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Endosperm-derived triploid plant regeneration in diploid *Actinidia kolomikta*, a cold-hardy kiwifruit relative

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ABSTRACT

Endosperm culture is a unique technique for producing a triploid plant from diploid plants. In the present study, endosperm of a diploid cold-hardy kiwifruit relative, *Actinidia kolomikta* (Maxim. & Rupr.) Maxim., was cultured to regenerate triploid plants and expand ploidy variation in this mostly diploid species. Endosperm derived from fruits harvested 6–10 weeks after flowering (WAF) was used for the initial explants to examine the effect of plant growth regulators on callus formation and organogenesis. The endosperm-derived calli induced in MS medium with above 1.0 mg L^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D) were soft, small, and translucent, and no morphogenic response was observed. Conversely, the endosperm-derived yellow-green calli induced in the medium with 2,4-D (0.1 mg L^{-1}) plus kinetin (1.0, 2.0, and 5.0 mg L^{-1}) were hard, compact, fast growing, and showed adventitious shoot primordia. These primordia elongated into shoots when the calli were transferred to MS medium supplemented with zeatin (1.0, 2.0, and 4.0 mg L^{-1}) under continuous light conditions. The shoots developed roots on half-strength MS medium lacking plant growth regulators. In total, 14 plants were obtained. Flow cytometry and chromosomal analysis confirmed that the regenerated plants were triploid, suggesting that the plant tissue originated from the endosperm. This study reveals that the endosperm of *A. kolomikta* has plant regeneration abilities and that the regenerated plants have the same ploidy level as the endosperm (triploid). These results may be of use for ploidy manipulation or polyploidy breeding in other species in the genus *Actinidia*.

1. Introduction

The endosperm is a distinctive tissue in its origin, development, and ploidy level (Thomas and Chaturvedi, 2008). It is formed inside the seeds of most flowering plants as part of the double fertilization process. In double fertilization, two sperm cells are carried by the pollen tube into the ovule: one sperm nucleus fertilizes the egg cell to form a zygote, and the other fuses with the two polar nuclei in the central cell to form an endosperm cell. Thus, the endosperm cell contains three sets of chromosomes, i.e., it is a triploid (3x) cell. The endosperm surrounds the embryo and provides the nutrition necessary for embryo development. Under the normal developmental process, it degenerates or persists in some seeds.

The triploid nature of the endosperm suggests that the direct production of triploid plants from diploid plants is possible if plants could be regenerated from cultured endosperm. Conventionally, triploid plants are produced by crossing diploid plants with tetraploids that were induced from diploid plants by chemical treatment, such as colchicine; however, this process is laborious and time-consuming (Thomas and Chaturvedi, 2008). Endosperm culture has been suggested as an alternative and efficient method for triploid plant production (Thomas and Chaturvedi, 2008; Hoshino et al., 2011). Regeneration of plants with even higher ploidy levels such as hexaploidy might be possible in tetraploid plants; and, in interploid crosses, the estimated ploidy levels of endosperm-derived plants would be expected to vary. To date, plant regeneration from cultured endosperm has been reported in a wide range of fruit crops, e.g. *Malus* (Shihkin and Shuchiung, 1977; Mu and Liu, 1978), *Citrus* (Wang and Chang, 1978; Gmitter et al., 1990), *Prunus* (Liu and Liu, 1980), *Emblica* (Sehgal and Khurana,

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; 2,4-D, 2,4-dichlorophenoxyacetic acid; WAF, week (s) after flowering.

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1985), *Lycium* (Gu et al., 1985), *Annona* (Nair et al., 1986), *Pyrus* (Zhao, 1988), *Diospyros* (Tao et al., 1996), *Passiflora* (Mohamed et al., 1996), *Morus* (Thomas et al., 2000), *Lonicera* (Miyashita et al., 2009), and *Carica* (Sun et al., 2011). These successes in endosperm culture should be applied in other various fruit crops as means of ploidy manipulation.

The genus *Actinidia* comprises 76 species and approximately 120 taxa (Ferguson and Huang, 2007) distributed across a wide geographical range from the tropics (latitude 0°) to cold, temperate regions (latitude 50°N) in Southeast and East Asia (Huang et al., 2004). The genus includes commercially grown kiwifruit, *A. deliciosa* (A. Chev.) C. F. Liang & A. R. Ferguson and *A. chinensis* Planch., and is therefore horticulturally important. Other species in the genus with potential value as fruit crops include *A. arguta* (Siebold & Zucc.) Planch. ex Miq., *A. eriantha* Benth., *A. kolomikta* (Maxim. & Rupr.) Maxim., and *A. rufa* (Siebold & Zucc.) Planch. ex Miq. Thus far, plant regeneration from cultured endosperm has been reported in a few plant species in the genus *Actinidia*: *A. chinensis* (Gui et al., 1982), *A. chinensis* × *A. melanandra* (Mu et al., 1990), *A. arguta* × *A. deliciosa* (Mu et al., 1990), *Actinidia* spp. (Machno and Przywara, 1997), and *A. deliciosa* (Góralski et al., 2005). Machno and Przywara (1997) reported that no morphogenic response was observed in calli induced from the endosperm of *A. kolomikta* cultured on MS medium supplemented with 2,4 dichlorophenoxy acetic acid (2,4-D, 2.0 or 4.0 mg L^{-1}) and kinetin (5.0 mg L^{-1}).

