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Production of a desirable *Brassica oleracea* CMS line using an alloplasmic *B. rapa* CMS line carrying *Diplotaxis erucoides* cytoplasm as a bridge plant

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Abstract

In *Brassica oleracea*, production of F_1 hybrid seeds mainly makes use of the improved *Ogura* cytoplasmic male sterile (CMS) line. However, reliance on one particular line is a risk, and it would be advantageous to develop other CMS lines. In this study, we transferred *Diplotaxis erucoides* cytoplasm to *B. oleracea* cultivars using an alloplasmic *B. rapa* CMS line as a bridge plant to avoid incompatibility between donor and recipient plants. The new *B. oleracea* CMS lines, which were derived by four generations of backcrossing, had small rudimentary anthers with no pollen grain and showed complete male sterility. There was no functional defect in other floral organs, and the ability to receive normal pollen did not appear to be impaired. Moreover, the *B. oleracea* CMS lines carrying *D. erucoides* cytoplasm had larger leaf areas and a normal plastochron. As a consequence, the *B. oleracea* CMS lines carrying *D. erucoides* cytoplasm have the potential to be valuable alternatives for use in commercial *B. oleracea* hybrid seed production.

KEYWORDS
alloplasmic line, *Brassica oleracea*, Brassicaceae, bridge plant, cytoplasmic male sterility, *Diplotaxis erucoides*, intergeneric hybridization

1 | INTRODUCTION

Brassica oleracea belongs to a plant family Brassicaceae, including 338 genera and 3,709 species (Warwick, Francis, & Al-Shehbaz, 2006), and composes the U triangle with *B. rapa* (AA), *B. nigra* (BB), *B. juncea* (AABB), *B. napus* (AACC) and *B. carinata* (BBCC) (U 1935). *B. oleracea* is one of the most important crop in the world and is highly valued in recent years because it contains a variety of glucosinolates (GSLs) that were reported to have potent anticancer activity and improve type 2 diabetes (Axelsson et al., 2017; Higdon, Delage, Williams, & Dashwood, 2007 & Li et al., 2010). F_1 hybrid seeds in *B. oleracea* are widely used because of their notably vigorous development caused by the heterosis (Tanaka & Niikura, 2006). Some F_1 hybrid seeds are produced using self-incompatibility (SI). However, it was reported that a few self-seed generates because SI system does not always work well under environmental fluctuations (Horisaki & Nikura, 2008). On the other hand, cytoplasmic male sterile (CMS) system is known to be less susceptible to environmental changes than SI system (Wan et al., 2014). It would be also responsible for the importance of CMS in F_1 hybrid breeding that amphidiploid *Brassica* species *B. napus* and *B. juncea* suppress SI (Yamagishi & Bhat, 2014).

The most major CMS in *B. oleracea* is referred as improved *Ogura* CMS discovered from Japanese radish (*Raphanus sativus* L.) (Bannerot, Loulidard, Cauderon, & Tempe, 1974; Dey, Bhatia, Sharma, Parkash, & Sureja, 2013 & Ogura, 1968). It is also used worldwide in F_1 seed production of *B. napus*, *B. juncea* and *R. sativus* (Dey, Sharma, Bhatia, Kumar, & Prakash, 2011; Dey, Sharma, Bhatia, Parkash, & Barwal, 2011; Kagami, Abamatsu, & Shiga, 1990). A variety

of alien cytoplasm has ever been introduced in *B. oleracea* such as that of *Diplotaxis muralis* (Shinada, Kikucho, Fujimoto, & Kishitani, 2006), *Moricandia arvensis* (Chamola, Balyan, & Bhat, 2013) and *Erucastrum canariense* (Chamola et al., 2013). However, these alloplasmic CMS lines have hardly ever been applied to F_1 hybrid seed production. Generally, availability of new CMS system in Brassicaceae crops raises the question of adverse effects associated with alien cytoplasm such as leaf chlorosis and poor female fertility (Yamagishi & Bhat, 2014). If new CMS system had no advantage compared to current CMS system, most major CMS systems would continue to be used in Brassicaceae crops. Therefore, the improved *Ogura* CMS system with no adverse effect has been relied on as a regular CMS system for the F_1 seed production in *B. oleracea* (Dey et al., 2013).

An epidemic of southern corn leaf blight against T-cytoplasmic maize led to the crises in the situation that hybrid seed production had largely depended on the particular CMS line (Havey, 2004). This case implied that it is at a potential risk just to keep using the particular CMS line in *B. oleracea* without developing an alternative CMS line.

