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Hydrolysable Tannin Production in *Oenothera tetraptera* Shoot Tissue Culture

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Abstract

Shoot-generating tissue cultures of *Oenothera tetraptera* that produce polyphenolic compounds were established. The tissues, which were subcultured on LS agar medium containing 10 μM IAA and 10 μM kinetin, produced galloylglucoses and monomeric and dimeric ellagitannins. Plants were regenerated from the tissues upon transfer from the growth regulator-containing medium to hormone-free medium. Plant regeneration from *Oenothera* shoots was accompanied by changes in the composition of hydrolysable tannins (galloylglucoses to oligomeric ellagitannins).

Key words: ellagitannin oligomer, oenothein B, *Oenothera tetraptera*, polyphenol, regeneration, tannin biosynthesis.

Abbreviations

GG, *O*-galloyl-β-D-glucose; HPLC, high-performance liquid chromatography; IAA, indole-3-acetic acid; LS, Linsmaier and Skoog; NP, normal phase; RP, reverse phase.

Introduction

Plants of the genus *Oenothera* (Onagraceae) are native to North and South America. Although *O. biennis* and several other *Oenothera* species grow naturally throughout Japan, *O. tetraptera* does not, owing to its limited ability to adapt to various environmental conditions. Recently, we isolated a new trimeric tannin with a unique acyl group, oenotherin T_1, from *O. tetraptera* leaves and from *O. laciniata* callus cultures (Taniguchi *et al.*, 2002). Large amounts of oenotheins A and B and related tannins were also produced from *O. laciniata* calli (Taniguchi *et al.*, 1998). Oenotheins A and B are oligomeric ellagitannins with macrocyclic structures (Hatano *et al.*, 1990; Yoshida *et al.*, 1991), which have potent anti-tumor activity (Miyamoto *et al.*, 1993a, b). Oenothein B also has antiviral effects against the herpes simplex virus (Fukuchi *et al.*, 1989).

Plant regeneration from *O. hookeri* tissue culture has been reported (Kuchuk *et al.*, 1998). Fatty acids, flavonoids, and phenolic acids (gallic acid and ellagic acid) have been produced from regenerated plants of other *Oenothera* species (Skrzypczak *et al.*, 1998; Thiem *et al.*, 1999). In this paper, we describe the regeneration of *O. tetrapera* plants from tissue culture, a step towards the production of biologically active compounds from this plant.

Materials and Methods

Tissue culture induction

O. tetraptera was grown in the Medicinal Plant Garden of the Faculty of Pharmaceutical Sciences at Okayama University. Leaves were excised and sterilized with 10% antiformin plus 0.5% Tween-80 for 15 min. Leaf sections were placed on LS (Linsmaier and Skoog, 1965) agar supplemented with 10 μM IAA, 10 μM kinetin and 3% sucrose in the dark at 25 ℃. The same growth conditions were used for *O. laciniata* callus culture (Taniguchi *et al.*, 1998). After one month, shoot-generating tissue was produced from the leaf segments.

