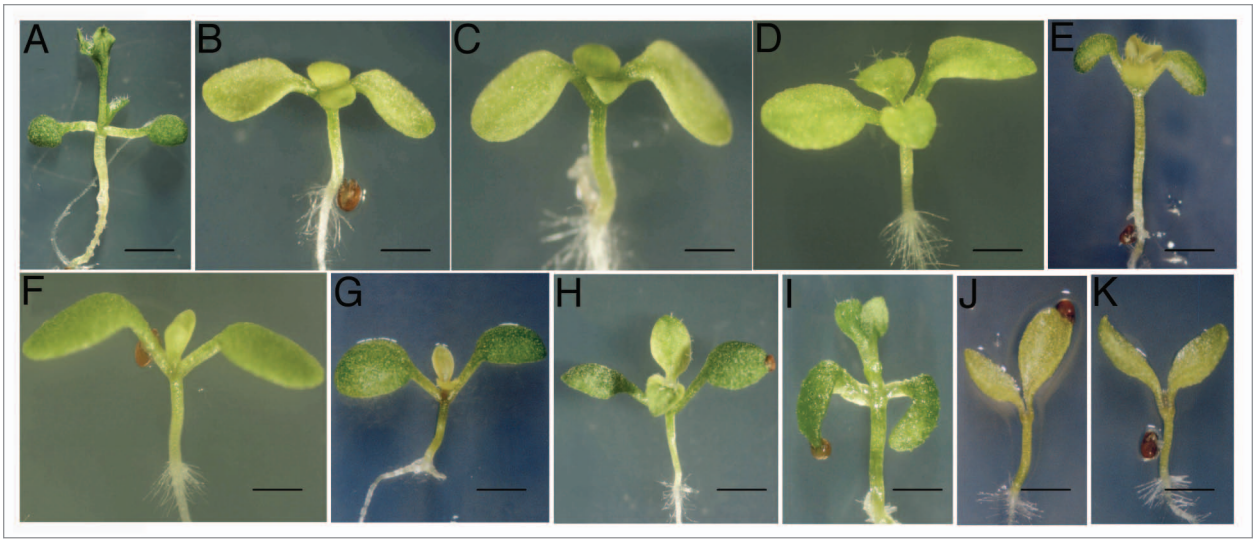

Figure 1. Genetic analyses of *sur2* and *trp* mutants. (A) *sur2-1*. (B) *trp2-1*. (C) *sur2-1 trp2-1* double mutant. (D) *trp2-8*. (E) *sur2-1 trp2-8* double mutant. (F) *trp3-1*. (G) *sur2-1 trp3-1* double mutant. (H) *trp3-100*. (I) *sur2-1 trp3-100* double mutant. (J) *trp1-1* (K) *sur2-1 trp1-1* double mutant. Seedlings were grown on an agar medium for 10 days after germination in the continuous light condition. Bars; 7 mm (A), 3 mm (B–D and F–H), 5 mm (E and I), 1.5 mm (J and K).

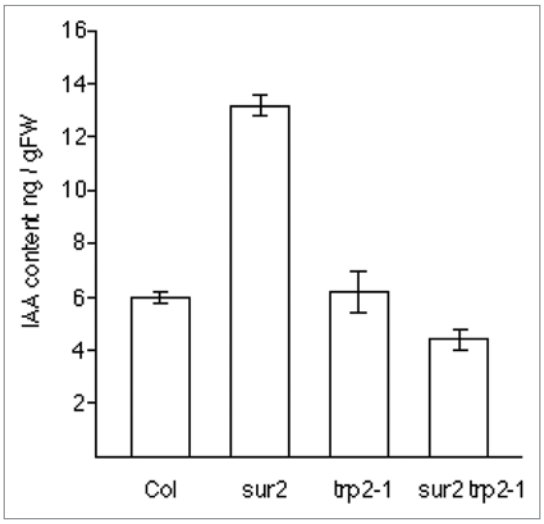

Figure 2. IAA contents of Col, *sur2, trp2-1* and *sur2 trp2-1* double mutant. Seedlings were grown on an agar medium for 5 days after germination. Mean value and standard deviation was calculated by three independent experiments.

was completely suppressed to the levels of wild type and *trp2-1* mutant. These results indicated that *the trp2, trp3* and *trp1* mutation is epistatic to the *sur2* mutation, suggesting that the Trp dependent IAA biosynthesis pathway is completely intercepted in the *trp2-1, trp3-1* and *trp1-1* mutants.

