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Full Length Research Paper

Efficient *in vitro* production of non-chimeric tetraploid in kale (*Brassica oleracea* L. var. *acephala*) and its evaluation as a functional crop

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Kale (*B. oleracea*var. *acephala*) is a kind of vegetables with high content of glucosinolates (GSL) such as glucoraphanin (GRA), which are known to be functional ingredients. In this study, efficient method of tetraploid production in kale was established by treating seeds, *in vitro*germinated5 day-old seedlings and *in vitro*-propagated plantlets having 3 or 4 leaves, with either colchicine or amiprophos-methyl (APM), followed by *in vitro* culture. The highest frequency of tetraploid plant production (24.0%) was obtained when the seeds were treated with APM at 9 mg l^{-1} for 24 hours. Moreover, non-chimeric tetraploid can be efficiently selected by measuring fresh weight 7 days after the treatment. These tetraploid plants had g bigger sizes of leaf, flower and pollen and more number of leaves than diploid, but showed comparable content of GRA to diploid.

Keywords: Amiprophos-methyl, *Brassica oleracea* var. *acephala*, colchicine, glucosinolate, *in vitro* culture, kale, tetraploid

Abbreviations: APM; amiprophos-methyl, GSL; glucosinolate, GRA; glucoraphanin

INTRODUCTION

Vegetables of *Brassica* species such as broccoli (*Brassica oleracea* var. *italica*), cabbage (*B. oleracea* var. *capitata*), kale (*B. oleracea*var. *acephala*) are known to have high amounts of functional substances such as glucosinolates (GSL) in addition to nutritional substances (Becerra-Moreno *et al*., 2013; Cao *et al*., 1996; Ou *et al*., 2002). Especially, kale has high amounts of GSL in addition to nutrients such as vitamins and minerals (Fahey *et al*., 2001; Podsędek, 2007) and is now widely used as the major ingredient for producing healthy foods and drinks such as vegetable juice.

GSL is hydrolyzed by myrosinase into isothiocyanate, which suppresses *Helicobacter pylori*, detoxifies carcinogen, and inhibits the cell division of carcinoma (Bonnesen *et al*., 2001; Kim *et al*., 2011; Nakamura *et al*., 2002; Srivastava and Singh, 2004; Talalay and Fahey, 2001; Zhang *et al*., 1994). Inhibitory effect of GSL on *Helicobacter pylori*-induced gastritis was also reported (Yamada *et al*., 2014).

Usefulness of polyploids has been reported for increasing the plant phenotype such as improved tolerance against water stress, extension of flowering period, and increase in organ size and in contents of secondary metabolites (Dhawan and Lavania, 1996; Fox and Duronio, 2013; Liu *et al*., 2011; Saleh *et al*., 2008). Especially, the increase in plant size and contents of secondary metabolites in induced polyploids has been reported in various species such as *Daturastramonium, D. innoxia* and *Hyoscyamusniger* (Berkov, 2001)*,Solanumcommersonii*(Caruso *et al*., 2011) and *Artemisia annua* (Banyai et al. 2010). Therefore, it is important to produce tetraploid in kale to evaluate its GSL content.

Generally, cholchicine, an alkaloid isolated from *Colchicum autumnale*, is widely used as a chromosome doubling agent to produce polyploids due to its action to bind tubulin, impairing its polymerization or promote microtubule depolymerization(Caperta *et al*., 2006). Other chromosome doubling agents, such as APM, butamifos, oryzalin, propizamide and trifluralin have also been used for the same purpose (Hansen and Andersen, 1996; Hansen and Andersen, 1998; Petersen *et al*., 2003) because they also inhibit spindle formation like colchicine, by inhibiting polymerization of tubulin and microtubule.

Thus, the objectives of this study were to establish efficient method of tetraploid production in kale by treating with antimitotic agents and to evaluate the amount of GSL and plant characteristics in the induced tetraploid plants.

MATERIALS AND METHODS

Plant materials

Seeds of kale “2010-91” (*Brassica oleracea*var. *acephala*L., 2n=18) produced at Nagano Vegetable and Ornamental Crops Experiment Station in Japan were used in this study. Seeds were surface-sterilized with 0.2% sodium hypochlorite solution for 10 min and rinsed

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with sterile distilled water 3 times. They were then inoculated onto 1/2 MS medium, which was 0.8% agar-solidified half-strength MS medium (Murashige and Skoog, 1962) supplemented with 20 mgl^{-1} sucrose, and incubated at 25±2°C under 16 h photoperiod at 30-40 $\mu mol\ m^{-2}\ s^{-1}$ with cool white fluorescent lamps. They were subcultured for multiplication by transferring shoot segments with 2–3 nodes to the fresh medium every month.