Culture conditions and strains

After one month, induced shoots were subcultured

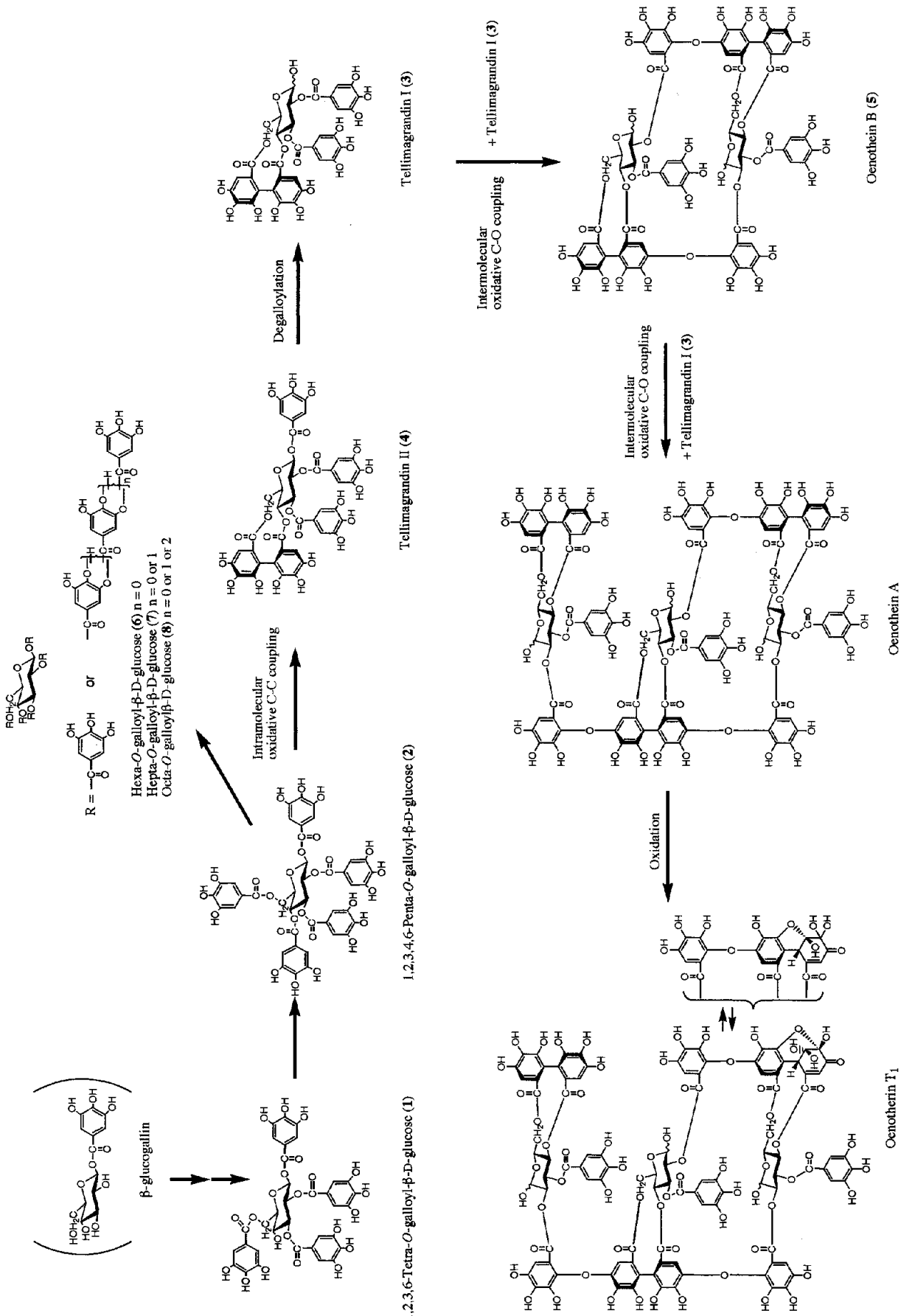

Fig. 1 Identity and biosynthetic relationship of hydrolysable tannins in *Oenothera tetrapterea* shoots and plantlets

on the LS agar medium above under white fluorescent light (3,000 lx., 12 h/day) at 25 ℃ for two years to establish the OtL strain.

OtL tissue was transferred to LS liquid medium containing the same growth regulators as the LS agar medium to generate suspended shoot-generating tissue (OtL liquid strain). These were subcultured every three weeks and grown on a rotary shaker (100 rpm) under fluorescent light. To examine the effect of light, 3.0 g of the OtL liquid strain was inoculated in 30 ml LS medium in 100-ml Erlenmeyer flasks and cultured either in the dark or with light. Tissue was harvested after 21 days in culture and analyzed by HPLC.

In a separate experiment, OtL tissue was transferred onto LS agar medium lacking growth regulators and subcultured each month on the same medium for 2 or 3 months (regulator-free OtF).

HPLC analysis of OtL tissue

MeOH extracts of OtL tissues were analyzed by NP- and RP-HPLC modes under the following conditions: NP-HPLC, YMC-pack SIL-60 (250 mm x 4.6 mm i.d.) column in a solvent consisting of *n*-hexane-MeOH-THF-HCO_2H (60:45:15:1) containing oxalic acid (500 mg/1.2 l), at a flow rate of 1.5 ml/min; RP-HPLC, YMC-pack ODS-A-303 (4.6 x 250 mm), in a solvent of 0.01 M H_3PO_4-0.01 M KH_2PO_4-CH_3CN (0-20 min, 9:9:2; 20-40 min, 9:9:2 to 3:3:2 linear gradient) at a flow rate of 1.0 ml/min at 40 ℃ . HPLC detection was done at 280 nm UV. GG identity in OtL extracts was further confirmed by methanolysis. Prior to methanolysis, the OtL extract (1.8 mg) was subjected to Sep-pak C18 (Waters), eluted with H_2O, then 20, 40, 60, 80, and 100% MeOH to remove large molecular weight tannins other than GGs. The 40 and 60% MeOH eluates, containing the GGs, were combined and added to 110 μl MeOH-AcOH (10:1) buffer (pH 5.6) at 37 ℃ for 19 h to produce 1,2,3,4,6-penta-GG (**2**), which was identified by NP- and RP-HPLC.