We suggested that the Trp auxotroph mutants have a defect in Trp dependent IAA biosynthesis pathway. To better characterize the function of Trp dependent IAA biosynthesis on gravitropism, which is known as an auxin related phenotype, we examined the effect of exogeneously applied auxin transport inhibitor, naphthylphthalamic acid (NPA). When wild-type plant was grown on a medium containing 1 μM NPA, reduced gravitropic response was observed (**Figs. 3A, B, 4A and D**), and the reduction of the gravitropic response in *trp2-1* and *trp3-1* were significantly enhanced (**Figs. 3C–F, 4B, C, E and F**), indicating that the *trp2-1* and *trp3-1* mutants showed hyper sensitivity to the NPA at least on the phenotype of gravitropism.

It is known that the DR5::GUS expression is observed at the root tip, and its accumulation is enhanced by an application of the NPA (Casimiro et al. 2001). NPA could block basipetal and acropetal auxin transport, and it is suggested that the enhancement of the GUS activity at the root tip by NPA application was caused by the IAA biosynthesis in vivo. To visualize the auxin spatial accumulation especially at the root tip, we examined the reporter gene activities of *DR5::GUS* and *AtAUX2-11::GUS* (Ulmasov et al. 1997, Wyatt et al. 1993). When plants were grown on an agar plate without NPA, similar GUS expression pattern were observed in the wild type, *trp2-1*, and *trp3-1* mutants (**Fig. 5A–C, G–I**). When they were grown on the agar plates including 1 μM NPA, the GUS activity in the *trp2-1* and *trp2-8* were significantly reduced in comparison to that of wild type (**Fig. 5D–F and J–L**), indicating that the IAA is synthesized via the Trp dependent IAA biosynthesis pathway at the root tip. This hypothesis is consistent to the result that the Trp auxotroph mutants showed hyper sensitivity to the NPA on the gravitropic response. Trp dependent IAA biosynthesis may be responsible for the normal gravitropism.

Discussion

Here we showed that the Trp biosynthesis is responsible for the *SUR2* function, and the Trp dependent IAA biosynthesis pathway at the root tip contributes for the appropriate root gravitropism.

SUR2 gene is shown to encode a cytochrome P450 reductase, and SUR2 is shown to catalyze the first committed step in indole glucosinolate biosynthesis by metabolizing indole-3-acetaldoxime

to its corresponding *aci*-nitro compound in vitro. *SUR2* is suggested to be a regulator of IAA biosynthesis by regulating the flux of indole-3-acetaldoxime into IAA and/or indole glucosinolate biosynthesis.

Here we showed that *trp2-1, trp3-1* and *trp1-1* mutation completely suppressed the *sur2* phenotypes. This is the first report to show that the Trp dependent IAA biosynthesis is completely intercepted in the loss of function mutant *trp2-1, trp3-1*. It is known that the *trp2-1* and *trp3-1* mutants accumulate conjugated types of IAA compared with that of wild type, and it is suggested that the Trp independent IAA biosynthesis pathway is activated in these mutants.

YUCCA and *TAA1* are responsible for Trp dependent IAA biosynthetic pathway regulating embryo development.[6,12] Together with *yucca* and *taa1* mutants, *trp2-1* and *trp3-1* mutants would be good materials to analyze the biological function of the Trp dependent IAA biosynthesis pathway.

