

Novel root culture system using a recessive mutant with a rooty phenotype

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Abstract Hairy roots induced by *Agrobacterium rhizogenes*-mediated transformation are widely used for the study of metabolic regulation and large-scale metabolite production. We developed an alternative root culture using an allelic mutant of *Arabidopsis superroot 1 (sur1)*. When the mutant was cultured in liquid modified Heller's medium developed for hairy root cultures, it grew rapidly into a globe-shaped rooty phenotype and was easily subcultured in the medium. This mutant is advantageous as the desired characteristics can likely be maintained via seeds because the rooty phenotype is recessive. To verify this postulate, heterozygous *sur1* plants were crossed with *fatty acid desaturase (fad)* mutants. The *sur1* homozygous mutants under *fad2* and *fad3* background accumulated high amounts of oleic acid and linolenic acid, respectively, and the mutant crossed with a *FAD3* overexpression transformant accumulated high amounts of α -linolenic acid. Thus, the *sur1* root culture system is an alternative tool for studying metabolic regulation and the production of useful compounds in roots.

Key words: *Arabidopsis*, fatty acid desaturase, hairy root, root cultures.

Plants produce many types of metabolites, such as terpenoids, alkaloids, and flavonoids, which are used as food additives, medicines, and industrial materials. Hairy roots induced by *Agrobacterium rhizogenes*-mediated transformation are widely used for the study of metabolic regulation and large-scale metabolite production because biosynthesis in hairy roots often mimics that of intact roots in the original plants (Uozumi 2004, Seki et al. 2005, Guillon et al. 2006). Cultures of adventitious roots induced by exogenous application of auxin are also applied in these types of studies. Root cultures are usually maintained by subculture at appropriate intervals, which are laborious and time-consuming, and also risk contamination by fungi or bacteria during the subculture process. Although several studies have reported on the cryopreservation of hairy roots, these preservation protocols are not generally applied to hairy roots (Teoh et al. 1996; Touno et al. 2006).

In the course of isolating T-DNA insertion mutation pools in *Arabidopsis*, prepared to generate transgenic *Arabidopsis* plants overexpressing *AtRBPI-GFP* in our previously work (Suzuki et al. 2000), we discovered a

single recessive mutant producing excess lateral roots following germination in a seed germination plate. The heterozygous mutant showed no visible abnormal phenotype and set seeds. Moreover, a preliminary experiment revealed that isolated lateral roots from the recessive mutant could grow and be subcultured in a liquid hormone-free medium. This finding led us to investigate the potential application of the "rooty" phenotype in the recessive mutant for a novel root culture system (Figure 1).

Arabidopsis seeds used in this study were surface-sterilized, germinated, and incubated on 1×Murashige–Skoog (MS) agar plates (0.8% agar, 3% sucrose) at 23°C under long-day conditions (16 h light/8 h dark) for 2 weeks unless otherwise stated. Although this mutant was isolated from our T-DNA insertion mutant pool, the mutation was not linked to the T-DNA insertion (data not shown) and exhibited several severe phenotypes; e.g., elongated hypocotyls with small and epinastic cotyledons were observed in 8-day-old seedlings (Figure 2), the development of excess adventitious and lateral roots occurred in 3-week-old seedlings (Figure 2), and a reduced number of leaves and the absence of an

Abbreviations: *alf1*, aberrant lateral roots formation 1; FAD (*fad*), fatty acid desaturase; HF medium, modified Heller's medium; *hls3*, *hookless 3*; IAA, indole-3-acetic acid; MS, Murashige-Skoog; *rt*, rooty; *sur1*, *superroot 1*; WT, wild-type
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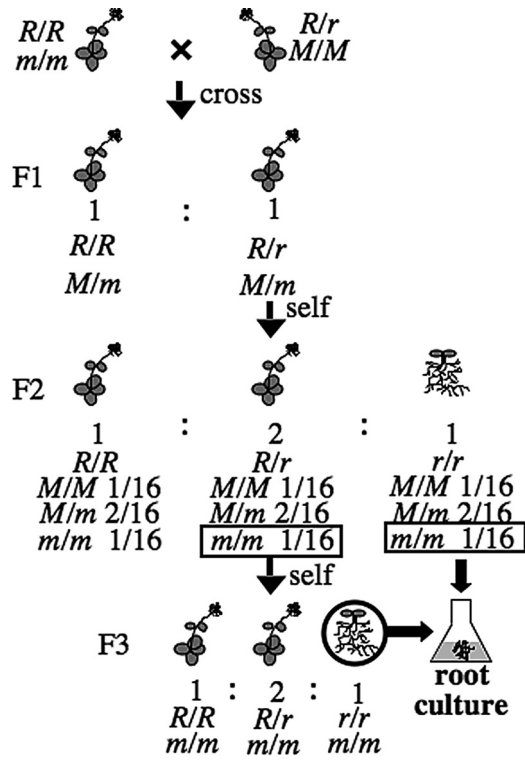


