In Vitro Regeneration of Castor (*Ricinus Communis* L.) Using Cotyledon Explants

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Abstract. An efficient plant regeneration protocol using cotyledon explants was established for castor (*Ricinus communis* L.), an important oilseed crop. Mature seed-derived cotyledon explants produced adventitious shoots when placed on Murashige and Skoog (MS) medium containing thidiazuron (TDZ). The rate of shoot regeneration was maximal (≈ 25 shoots per explant) when explants were cultured on shoot induction medium supplemented with 5 μM TDZ and preincubated in the dark for the first 7 days before transferring to the day/night cycle (16/8 h). Only the proximal ends of cotyledon explants produced adventitious shoots, although green calli were observed in cotyledon veins. After 4 weeks in culture, explants with well-developed shoot buds were transferred to MS medium without plant growth regulators for the shoot elongation and development. At ≈ 4 months after culture initiation, shoots (2 cm in length) were transferred to root induction medium (MS medium supplemented with 5 μM indole-3-butyric acid) where they developed roots in 4 to 6 weeks. Plantlets were transferred to soil and acclimatized to greenhouse conditions. Histological analysis showed the adventitious induction of the shoots originated from the cortical and epidermal cell layers of the cotyledon explants.

Castor (*Ricinus communis* L.), a semi-tropical perennial plant, is a valuable oilseed crop. It is the only commercial source of ricinoleic acid that is used for numerous industrial products (e.g., lubricants, paints, coatings, and plastics) (Caupin, 1997). However, the seed contains the ricin toxin (reviewed in Hartley and Lord, 2004; Lord et al., 1994) and hyperallergenic storage proteins (2S albumins) (reviewed in Pastorello et al., 2001; Shewry et al., 2002). When introduced into cells, the ricin toxin inactivates ribosomes, resulting in inhibition of protein synthesis (Endo and Tsurugi, 1987). 2S albumin was identified as the major allergen of castor seeds, bringing about an immunoglobulin E (IgE) response in 96% of castor-sensitive patients (Thorpe et al., 1988). These hazardous proteins pose serious

health concerns to farmers and oil plant workers greatly limiting its cultivation.

These hazardous proteins could potentially be reduced through genetic engineering. However, castor is highly recalcitrant to in vitro plant regeneration and genetic transformation. Recently, *Agrobacterium*-mediated transformation was reported in castor (Malathi et al., 2006; Sujatha and Sailaja, 2005) using meristems of embryonic tips as explants. However, the rates of putative transformant recovery were very low (0.08% and 0.42%, respectively). Because a number of independent transformants are typically required to identify a line with an appropriate level of transgene expression, the current transformation efficiency of castor needs to be improved. Considering the high rates of plant regeneration using embryonic tips (an average of 40 shoots per explant; Sujatha and Reddy, 1998), it is likely that the meristematic cells involved in plant regeneration were difficult to transform using *Agrobacterium*. To increase the transformation efficiency in castor, we are developing protocols for plant regeneration through adventitious shoot formation by testing various tissues from mature seed, including endosperm, embryo root, hypocotyl, and cotyledon. We found that hypocotyl (Ahn et al., 2007) and cotyledon (this report) were capable of producing adventitious shoots from cortex tissues at similar rates (≈ 24 and 25 shoots per explant, respectively). Additionally, the cotyledon also used epidermis to produce adventitious shoots, which would

provide accessible surface targets for the *Agrobacterium*-mediated or biolistic transformation of castor.

Materials and Methods

Explant preparation and culture initiation. Castor seeds (*Ricinus communis* L. PI PI215769) were obtained from the U.S. Department of Agriculture, Germplasm Resources Information Network, Southern Regional Plant Introduction Station (Griffin, GA). Seeds were decoated and surface-sterilized in a 5% (v/v) commercial bleach solution (5.25% sodium hypochlorite) for 15 min followed by five rinses in sterile deionized water. Cotyledon explants were aseptically isolated from embryo and placed vertically (distal side in contact with medium) on the shoot induction media [MS medium (Murashige and Skoog, 1962), 30 g·L⁻¹ sucrose, 0.5 g·L⁻¹ 2-(4-morpholino) ethanesulfonic acid (MES), pH 5.7] supplemented with 1, 5, or 10 μM thidiazuron (TDZ). For the first week in culture, half of the explants were preincubated in the dark and the other half were incubated under the day/night cycle (16/8 h) at 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ supplied by cool-white fluorescent lamps (Sylvania, Danvers, MA) at constant 26 °C.

Dry weight of cotyledon explants. Light grown (control) and dark preconditioned cotyledon explants (40 each) on 5 μM TDZ were harvested at the 7 d of culture initiation and dried at 70 °C for 3 d to measure the dry weight of the explants. The data were analyzed by the *t* test ($P < 0.01$) using Sigmaplot (Systat Software, Point Richmond, CA).