Quantitative analysis of tannins in culture and in seedlings

OtL and OtF tissues and acclimated plantlets were homogenized in MeOH. After centrifugation, aliquots of the supernatants were dried. Quantities of 1,2,3,6-tetra-GG (**1**), 1,2,3,4,6-penta-GG (**2**), tellimagrandin I (**3**), tellimagrandin II (**4**) and oenothein B (**5**) were determined by their HPLC peak areas, in comparison to standard amounts of the same compounds. Quantities of hexa- (**6**), hepta- (**7**) and octa-GGs (**8**) were estimated based on their respective peaks on NP-HPLC as compared to the 1,2,3,4,6-penta-GG (**2**) peak. Control seedlings were obtained from the garden and were treated similarly for quantitative analysis of tannins.

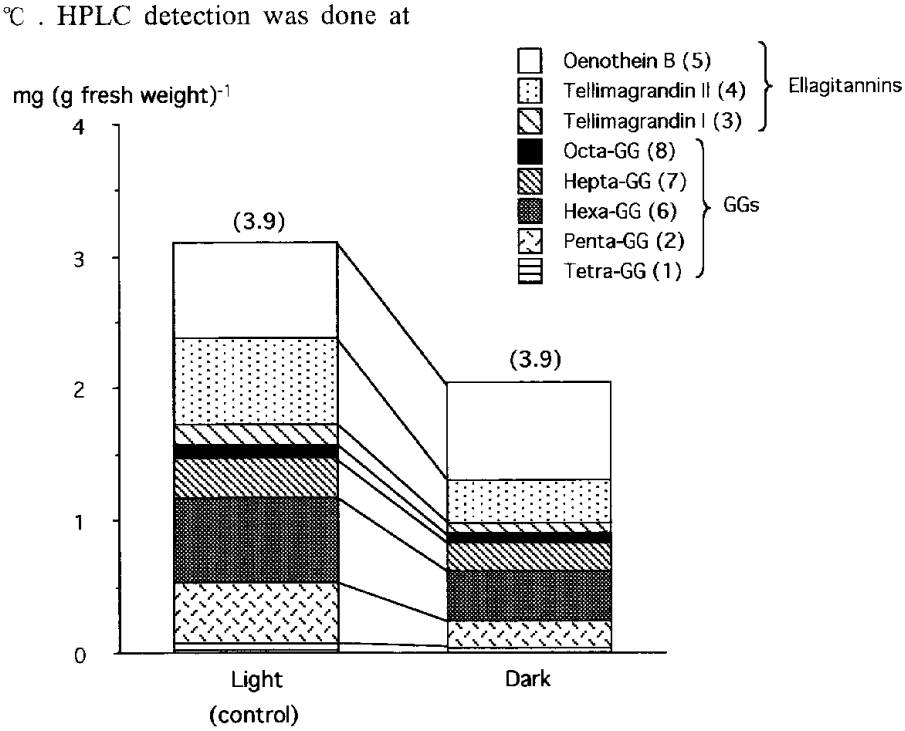

Fig. 2 Effects of light on cell growth and content of each tannin in shoot-generating *Oenothera tetraptera* tissues (OtL). Shoot tissues were cultured in LS liquid medium as described in Materials and Methods and harvested 21 days after inoculation. Cell growth (g fresh weight per flask) is shown in parentheses.

Results and Discussion

Constituents of OtL shoot-generating tissues

HPLC analysis of MeOH extracts of the OtL tissue revealed that it produced the following tannins: galloylglucoses [1,2,3,6-tetra-GG (**1**) and 1,2,3,4,6-penta-GG (**2**)], ellagitannin monomers [tellimagrandin I (**3**) and tellimagrandin II (**4**)], and an ellagitannin dimer [oenothein B (**5**)] (RP- and NP-HPLC). The NP-HPLC also indicated the presence of other tannins with molecular weights greater than that of penta-GG (**2**) but lower than that of oenothein B (**5**), as judged by their retention times (Okuda *et al.*, 1989). Those tannins were found to be hexa- (**6**), hepta- (**7**), and octa-GGs (**8**), based on methanolysis of the OtL extract, which produced 1,2,3,4,6-penta-GG (**2**). Structural comparisons of these compounds and oligomeric tannins based on their biosynthetic pathways are shown in **Fig. 1**. The GGs we identified (**1, 2, 6, 7** and **8**) were a series of products whose biosyntheses are derived from β-glucogallin (Niemetz *et al.*, 1999). Tellimagrandin II (**4**), a key intermediate in ellagitannin production, is thought to be derived from **2** by intramolecular oxidative C-C coupling of galloyl