When plants were grown on agar plate with NPA, enhanced IAA accumulation was observed at root tip, and it would be a result by de novo IAA biosynthesis, not by IAA transport from the shoot. GUS expression levels of the *DR5::GUS* and *AtAUX2-11::GUS* were reduced in the *trp2-1* and *trp3-1* mutants, when plants were grown on agar plates with NPA. This result is consistent to the result that *trp2-1* and *trp3-1* showed hyper sensitivity to NPA on the root gravitropic response. These results suggested that the Trp dependent IAA biosynthesis pathway mainly contribute to the IAA biosynthesis in the root tissues. As for the shoot tissues, both of the Trp dependent and independent IAA biosynthesis pathways would be activated, and the IAA would be transported from the shoot tissues to root tissues contributing lateral root formation and/or root gravitropisms. This hypothsis is consistent to the result of the inhibition of lateral root formation by the NPA application to the hypocotyl.[11]

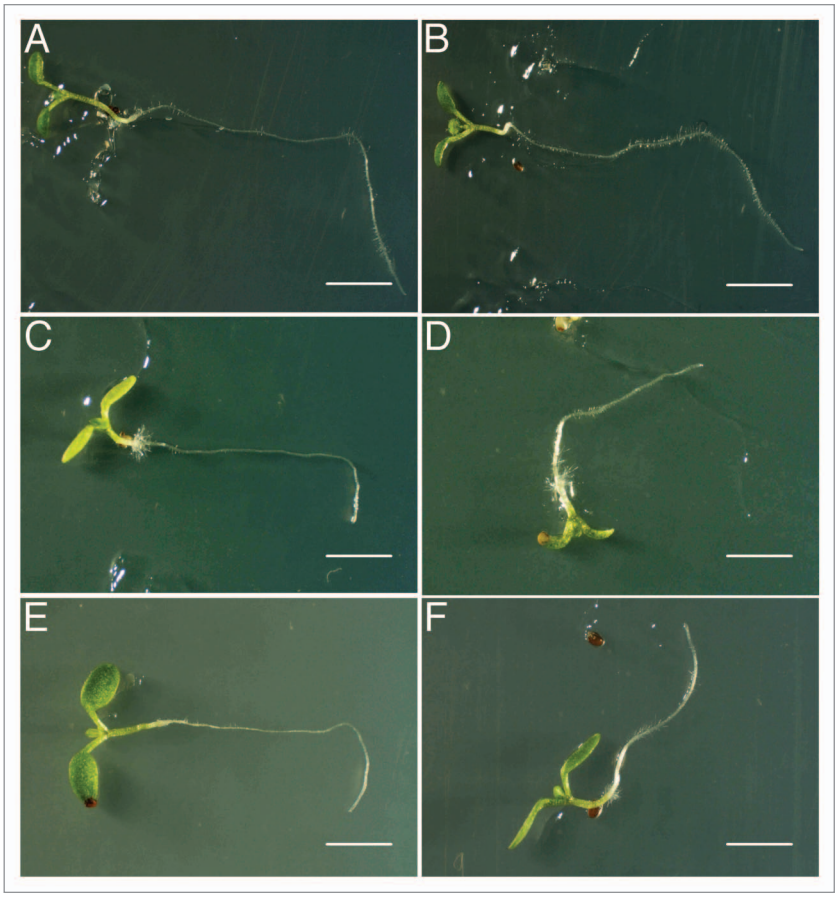

Figure 3. *trp* mutants showed remarkable sensitivity to NPA. (A and B) wild type. (C and D) *trp2-1*. (E and F) *trp3-1*. (A, C and E) Mock treatment. (B, D and F) 1μM NPA treatment. After the incubation for 7 days on agar plates in a vertical position, the plates were rotated 90 degrees and incubate for 2 days. Bars; 1 cm (A and B), 5 mm (C–F).