Adventitious shoot induction. After 7 d of the dark preconditioning, explants were transferred to the day/night cycle described previously. After an additional 3 weeks on shoot induction medium, explants with well-developed adventitious shoot buds at the proximal ends were placed on shoot development medium [MS medium (pH 5.7, 30 g·L⁻¹ sucrose, 0.5 g·L⁻¹ MES) without plant growth regulators]. When shoots reached 5 mm in length at ≈ 2 months, each adventitious shoot mass was divided into three to four shoot clumps. The number of shoots per cotyledon explant was counted at 4 months, when most shoots reached ≈ 2 cm in length. The data sets for adventitious shoot formation (Fig. 1) were analyzed by the *t* test ($P < 0.05$) using Sigmaplot (Systat Software). Cultures were transferred to fresh medium every 2 weeks. All the plant growth regulators were purchased from Phytotechnology (Shawnee Mission, KS). They were filter-sterilized and added to the autoclaved media. Experiments were repeated two to three times with 30 to 40 explants per condition.

Rooting. Regenerated shoots that were ≈ 2 cm in length were transferred to root induction medium [MS medium (pH 5.7, 30 g·L⁻¹ sucrose, 0.5 g·L⁻¹ MES) supplemented with 5 μM indole-3-butyric acid (IBA)]. After 4 to 6 weeks, rooted plantlets were transferred to a peat-vermiculite growth mixture and cultured in a greenhouse at temperatures ranging

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Fig. 1. Optimization of adventitious shoot production. Cotyledon explants dissected from mature seeds were placed on shoot induction media containing 1, 5, or 10 μM thidiazuron (TDZ). For the first week of culture initiation, cotyledon explants were cultured under the day/night (16/8 h) cycle (light) or in the dark (dark) at constant 26 °C. At 8 d, dark preconditioned explants were also transferred to the day/night cycle. After 4 weeks on the shoot induction media, explants with adventitious shoot buds were transferred to shoot development medium (Murashige and Skoog medium without plant growth regulators). The number of shoots regenerated per explant was calculated at 4 months when most of the shoots reached ≈ 2 cm in length. Values followed by the same letter are not significantly different at the level of 5% according to the *t* test (Sigmaplot; Systat Software, Point Richmond, CA). T1, 1 μM TDZ; T5, 5 μM TDZ; T10, 10 μM TDZ.

from 18 to 28 °C (night/day) under a 15/9 h (day/night) photoperiod with light supplemented by metal halide lighting at a photon flux density of 1000 to 1250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Transparent plastic covers were placed over the plantlets for the first 2 weeks for acclimatization.

Histological analysis. Cotyledon explants were dissected from dry seeds as described previously and placed vertically (distal side in contact with medium) on the shoot induction medium (MS medium supplemented with 5 μM TDZ). They were preincubated in the dark for 7 d and then placed under the day/night cycle (16/8 h) at constant 26 °C for another 7 d. In some cases, cotyledon petioles developed from the cotyledon explants and reached 2 to 3 mm in length at the end of the 7-d dark preincubation. The junction (5 mm) of cotyledon and cotyledon petiole with well-developed adventitious shoot buds was obtained at 10 and 14 d after culture initiation. Histological analysis was performed as described previously (Ahn et al., 2007). Briefly, tissues were incubated in fixing solution (formaldehyde, acetic acid, and ethanol) overnight and dehydrated in a series of ethanol solutions with increasing concentrations. Embedding was performed with Technovit 7100 kit (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. Cross-sections (3 μm) were obtained using a manual microtome and adhered to glass slides using water. Tissues

were stained with 0.1% toluidine blue (Serva, Heidelberg, Germany) and mounted using a synthetic medium (Permount; Fisher Scientific, Fair Lawn, NJ). Sections were visualized using a Zeiss Axiophot microscope (Zeiss, Jena, Germany).

Results

Effect of dark preconditioning and thidiazuron on adventitious shoot induction.

Our previous study showed that the dark preconditioning of hypocotyl explants in castor increased the size of the explants and the rate of plant regeneration, and TDZ induced more adventitious shoots than 6-benzylaminopurine (Ahn et al., 2007). To test the effect of dark preconditioning and different TDZ concentrations on explant growth and subsequent adventitious shoot induction, cotyledon explants were cultured on shoot induction media containing TDZ at 1, 5, or 10 μM with or without 7 d dark preconditioning. As shown in Figure 1, the light-grown control explants produced adventitious shoots at an average number of two, four, and seven, respectively. When explants were dark preconditioned, the average number of shoots regenerated increased slightly at 1 μM TDZ (six shoots per explant). However, the increase was not statistically significant based on the *t* test ($P < 0.05$). A dramatic enhancement in plant regeneration was observed on 5 μM TDZ. Dark preconditioned explants produced an average of 25

shoots per explant, representing an ≈ 6 -fold increase compared with the light-grown ones. At 10 μM TDZ, dark preconditioning did not enhance plant regeneration efficiency. Thus, shoot production was maximal when cotyledon explants were preincubated in the dark during culture initiation and treated with 5 μM TDZ.