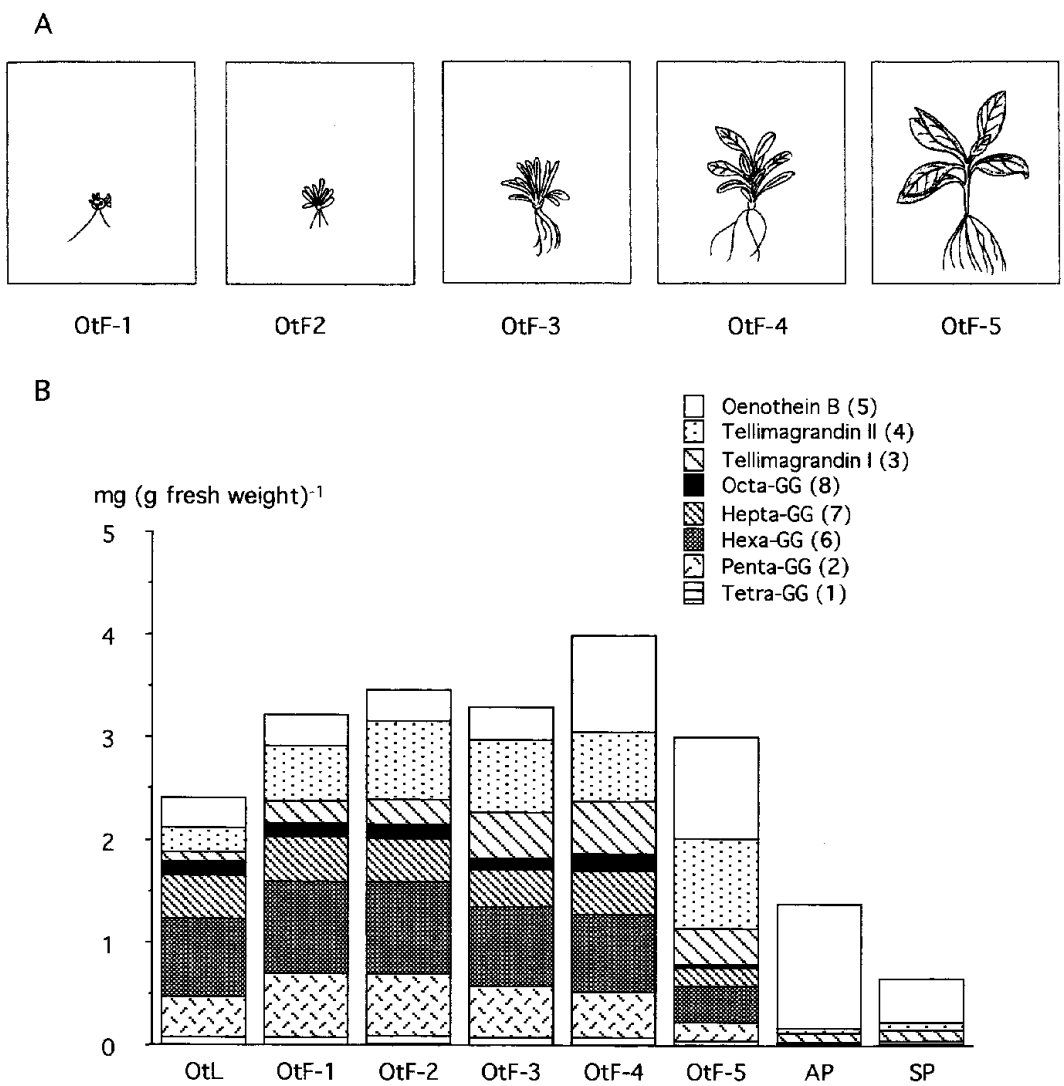

Fig. 3 (A) Morphology of OtF shoots and plantlets. Shoots and plantlets of the OtF tissues were obtained upon transfer of OtL shoots to plant regulator-free LS agar and were subcultured every month onto the same medium for 2 or 3 months. The OtF tissues were classified into five developmental stages (OtF-1 to OtF-5). The OtF-1 morphology was similar to that of OtL, and OtF-1 was less than 1.0 cm long. OtF-2 was between 1.0 and 1.5 cm long, with partially differentiated leaves. OtF-3 had narrow vitreous leaves 2-3 cm long. OtF-4 had differentiated stems and roots, and its leaves were no longer vitreous. OtF-4 was subcultured for an additional month on LS hormone-free medium to produce OtF-5. Bar = 1 cm. (B) Tannin contents in OtL, OtF-1 to OtF-5, acclimated plantlets (AP) and seedlings (SP).

groups (Haslam, 1989). Degalloylation of **4** gives **3**. Intermolecular C-O oxidative coupling of two molecules of **3** produces a dimer (**5**), and further intermolecular coupling of **3** and **5** produces oenothein A. Oenotherin T_1 is produced by the oxidation of oenothein A in plants.

Among the identified compounds in the cultured tissues, tetra- to octa-GGs (**1, 2, 6, 7** and **8**) were not detected in extracts from intact leaves, whereas the ellagitannins (**3, 4** and **5**) were found in leaves (data not shown).

Effects of light

OtL liquid strain tissues were cultured in the dark or in light for three weeks. Shoots cultured in the dark were brown, whereas OtL shoots grown in light were green. The effects of light on tannin production are summarized in **Fig. 2**. Cell growth and oenothein B (**5**) production were not affected by light. However, production of hydrolysable tannin monomers (both GGs and ellagitannin monomers) was lower in the dark.

Ellagitannin production and cell growth in *in vitro* cultures of *Heterocentron* roseum have been