Figure 1. Schematic diagram of the root culture system using a mutant producing excess roots. *r*: mutated locus producing excess roots. *m*: mutated locus producing useful metabolites.

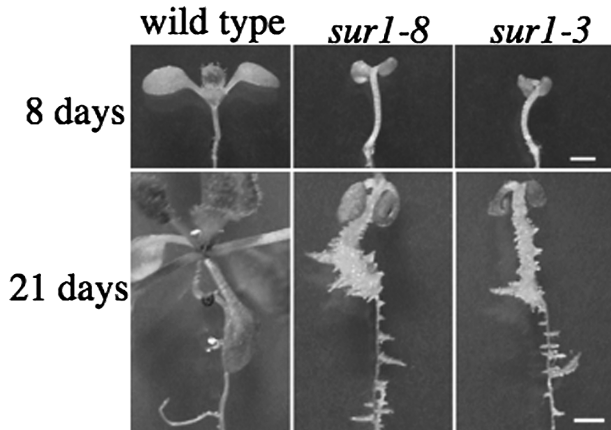


Figure 2. Phenotypes of *sur1* mutants. Seedlings grown on solid MS medium. Eight-day-old seedlings (top) and 3-week-old seedlings (bottom) of the wild type (WT), *sur1-8*, and *sur1-3* (left to right). Scale bar = 5 mm.

inflorescence were seen after several weeks of culture. These phenotypes were reminiscent of auxin effects. Some hyper-auxin mutants are well-known, including *yucca* (Zhao et al. 2001), *superroot 1 (sur1)/rooty (rty)/aberrant lateral roots formation 1 (alf1)/hookless 3 (hls3)* (Boerjan et al. 1995; Celenza et al. 1995; King et al. 1995; Lehman et al. 1996), and *sur2/rnt1* (Delarue et al. 1998; Barlier et al. 2000; Bak et al. 2001). Hereafter, *sur1/rty/alf1/hls3* and *sur2/rnt1* are referred to as *sur1* and *sur2*, respectively. Since the phenotype of our mutant was quite similar to *sur1-3* (Figure 2), we examined the sequence of the *SUR1* locus (Gopalraj et al. 1996) of this mutant to test the hypothesis that our mutant was allelic with *sur1*. A 36-bp deletion and 4-bp insertion were found in the fifth exon of *SUR1* (Figure 3). To determine whether the phenotypes of this mutant were caused by a mutation of *SUR1*, allelic examination was performed. The F1 plants from a cross between heterozygous mutants and *sur1-3* segregated for both wild-type (WT) plants (36 plants) and rooty plants (12 plants). Therefore, because this mutant is a new allele of *sur1*, we designated our mutant *sur1-8*.

The levels of endogenous free and conjugated auxin in *sur1* are higher than normal (Boerjan et al. 1995). In addition, *SUR1* is the *C-S* lyase in glucosinolate biosynthesis (Mikkelsen et al. 2004), and indole-3-acetoaldehyde is metabolized not only to indole-3-acetic acid (IAA) but also glucosinolate. As glucosinolate biosynthesis is defective in *sur1*, it is believed that the accumulated aldoximate is channeled to IAA, leading to a high-auxin phenotype in *sur1*. Thus, the development of excess adventitious and lateral roots in *sur1-8* mutants is attributable to an increased level of auxin. The 36-bp deletion and 4-bp insertion in the *SUR1* gene may be the “footprint” of the T-DNA insertion and dislocation.

Although Heller’s medium is generally used for root culture, modified Heller’s medium (HF medium) is better for hairy root culture (Mano et al. 1989). To determine whether HF medium was suitable for *sur1-8* root culture, the growth of *sur1-8* and WT plants was compared between HF and MS media. Then *sur1-8* and WT seeds were germinated on MS agar plates for 8 days and transferred to liquid HF or MS medium. After 1 week in rotary culture, more lateral roots had developed in *sur1-8* than in WT plants. Moreover, the lateral roots of *sur1-8* plants were shorter and thicker than those of WT plants.