Effect of dark preconditioning on explant development. During the initial 7 d in culture, the light-grown (control) explants did not enlarge much and accumulated chlorophyll slowly (Fig. 2A). Approximately 10% of the light-grown explants failed to produce chlorophyll until 4 weeks after culture initiation (data not shown). On the other hand, the dark preconditioned explants were much bigger than the light-grown ones (Fig. 2B). When their dry weights were compared, the former was ≈ 6 times heavier than the latter (Fig. 3). Although these data were shown for cotyledon cultures with 5 μM TDZ, similar results were obtained for the cultures with 1 or 10 μM TDZ (data not shown).

Adventitious shoot induction and plantlet formation. When the dark preconditioned cotyledon explants were transferred to the day/night cycle, they rapidly accumulated chlorophyll within a couple of days (data not shown) and gradually developed adventitious shoot buds at their proximal ends. Figure 2C shows a dark preconditioned explant with initial shoot buds (3 weeks after culture initiation, 5 μM TDZ). In some cases, cotyledon petioles also developed and adventitious shoot buds were formed at the junction of cotyledon and petiole. The shoot buds developed more vigorously on the adaxial surface of cotyledon explants. More than 90% of the dark preconditioned explants were responsive and produced adventitious shoot buds (data not shown). Small shoots started to be visible at 4 weeks after culture initiation (Fig. 2D). Then, explants were transferred to MS medium without plant growth regulators for shoot elongation. The adventitious shoots reached ≈ 5 mm in length at 2 months after culture initiation (Fig. 2E) and 1 cm at 3 months (Fig. 2F). When shoots reached 2 cm in length, at ≈ 4 months, individual shoots were transferred to root induction medium (MS medium supplemented with 5 μM IBA) and developed the root system in 4 to 6 weeks (Fig. 2G). The rooted plantlets were transplanted to a commercial peat-vermiculite growth mixture and successfully acclimatized in the greenhouse (Fig. 2H).

Histological survey. Transverse sections of the cotyledon explants developing adventitious shoot buds were examined to learn the origin of the regenerated shoots. In some cases, petioles developed from the cotyledon explants, and adventitious shoot buds were formed at the junction of cotyledon and petiole. Figure 4 shows the histological characteristics of the junction tissue producing adventitious shoot buds during the first 2 weeks of culture initiation. At 10 d, the cross-section of the junction tissue retained

Fig. 2. Adventitious shoot induction and plantlet establishment in castor. Cotyledon explants dissected from mature seeds were placed on Murashige and Skoog medium supplemented with $5 \mu\text{M}$ thidiazuron (TDZ). For the first week of culture initiation, (A) half of the explants were placed directly under the day/night cycle (16/8 h) and (B) the other half were preconditioned in the dark at constant 26°C . Pictures were taken at 7 d. From the second week, the dark preconditioned explants were also cultured under the day/night cycle. Adventitious shoot buds were induced at the proximal ends of cotyledon explants [(C), 3 weeks after culture initiation; (D), 4 weeks] and developed into multiple shoots [(E), 2 months; (F), 3 months] without morphological defects. (G) Regenerated shoots developed root system in 4 to 6 weeks when treated with $5 \mu\text{M}$ indole-3-butyric acid. (H) Plantlets were successfully acclimatized in the soil. Bars = 1 cm.

a typical structure of petiole where a concaved adaxial surface was still distinguishable (Fig. 4A). Active cell division was observed in the epidermal and cortical cell

layers of the adaxial side of the tissue. At 14 d, the concaved surface expanded outward and developed protrusions (Fig. 4B). When examined closely, leaf primordium-like struc-

tures were observed in the protrusions (Fig. 4C). Our results indicate that the adventitious shoot buds originate from the cortical and epidermal cell layers of the adaxial side of the cotyledon-petiole junction.