Diplotaxis erucoides used as a cytoplasmic resource in this study is phylogenetically close to *Brassica* cultivated species (Lysak, Koch, Pecinka, & Schubert, 2005; Pradhan, Prakash, Mukhopadhyay, & Pental, 1992; Quiros, Ochoa, & Douches, 1988 & Vyas, Prakash, & Shivanna, 1995). *D. erucoides* cytoplasm conferred CMS in *B. juncea* (Bhat, Vijayan, Dwivedi, & Prakash, 2006) and *B. rapa* (Peng et al., 2015; Wan et al., 2013). We have ever tackled to produce *B. oleracea* line carrying *D. erucoides* cytoplasm by means of intergeneric crossing between *D. erucoides* and *B. oleracea*, and successive backcrossing with *B. oleracea*. However, we have never achieved an elimination of whole set of *D. erucoides* nuclear genome probably because a few *D. erucoides* chromosome is indispensable for maintaining female fertility (Jeong, 2008). Vyas et al. (1995) also reported that BC_1 progeny had not been yielded in the crossing between female *D. erucoides* and male *B. oleracea*. In order to avoid this difficult situation, we used *B. rapa* that maintain compatibility to *B. oleracea* as a bridge plant.

In this study, we developed the alloplasmic *B. oleracea* CMS line carrying *D. erucoides* cytoplasm using the alloplasmic *B. rapa* lines carrying *D. erucoides* cytoplasm as a bridge plant and revealed some superior characteristics of this CMS line.

2 | MATERIALS AND METHODS

2.1 | Plant materials

The seed parent used was *B. rapa* CMS line carrying *D. erucoides* cytoplasm (2n = 20, AA, Figure S1). The pollen parents used were eight cultivars of autoplasmic *B. oleracea* L. (2n = 18, CC), which are readily available in crossings because these are grown in our greenhouse: var. *capitata* cv. 'Hathukoi,' cv. 'Koiji,' cv. 'Youshin,' cv. 'Nakatekanran,' var. *gammifera* 'U-M1,' var. *acephala* cv. 'Aojiru-kale,' var. *gongylodes* 'U-KR1,' 'U-KW1.' 'Hathukoi' and 'Koiji' were provided by the Tohoku Seed Company, Japan. 'Nakatekanran' and 'Aojiru-kale' were provided by the Utane Seed Company, Japan. 'Youshin,' 'U-M1,' 'U-KR1' and 'U-KW1' were stocked from the Laboratory of Plant Breeding, Utsunomiya University, Japan.

2.2 | Production of hybrid progeny and amphidiploid plant

Interspecific hybridization between *B. rapa* CMS line carrying *D. erucoides* cytoplasm and *B. oleracea* was performed using a conventional bud pollination. Flower buds were emasculated one day before flowering, immediately pollinated with fresh pollen and then bagged for approximately one week. In a step to produce amphidiploid plants, apical meristem tissue at plants extending major leaves has been wrapped with cotton soaked with 0.1% colchicine solution for 48 hr. After this treatment, the other axillary buds except for treated apical meristem were removed. In BC_2 generation, we used a multiple pollination method, which pollinates a seed parent with pollen of eight different *B. oleracea* cultivars on four consecutive days from the date of the bud pollination.

2.3 | Investigation of chromosome number in hybrid progeny

Meiotic chromosome behaviour in pollen mother cells of F_1 plants was observed using the 1% acetic orcein smear method. The root tips of plants extending major leaves were preserved in 8-hydroxyquinolin solution during 5 hr and then were fixed in Farmer (ethanol:acetic acid = 3:1) at 4°C all night. Somatic chromosome number in the root tip cells of BC_1 plants and BC_2 plants was examined using the Feulgen stain squash method followed by 1% acetocarmine staining.

2.4 | PCR analysis

Total DNA of plants was extracted according to the CTAB method (Doyle & Doyle, 1987). We examined PCR analysis using following *orf108*-specific primers (forward primer; CCCGAAAATCAACTTC-TACTTATGAATAC, reverse primer; CTAAACCCCCCCGCCCGT-TAAA) reported by Naresh et al. (2016). The 10 μl reaction mixtures contained 10 × Buffer for KOD-Plus-Ver. 2 (TOYOBO CO., LTD., Osaka, Japan), 2 mM dNTPs, 25 mM $MgSO_4$, primers (10 μM each), KOD-Plus-polymerase (1 U/μl) in 0.2-ml tubes. PCR was carried out using a Program Temp Control system PC-320 (ASTEC Co., Fukuoka, Japan). PCR conditions were 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, and finally 72°C for 1 min. PCR products were mixed with loading dye and applied to a 2.0% agarose gel. After the electrophoresis, the gel was observed on a transilluminator after ethidium bromide staining.

2.5 | Histological observation of anther development

Flower buds of approximately 2.0–10.0 mm were collected from the top of the main stem. Collected fresh buds were fixed in FAA (formalin: acetic acid: ethanol = 1:1:18) at 4°C all night. Fixed flower buds were dehydrated using a graded ethanol series (50%, 70%,