Induction of tetraploids

Surface-sterilized seeds, 5 day-old seedlings and 2 week-old plantlets derived from one seed having 3 or 4 leaves obtained 2 weeks after the subculture, were used for chromosome doubling treatment. Seeds were immersed in 25, 50, 250, 500, 1000 mg l^{-1} filter-sterilized colchicine and 3, 6, 9 mg l^{-1} autoclaved APM solutions at different concentrations in culture bottles (6 cm×13 cm) (Nihon Yamamura Glass Co., Ltd., Japan), and kept on the reciprocal shaker at 120 rpm for 24 hours. Treated seeds were washed three times with sterile distilled water and transferred to 1/2 MS medium. After 7 days of cultivation, seedlings obtained were individually subjected to measure the fresh weight and were subcultured every month as described above. Ploidy level was checked 3 and 6 months after the treatment.

Five day-old seedlings and 2 week-old plantlets were also immersed in each antimitotic agent, washed with sterilized distilled water and transferred to 1/2 MS medium. Individual seedlings and plantlets were subcultured every month by the standard method. Ploidy levels of these plant materialswere checked 3 months after the treatment and were further checked every 6 months until acclimatization. Diploid and tetraploid plants derived from the treatments of 2 week-old plantlets were maintained *in vitro* for more than 2 years by cutting the plantlets with 3 or 4 nodes into 1 or 2 nodal segments every month on 1/2 MS medium.

Flow cytometric analysis

After the chromosome doubling treatment, ploidy levels of the treated plants were checked with newly growing leaves by flow cytometry according to the method of Mishiba and Mii(2000) with slight modification using a Partec PA cytometer equipped with a mercury lamp (Partec, Germany). Ploidy levels were determined by comparing the position of dominant peaks at G0-G1 phase of the cell cycle between the original diploid plant and the treated plants. For staining released nuclei, about 0.1 g fresh weight tissues were chopped with a razor blade in 1.0 ml of solution composed of 100 mMTris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v) polyvinyl-pyrrolidone (PVP), 0.1% (v/v) Triton X-100 and 2 mg l^{-1} 4,6-diamidino-2-phenylindole (DAPI), pH 7.5, in a plastic Petri dish, and filtered through a 30 µm nylon mesh. The suspension of nuclei was subjected to flow cytometric analysis for determining the relative nuclear DNA contents on a linear scale histogram.

Cultivation and characterization of diploid andtetraploid plants

Each 10 diploid and tetraploid plants derived from one seed were sufficiently washed withrunning tapwater to

remove the adhering medium, planted to commercial soil (Lixil Viva co., Japan) in 9 cm pots, and grown in the greenhouse without any special acclimatization treatment. After two weeks of cultivation, these plants were transferred to 30 cm pots and were further grown in the greenhouse (December 2012 to March 2013). At flowering stage after 3 months of cultivation, plant height, leaf size, petiole length, stem size, and number of leaves were measured for phenotypic characterization.

Preparation of the extracts for evaluating glucosinolate contents

Extraction method of Takahata et al. (2006) was modified. Firstly, 200 µg of freeze-dried leaves of diploids and tetraploids were powdered by Multi-beads shocker (Yasui Kikai Co., Japan), added with 1 ml of 80% methanol and then incubated 10 min at 60°C for deactivation of myrosinase. Then samples were centrifuged with 15,000 rpm at 25°C for 5 min and supernatants were collected to new tubes. Extraction was repeated further two times for the residues, and totally 3 ml of methanol extracts were obtained. The crude extracts were applied to ion exchange column DEAE–Sephadex® A-25 (GE Healthcare Japan Co., Japan). The column was washed with 5 ml of distilled water and was treated with 75 µlsulfatase at 25°C overnight for desulphonation. Samples containing GSL were eluted with 5 ml of distilled water and filtrated through 0.20 µm syringe filter unit (Merck Ltd., USA) before analysis.

Glucoraphanin analysis by LC-MS/MS

Content of glucoraphanin (GRA) was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (LCMS-8030, Shimadzu, Japan) because it was reported that *B. oleracea* crops such as kale produce GRA as a major component of GSL (Kushad *et al.*, 1999). Commercially available GRA (Cayman Chemical Co., USA) was used as a standard sample. TSKgel ODS-100V 3µm (column size 2.0 mm×150 mm) (Tosoh co., Japan) was used as the analytical column. TSKgel Guard Cartridge Holder and TSKgel guardgel ODS-100V 3µm (column size 2.0 mmI.D.×1.0 cm) (Tosoh Co., Japan) were used as guard holder and cartridge, respectively. The mobile phase selected was (A) water and (B) acetonitrile (20%, v/v) applied at a flow rate of 1 ml min^{-1} in a gradient mode as follows: (i) 0 min (A/B: 100/0, v/v); (ii) 0–10 min (A/B: 10/90, v/v); (iii) 10–14 min (A/B: 100/0, v/v); (iv) 14–15 min (A/B: 100/0, v/v). The injection volume and column temperature were set at 2 µl and 35°C, respectively. The range of detection wavelength selected after examining the UV spectra collected was 190-340 nm as most of the glucosinolate absorption maxima were at this wavelength range. Full-scan LC–MS spectra were obtained by scanning from m z^{-1} 100 to 1000. The compound was identified by comparing the PDA, MS and MS/MS data (retention time, spectra, ions, and confirmation transition) with those obtained from a GRA standard solution.