Materials and Methods

Plant material and growth conditions. Arabidopsis plant seeds were sown on the surface of agar plates with half-strength MS medium, and incubated at 4°C for 3 days. Plants were grown in a laboratory room under continuous illumination of 50–100 μE m^{-2} s^{-1} at 22°C. For the chemical application, 1 μM of NPA was

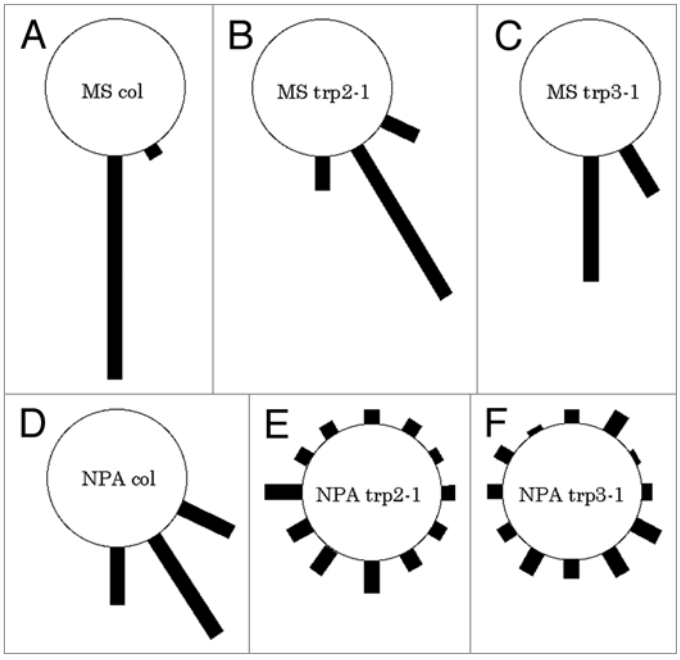

Figure 4. Wild-type (Col), *trp2* and *trp3* were germinated on MS agar plate (A–C), or a agar plate containing 1 μM NPA (D–F). The orientation of root growth of 30 seedlings was measured after 7 days. Each root orientation was assigned to one of twelve 30 degree sectors. The length of each bar represents the percentage of seedlings showing direction of root growth within that sector.