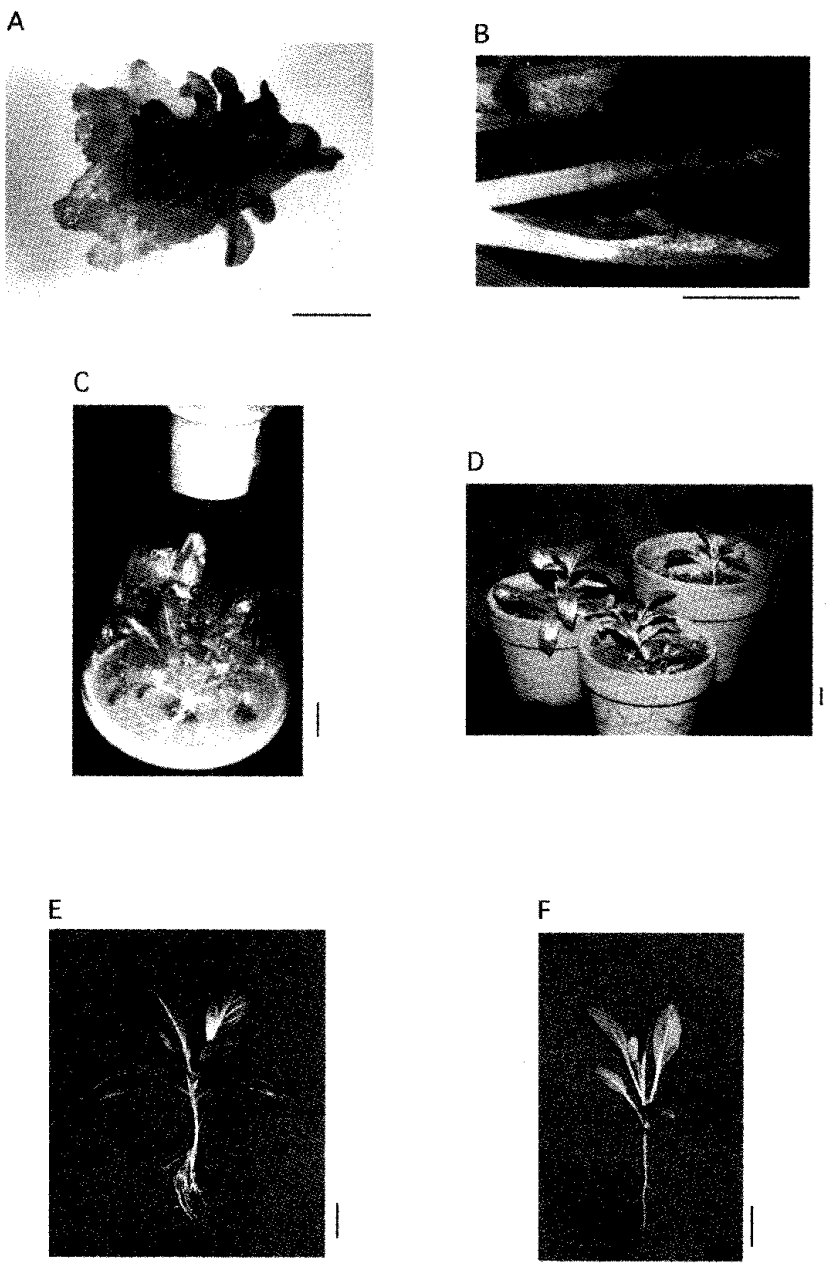

Fig. 4 Plant regeneration of *Oenothera tetraptera*. (A) Shoot-generating tissues of *Oenothera tetraptera* (OtL) were subcultured on LS agar containing 10 μM IAA, 10 μM kinetin and 3% (w/v) sucrose under illumination (3,000 lx., 12 h/day). (B) OtF-3 vitreous leaves. (C) Regenerated plantlet (OtF-5). (D) Regenerated plantlet, one month after transfer to soil. (E) Acclimatized plantlet harvested from soil. (F) *Oenothera tetraptera* seedling. Bar = 0.2 cm in (A), 1 cm in (B) to (F).

shown to be stimulated by light (Yazaki and Okuda, 1995). Recently, chloroplasts were reported to be a major site for the formation and deposition of hydrolysable tannins, particularly GGs (Grundhofer and Gross, 2001; Grundhofer *et al.*, 2001). These findings suggest that the accumulation of tannins in chloroplasts may help to absorb UV light and protect the photosynthetic machinery.

Regeneration of OtL shoots

We investigated further the regeneration of plantlets from OtL tissue. OtL tissue subcultured for two or three months on regulator-free medium produced shoots with various morphological features (OtF strain). **Fig. 3A** shows the morphological variations of the OtF tissues, which were classified according to their size and developmental stage (OtF-1 to OtF-5). Characteristic features and sizes of the stages are described in the legend. **Fig. 4** describes the changes that occur during the development of plantlets from shoot-generating tissues (OtL) through the various OtF stages. Leaves, stems, and roots differentiated, and vitreousness decreased from OtF-1 to OtF-4. OtF-5 was generated upon additional subculture of OtF-4 plantlets for one month on LS hormone-free medium (**Fig. 4C**). The OtF-5 plantlets were then transferred to pots and grown for one month to produce regenerating plantlets (**Fig. 4D, E**). Leaves of OtF-5 plantlets (**Fig. 4E**) were similar to seedling leaves (**Fig. 4F**), whereas their root shapes differed.