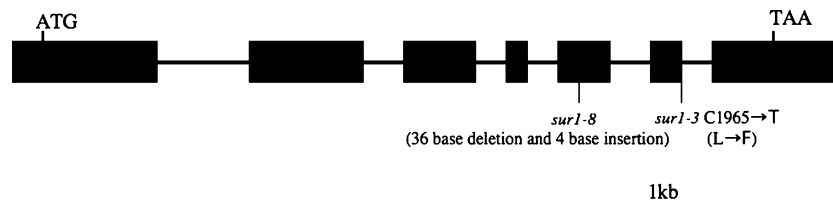


Figure 3. Structure of the *SUR1* gene. Mutation sites of *sur1-3* and *sur1-8*.

In *sur1-8* plants, more lateral roots developed in HF medium than in MS medium (Figure 4A). In WT plants, no difference was observed between HF medium and MS medium. After 1 month in rotary culture, the roots of *sur1-8* plants in HF medium were globe-shaped, and with appropriate transplants, the globular *sur1-8* cultured roots were 2 cm in diameter after 2.5 months of culture (Figure 4B). As this phenotype was similar to “marimo,” a globular alga (*Aegagrophila*) that inhabits Lake Akan in Japan, we called the globular *sur1-8* cultured roots “white marimo.” The cultured roots continued to grow even after 18 months of culture. The dry weight of *sur1-8* cultured roots was 3.5 and 38.7 mg/plant after 3 and 6 weeks of culture, respectively. Although the mechanisms by which *sur1-8* cultured roots become globular are not yet understood, we speculate that this occurs because the roots frequently ramify and twist.

To determine whether *sur1-8* cultured roots are practical for metabolite production systems, we generated double mutants, some fatty acid biosynthetic mutants in a *sur1-8* background, and examined fatty acid accumulation in these mutants. Since the *FAD2* and *FAD3* genes are responsible for fatty acid desaturation in roots, three mutants were used to construct double mutants with *sur1-8*: the *fad2-1* mutant defective in the

FAD2 gene encoding an enzyme that catalyzes desaturation from oleic acid (18:1) to linoleic acid (18:2; Okuley et al. 1994), the *fad3-2* mutant defective in the *FAD3* gene encoding an enzyme that catalyzes desaturation from linoleic acid (18:2) to α -linolenic acid (18:3; Browse et al. 1993), and *35S::BnFAD3* plants that overexpress the *Brassica napus FAD3* gene (Sakamoto et al. unpublished data). As the mutation site of *fad2-1* has not yet been identified, it was determined by sequencing the *FAD2* cDNA synthesized from total RNA extracted from *fad2-1* seedlings prior to generation of the *fad2-1 sur1-8* double mutant. We found that the 310th base, counted from the translational initiation point, was a G substituted for an A. Double mutants were grown on MS agar plates for 2 weeks, transplanted into liquid HF medium, and cultured for an additional 63 days. Then the fatty acids of approximately 1 mg dry weight of each double mutant were extracted and quantitatively analyzed based on Kodama et al. (1994). As shown in Figure 5, oleic acid, linoleic acid, and α -linolenic acid accumulated in the *sur1-8 fad2-1* double mutants, *sur1-8 fad3-2* double mutants, and *sur1-8 35S::BnFAD3*, respectively. The 70.3% accumulation of oleic acid in *sur1-8 fad2-1* plants was comparable to the 55.9% accumulation in the roots of *fad2-1* plants (Okuley et al. 1994). The 63.8% accumulation of linoleic acid in *sur1-8 fad3-2* plants was comparable to the 61.9% accumulation in the roots of *fad3-2* plants (Browse et al. 1993). The total fatty acid content was 6.36, 4.71, 5.92, and 5.49 mg g⁻¹ dry weight in *sur1-8*, *sur1-8 fad2-1*, *sur1-8 fad3-2*, and *sur1-8 35S::BnFAD3*, respectively, indicating that total fatty acid content is approximately equal in these mutants.