Actinidia kolomikta (Miyama matatabi in Japan) is a dioecious and mostly diploid species (2n = 2x = 58) native to East Asia. In Japan, it is distributed in relatively cold temperate regions from central to northern Honshu and Hokkaido, and is highly cold tolerant. Therefore, it is of interest as a breeding material for kiwifruit cultivation in cold temperate climates. The leaves are attractive owing to their green to pink-colored variegation seen during flowering season and this species therefore also has potential as an ornamental plant. The smooth-skinned, edible fruits are rich in taste and flavor and contain a high amount of vitamin C (ascorbic acid): up to 800 mg per 100 g fresh weight at maturity (Asakura and Hoshino, 2016). However, the fruits are usually very small (about 1–3 g at fresh weight) and have a short storage life. Therefore, further crop improvement through breeding is necessary for the commercial cultivation of this species. This includes not only crossing *A. kolomikta* to kiwifruit or other related species, but also improving the species via elite selection, genetic transformation, somatic hybridization, mutagenesis, and ploidy manipulation.

Thus far, there have been no reports on the characteristics of triploid *A. kolomikta* plants. Gui et al. (1993) evaluated fruits and vegetative characteristics of endosperm-derived kiwifruit (*A. chinensis*) and reported that a few endosperm-derived triploid plants obtained were fertile and not parthenocarpic, and one triploid line had relatively small fruits compared to its parental line. This might be attributed to the seedless nature of the triploid plants. In such a case, one more increase in ploidy, i.e., to hexaploidy, by chromosome doubling through conventional colchicine or oryzalin treatment might be necessary. Wu et al. (2012) reported that colchicine-induced autotetraploid plants of *A. chinensis* showed a significant increase (up to 50–60%) in fruit size. Hexaploidy in *A. kolomikta* would be a desirable ploidy level for interspecific hybridization with *A. deliciosa*, a natural hexaploid species of kiwifruit that is grown worldwide, as unbalanced ploidy level in the parents could be a factor in failure of interspecific hybridization in *Actinidia* (Hirsch et al., 2001). Hence, one more increase in ploidy could bypass the barriers during crossing as well as increase fruit size. Efficient production of triploid plants by endosperm culture could be useful as a base for the production of hexaploid plants. In the present study, we sought to establish triploid plant regeneration from cultured endosperm in *A. kolomikta*.

2. Materials and methods

2.1. Plant materials

A single diploid female *A. kolomikta* vine (accession number: TY979) growing on Mt. Teine at Sapporo, Hokkaido, Japan was open-pollinated from late June to early July 2015. Fruit produced by the plant was collected for use in laboratory experiments.

2.2. Effects of developmental stages and preparation of the explants on callus induction

Here, we investigated effects of developmental stages and preparation of the explants on callus induction from endosperm. The fruit was collected weekly from 1 to 10 weeks after flowering (WAF). The fruit was surface-sterilized in sodium hypochlorite solution (1% active chlorine) with two drops of polyoxyethylene sorbitan monolaurate (Tween 20) for 10 min and then rinsed 3 times in sterilized distilled water. Sterilized fruit was placed in a Petri dish, and the seeds were excised under a dissection microscope. These processes of sample preparation are shown in Fig. 1. In seeds collected at 1–5-WAF, the endosperm and embryo were effectively inseparable; therefore, intact immature seeds were used as the explant material. In seeds collected at 6–10-WAF, the seed coat was removed to allow the embryo to be excised by pressing the seed gently from both lateral sides with forceps under a dissection microscope. The remaining endosperm was used as explant material. The explants were cultured on MS medium (Murashige and Skoog, 1962) with 2.0 mg L^{-1} 2, 4-D and 5.0 mg L^{-1} kinetin to test for callus induction. More than 10 explants were used in each experiment (10–15 explants per plastic Petri dish [90 mm × 20 mm]; 30 Petri dishes in total) and were cultured at 22 °C in total darkness. Cultures were transferred to fresh medium every 5–6 weeks. After 12 weeks of culture, callus induction and organogenesis were recorded.

2.3. Effect of plant growth regulators on callus induction from 7-WAF endosperm

MS media containing 20 combinations of plant growth regulators (0, 0.1, 1.0, 2.0, or 4.0 mg L^{-1} 2, 4-D as an auxin in combination with 0, 1.0, 2.0, or 5.0 mg L^{-1} kinetin as a cytokinin) were examined for callus induction, based on various studies on endosperm culture in the genus *Actinidia* (Gui et al., 1982; Mu et al., 1990; Machno and Przywara, 1997; Góralski et al., 2005). These experiments used 7-WAF endosperm. More than 10 explants (10–15 explants per plastic Petri dish; 22 Petri dishes in total) were cultured in each experiment. Other culture conditions were the same as in the experiments described above.

2.4. Effect of zeatin on shoot formation and elongation

Endosperm cultures using callus induction media were monitored for shoot primordium formation using a dissection microscope. To promote shoot elongation and stimulate additional shoot formation in calli, the cytokinin zeatin was included in the culture medium. Calli greater than ca. 5 mm in diameter were transferred to MS medium supplemented with 1.0, 2.0, and 4.0 mg L^{-1} zeatin. The cultures were incubated at 22 °C under continuous light conditions (35 μmol m^{-2} s^{-1}). The calli were transferred to fresh medium every 5–6 weeks. After 12 weeks of culture, the development of shoot formation from calli, or shoot elongation from calli with shoot primordia, was assessed.