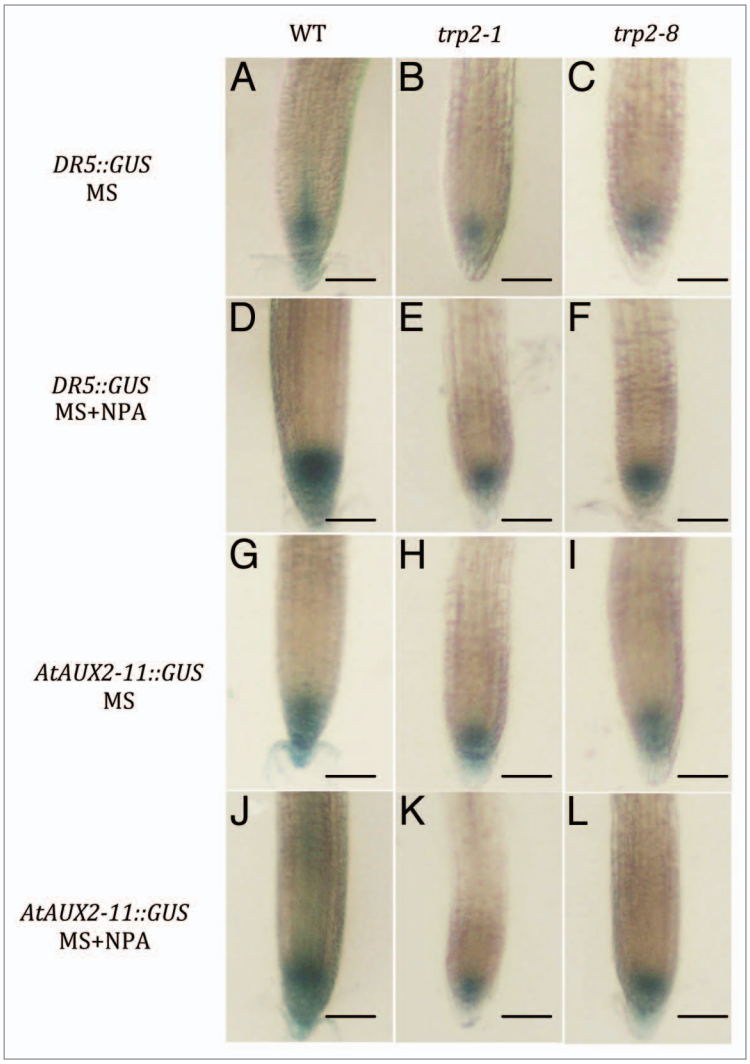

Figure 5. Histochemical staining for GUS activity at the region of root tip in the *DR5::GUS* (A–F) and *AtAUX2-11::GUS* (G–L) transgenic plants. Wild type (A, D, G and J), *trp2-1* (B, E, H and K), and *trp* 2–8 (C, F, I and L). Teedlings were on MS agar medium (A–C and G–I) or on agar medium containing 1 μM NPA (D–F and J–L) for 5 days and stained with 2 mg/ml X-Gluc. Bars; 100 μm.

added after the autoclave and amino acids, and indole were added before the autoclave.

Quantification of IAA level. For gas chromatography, single-ion-monitoring mass spectrometry (GC-SIM-MS) analyses, fresh plant material (corresponding to a pool of 10 seedlings grown on agar plates for 10 days in a continuous light condition) was carefully weighed, frozen in liquid nitrogen and stored at -80°C. For extraction of free IAA, the material was ground in liquid nitrogen using a mortar and pestle. After addition of about 0.6 ng of [$^{13}C_6$] IAA (Cambridge Isotope Lab, MS) as an internal standard, the material was extracted in 80% ether and 0.1 mg/ml 2, 6-Di-tret.-butyl-4-methylphenol (BHT) for 60 min. After centrifugation, the supernatant was collected. The pellet was re-extracted again for 90 min, and the supernatant was brought to a water phase in a rotary evaporator. After the pH adjustment to 3.0, the aqueous phase was then partitioned three times against ether containing 0.01 mg/ml BHT. The combined ether phase, which contained ether-soluble acidic and neutral substances, was concentrated until about 800 μl under a nitrogen stream. After the pH adjustment to 10.0 by 1 N NaOH, vortex for 10 min and centrifuge it. Organic phase was discard and repeat this extract twice. After the pH adjustment to 3.0, same volume of ether containing 0.01 g/ml BHT was added. Repeat this extraction twice and dried under a nitrogen stream, and dissolved in methanol. IAA was purified by passing it through a HPLC using a Nucleosil N(CH3)2 column (Senshu, Tokyo, Japan). The mobile phase was 100% methanol and 0.03% acetic acid. Purified solution was dried under a nitrogen stream and trimethylsilylated with MSTFA at 60°C for 15 min.

Splitless injections were made into a GC-SIM-MS system (QP5050A, Shimazu, Tokyo, Japan), equipped with a capillar (DB-1, 0.25 mm i.d. x30 m, film thickness 0.25 μm; J&W Scientific, CA). A linear temperature gradient was applied from 80 to 280°C with a increase of 20°C/min. The injection temperature of the GC was 250°C, the ion source temperature of the MS was 250°C, and a helium flow of 1.2 ml/min. was applied. The ionization potential was 70 electron-volt, and the scan time was 0.2 sec. The percentages of molecules of IAA labeled with ^{13}C were calculated from the relative intensities of m/z 202 to 208, and 319 to 325 ions after subtraction of background. Peak-area ratios for ion pairs were corrected for the abundance of natural isotopes.

Histochemical β-galactosidase assay. Plant tissue was fixed with 1% glutaraldehyde for 6 h at room temperature. The fixative was then removed and replaced with 1 mg/ml X-Gluc. Tissues were incubated at 37°C for 3 h. The materials were soaked in 95% ethanol and observed by Leica M420 microscope and photographed by Olympus DP50 digital camera.

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Note

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