Note three important characteristics of the *sur1* root culture system. First, *sur1* contains fewer glucosinolates than the WT, and excess intake of glucosinolates leads to thyroid hypertrophy (Etienne and Dourmad 1994). Thus, the *sur1* root culture system is thought to be safe for producing medical intermediates or foods. Second, the

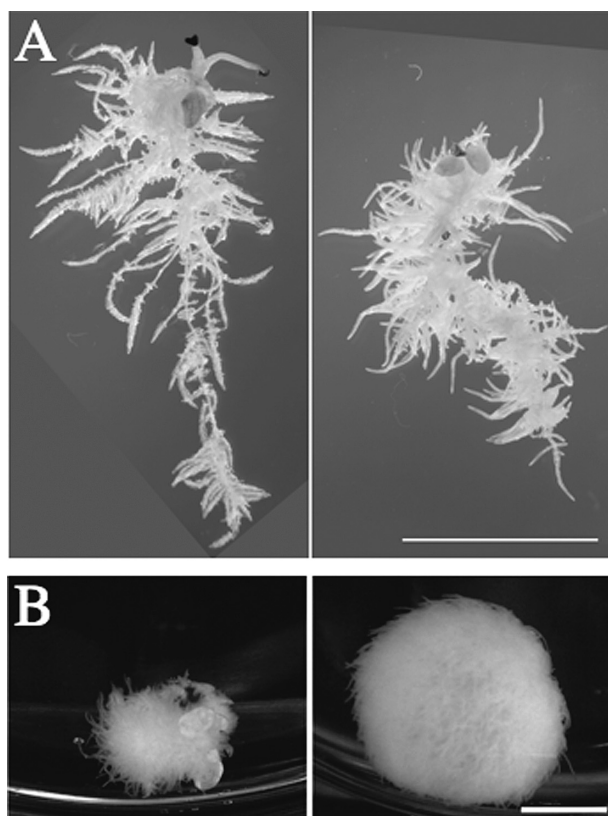


Figure 4. Phenotypes of *sur1-8* in liquid culture. (A) After 1 week of rotary culture in MS liquid medium (left) and HF liquid medium (right). Scale bar=1 cm. (B) After 1 (left) and 2.5 months (right) of rotary culture. Scale bar=1 cm.

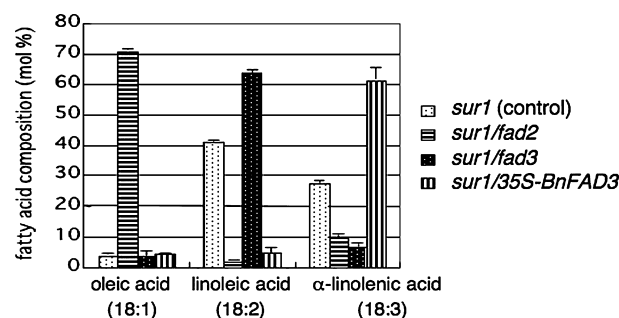


Figure 5. Fatty acids composition in cultured root of double mutants. Fatty acids were extracted and quantitatively analyzed from approximately 1 mg dry weight of each double mutant based on Kodama et al. (1994). Relative value of each fatty acid was shown. Extraction and analysis were at least three times.

sur1 culture system is convenient. As this culture system can be maintained by seeds in a *sur1* heterogenous line, this system need not be continually cultured, unlike other culture systems. Glucosinolate metabolism in which SUR1 is involved is found in the species of the order Brassicales, which include *Brassica* crops of economic and nutritional importance (Grubb and Abel 2006). It is possible that the defective of the SUR1 orthologue in these plant species would show the similar rooty phenotype as *sur1* in *Arabidopsis*, although it should be experimentally determined. The last characteristic is that *sur1* mutant is not transgenic plant. Higher IAA accumulating rooty plants have been previously reported (Sitbon et al. 1992). However, these plants were transgenic plants harboring bacterial genes. The *sur1* root culture system is useful for the production of metabolites by using non-GMO (genetically modified organism). We conclude that the *sur1* root culture system is an alternative tool for studying metabolic regulation and the production of useful compounds in roots.