Discussion

This report describes the adventitious shoot formation and plant regeneration from cotyledon explants in castor. Plant regeneration has been achieved by using cotyledons as explants in a number of plant species. In most cases, cotyledon tissues were obtained from young seedlings such as in squash (*Cucurbita pepo*; Ananthakrishnan et al., 2003), bottle gourd (*Lagenaria siceraria* Standl.; Han et al., 2004), and an oilseed crop, niger [*Guizotia abyssinica* (L. f.) Cass.; Murthy et al., 2003]. In castor, we used cotyledon explants obtained directly from mature seeds, like in black locust (*Robinia pseudoacacia* L.; Zaragozá et al., 2004) and radiate pine (*Pinus radiata* D. Don; Grant et al., 2004). The proximal end of the cotyledon explant (the junction of cotyledon and cotyledon petiole) was proven to be highly regenerative in castor. Calli were often produced along the cotyledon vein but did not regenerate into shoots (data not shown). Similarly, the proximal tissues of cotyledon explants were much more responsive than the distal ones in soybean (*Glycine max*; Mante et al., 1989), squash (Ananthakrishnan et al., 2003), and bottle gourd (Han et al., 2004).

We have shown here that the dark preconditioning of cotyledon explants increased the size of explants and the number of adventitious shoots regenerated. The enlargement of dark preconditioned explants was observed at all the TDZ concentrations tested (1 , 5 , and $10 \mu\text{M}$). However, the number of shoots regenerated increased significantly (≈ 6 -fold) only when adventitious shoots were induced on $5 \mu\text{M}$ TDZ. When treated with $1 \mu\text{M}$ TDZ, the dark preconditioned explants did not induce shoot buds at a significantly higher level than the light-grown control ones. On $10 \mu\text{M}$ TDZ, numerous shoot buds were initially induced from the dark preconditioned explants. However, most of the shoots remained small in elongation medium free of TDZ and did not elongate further to develop into plantlets. Thus, the maximal plant regeneration efficiency was achieved by using the dark preconditioning of explants and optimizing the TDZ concentration for the adventitious shoot induction and subsequent development. Previously, the dark preconditioning of explants also enhanced the plant regeneration in castor when hypocotyl tissues were used as explants (Ahn et al., 2007).

In other plant species, there are a number of studies reporting the beneficial effect of the dark preconditioning of explants on the subsequent plant regeneration through organogenesis such as in watermelon (*Citrullus lanatus*; cotyledon explants; Compton, 1999) and Chinese plant jujube (*Zizyphus jujube* Mill.; leaf explants; Gu and Zhang, 2005).

Fig. 3. Dry weight of cotyledon explants. Explants dissected from mature seeds were cultured under the day/night (16/8 h) cycle (light) or in the dark (dark) for 7 d. Then, ≈ 40 explants per condition were harvested and dried at 70 °C. After 3 d, the dry weight of each explant was measured. The bars represent the SE of the means. Values followed by the same letter are not significantly different at the level of 1% according to the *t* test (Sigmaplot; Systat Software, Point Richmond, CA).

The exact mechanism of the enhanced shoot bud production by using dark preconditioning of explants is not clearly understood. As we discussed previously (Ahn et al., 2007), studies suggested that explants experience changes during the dark preconditioning on a molecular or cellular level, resulting in the enhancement of plant regeneration. Several characteristics of dark-grown tissues such as 1) preservation of light-sensitive endogenous or exogenous plant growth regulators (Hutchinson et al., 2000), 2) a higher level of undifferentiated cells (Herman and Hess, 1963), and 3) reduced cell wall thickness or cell wall deposits (Herman and Hess, 1963) may confer the regenerative capacity on the dark-grown cells.

TDZ has been found to be the most effective plant growth regulator to induce shoots from various explants in castor (Ahn et al., 2007; Sujatha and Reddy, 1998). Our previous study using hypocotyl explants revealed that TDZ induced adventitious shoots from the cortex (Ahn et al., 2007), similar to what we found in this study using cotyledon explants in which the surface tissues, including the cortex and epidermis, were involved in the formation of adventitious shoots. The adventitious shoot formation at the outer cell layers of cotyledon explants could be beneficial for the *Agrobacterium*-mediated or biolistic transformation of castor when applied, because those cells are readily accessible to the *Agrobacterium* or particles bombarded. We believe that this highly efficient and simple plant regeneration protocol will be useful for the development of efficient systems for genetic transformation in castor.

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Fig. 4. Histological analysis of adventitious shoot formation. Cotyledon explants were dissected from dry seeds and placed on shoot induction medium (Murashige and Skoog medium supplemented with 5 μ M thidiazuron). They were preincubated in the dark for 7 d and then placed under the day/night cycle (16/8 h) at constant 26 °C for another 7 d. Cross-sections of the cotyledon–petiole junction with adventitious shoot buds obtained (A) at 10 d after culture initiation and (B and C) at 14 d are shown. lp = leaf primodium-like structure. Bars = 500 μ m.

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