Constituents of shoots at different stages

We then analyzed tannins in shoots of different stages. The results are summarized in **Fig. 3B**. The morphological changes were accompanied by changes in tannin composition. OtL produced mainly GGs (**1, 2, 6, 7** and **8**). GG composition did not vary greatly among OtL and OtF-1 to OtF-4. Total GG content was significantly lower in OtF-5 and plantlets, however. Ellagitannin monomers (**3** and **4**) increased as shoots developed and then decreased in the plantlets. In contrast, oenothein B (**5**) content in OtL and in OtF-1 to OtF-3 started low and increased, along with a loss in vitreousness. Plantlets produced mainly oenothin B (**5**), as much as two times that made in seedlings. Trimeric tannins (oenothein A and oenotherin T_1) were also detected (ca. 0.1 mg per g fresh weight) by HPLC in both plantlets and seedlings; these trimeric tannins were detected in only trace amounts at other stages.

Changes in the major tannin structures in the various stages were in accord with the biosynthetic pathway: GGs (OtL, OtF1 to OtF-4) to ellagitannin monomer (OtF-5) to dimers (plantlets), accompanied by morphological changes (see **Fig. 1**). Our experiments showed that GGs were produced in small shoots and in developed shoots, but not in fully grown leaves. Similar observations were reported for tannin production in *Cornus officinalis* cell culture (Yazaki and Okuda, 1993), which demonstrated that GGs were produced in undifferentiated cells and ellagitannins were produced in the intact plant. The pathways from **2** to **6, 7,** and **8** and for ellagitannin monomer formation (**2** to **3** and **4**) in *Oenothera* tissues were activated by light. The mechanism by which light and organ development related to tannin production is still unclear.

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