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References

- Bak S, Tax FE, Feldmann KA, Galbraith DW, Feyereisen R (2001) CYP83B1, a cytochrome P450 at the metabolic branch point in auxin and indole glucosinolate biosynthesis in *Arabidopsis*. *Plant Cell* 13: 101–111
- Barlier I, Kowalczyk M, Marchant A, Ljung K, Bhalerao R, Bennett M, Sandberg G, Bellini C (2000) The *SUR2* gene of *Arabidopsis thaliana* encodes the cytochrome P450 CYP83B1, a modulator of auxin homeostasis. *Proc Natl Acad Sci USA* 97: 14819–14824
- Boerjan W, Cervera M-T, Delarue M, Beeckman T, Dewitte W, Bellini C, Caboche M, Onckelen HV, Montagu MV, Inzé D (1995) *superroot*, a recessive mutation in *Arabidopsis*, confers auxin overproduction. *Plant Cell* 7: 1405–1419
- Browse J, McConn M, James D, Miquel M (1993) Mutants of *Arabidopsis* deficient in the synthesis of α -linolenate. *J Biol Chem* 268: 16345–16351
- Celenza JL, Grisafi PL, Fink GR (1995) A pathway for lateral root formation in *Arabidopsis thaliana*. *Gene Dev* 9: 2131–2142
- Delarue M, Prinsen E, Onckelen HV, Caboche M, Bellini C (1998) *Sur2* mutations of *Arabidopsis thaliana* defines a new locus involved in the control of auxin homeostasis. *Plant J* 14: 603–611
- Etienne M, Dourmad JY (1994) Effect of zearalenone or glucosinolates in the diet on reproduction in sows-A review. *Livestock Product Sci* 40: 99–113
- Gopalraj M, Tseng T-S, Olszewski N (1996) The *Rooty* gene of *Arabidopsis* encodes a protein with the highest similarity to aminotransferases. *Plant Physiol* 111: 114
- Grubb CD, Abel S (2006) Glucosinolate metabolism and its control. *Trends Plant Sci* 11: 89–100
- Guillon S, Tremouillaux-Guiller J, Pati PK, Rideau M, Gantet P (2006) Harnessing the potential of hairy roots: dawn of a new era. *Trends Biotechnol* 24: 403–409
- King JJ, Stimart DP, Fisher RH, Bleecker AB (1995) A mutation altering auxin homeostasis and plant morphology in *Arabidopsis*. *Plant Cell* 7: 2023–2037
- Kodama H, Hamada T, Horiguchi G, Nishimura M, Iba K (1994) Genetic enhancement of cold Tolerance by expression of a gene for chloroplast ω -3 fatty acid desaturase in transgenic tobacco. *Plant Physiol* 105: 601–605
- Lehman A, Black R, Ecker JR (1996) *HOOKLESS1*, an ethylene response gene, is required for differential cell elongation in the *Arabidopsis* hypocotyl. *Cell* 85: 183–194
- Mano Y, Ohkawa H, Yamada Y (1989) Production of tropane alkaloids by hairy root cultures of *Duboisia leichhardtii* transformed by *Agrobacterium rhizogenes*. *Plant Science* 59: 191–201
- Mikkelsen MD, Naur P, Halkier BA (2004) *Arabidopsis* mutants in the *C-S* lyase of glucosinolate biosynthesis establish a critical role for indol-3-acetaldoxime in auxin homeostasis. *Plant J* 37: 770–777
- Okuley J, Lightner J, Feldmann K, Yadav N, Lark E, Browse J (1994) *Arabidopsis FAD2* gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *Plant Cell* 6: 147–158
- Seki H, Nishizawa T, Tanaka N, Niwa Y, Yoshida S, Muranaka T (2005) Hairy root-activation tagging: a high throughput system for activation tagging in transformed hairy roots. *Plant Mol Biol* 59: 793–807
- Sitbon F, Hennion S, Sundberg B, Little CHA, Olsson O, Sandberg G (1992) Transgenic tobacco plants coexpressing the *Agrobacterium tumefaciens iaaM* and *iaaH* genes display altered growth and indoleacetic acid metabolism. *Plant Physiol* 99: 1062–1069
- Suzuki M, Kato A, Komeda Y (2000) An RNA-binding protein, AtRBP1, is expressed in actively proliferative regions in *Arabidopsis thaliana*. *Plant Cell Physiol* 41: 282–288
- Teoh KH, Weathers PJ, Cheetham RD, Walcerz DB (1996) Cryopreservation of transformed (hairy) roots of *Artemisia annua*. *Cryobiol* 33: 106–117
- Touno K, Yoshimatsu K, Shimomura K (2006) Characteristics of *Atropa belladonna* hairy roots cryopreservation by vitrification method. *Cryo Lett* 27: 65–72
- Uozumi N (2004) Large-scale production of hairy root. *Adv Biochem Eng Biotechnol* 91: 75–103
- Zhao Y, Christensen SK, Fankhauser C, Cashman JR, Cohen JD, Weigel D, Chory J (2001) A role for flavin monooxygenase-like enzymes in auxin biosynthesis. *Science* 291: 306–309