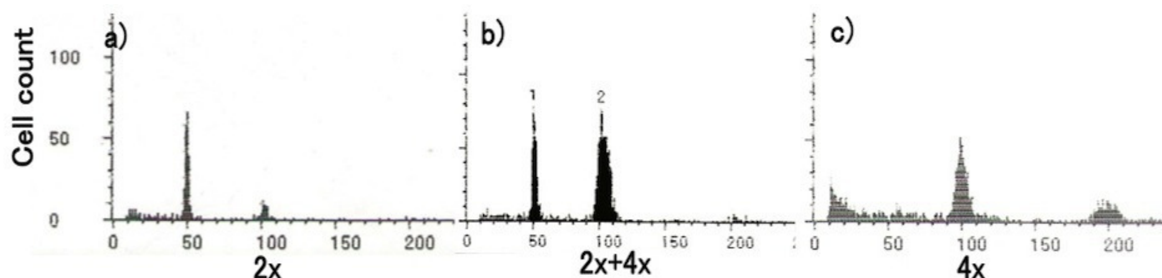

Figure. 1 Flowcytometric analysis of kale plants 3 months after chromosome doubling treatment. a) diploid, b) mixoploid with diploid and tetraploid, c) tetraploid.

RESULTS

Tetraploid production

Flow cytometric analysis revealed that both colchicine and APM treatments yielded tetraploids and mixoploids with 2x and 4x tissues at appropriate concentrations (Figure 1). In the seed treatment, APM was more effective to induce tetraploid than colchicine (Table 1). The highest frequency of tetraploids (24.0%) was obtained at 9 mg l^{-1} APM, followed by 8.7% at 6 mg l^{-1} APM, whereas colchicine treatment gave the frequencies of 4.7 and 6.7% at 500 and 1000 mg l^{-1}, respectively. In the treatment to 5 day-old seedlings, no obvious difference was found in the effects of colchicine and APM, and tetraploids were induced at the frequencies of 2.7 and 9.3% at 25 and 50 mg l^{-1} colchicine and 10.7% at 3 mg l^{-1} APM treatments, respectively (Table 1). In the treatment to 2 week-old plantlets after subculture showed a similar response to 5 day-old seedlings; tetraploids were induced at 3.9 and 7.8% at 50 and 250 mg l^{-1}colchicine, respectively, whereas 10.7% at 3 mgl^{-1} APM treatment (Table 1). In seed treatments, obvious difference was found in morphology among the seedlings 7 days after the treatments with both chromosome doubling agents as shown in Figure 1. All of the seedlings with stunted and swelled hypocotyls had less than 0.1 g fresh weight and revealed to be 100% tetraploid, whereas normally elongated seedlings with more than 0.15 g fresh weight were 100% diploid (Figure 2). The morphologically intermediate seedlings had the intermediate fresh weight between these two categories of seedlings and were detected to be chimera of diploid and tetraploid cells.

Characteristics of diploid and tetraploid

In *in vitro* condition, tetraploid kale had bigger leaves, thicker stem and more roots compared to diploid (Figure 3a, b). Both diploid and tetraploid plants were successfully grown in the greenhouse without any acclimatization treatment. After cultivation for 3 months in

Table 1 Effect of colchicine and amiprophos-methyl treatment on survival rate and chromosome doubling in kale with different developmental stages.

Plant material	Antimitotic agent	Concentration (mg l^{-1})	Survival rate (%)	A: Tetraploid (%)	B: Mixoploid (%)	A+B (%)
Seed	Colchicine	0	100±0.0 a	0.0 e	0.0 c	0.0 e
		25	100±0.0 a	0.0 e	0.0 c	0.0 e
		50	100±0.0 a	0.0 e	0.0 c	0.0 e
		250	100±0.0 a	0.0 e	0.0 c	0.0 e
		500	94.7±1.9 b	4.7±0.9 cd	2.7±1.9 ab	7.3±1.9 c
		1000	82.7±1.6 c	6.7±0.9 c	1.3±1.9 b	8.0±1.6 c
	Amiprophos-methyl	3	50.7±2.5 f	0.0 e	0.0 c	0.0 e
		6	22.0±2.5 i	8.7±0.9 bc	4.0±1.6 a	12.7±2.5 b
		9	26.0±4.1 hi	24.0±3.3 a	1.3±0.9 b	25.3±4.1 a
Five day-old seedling	Colchicine	0	61.3±1.9 e	0.0 e	0.0 c	0.0 e
		25	41.3±6.8 g	2.7±1.9 d	0.0 c	2.7±1.9
		50	26.7±1.9 h	9.3±1.9 b	0.0 c	9.3±1.9 bc
		250	14.7±1.9 j	0.0 e	0.0 c	0.0 e
		500	9.3±1.9 k	0.0 e	0.0 c	0.0 e
		1000	0.0 l	0.0 e	0.0 c	0.0 e
	Amiprophos-methyl	3	53.3±3.8 f	10.7±1.9 b	0.0 c	10.7±1.9 b
		6	6.7±1.9 k	0.0 e	0.0 c	0.0 e
		9	0.0 l	0.0 e	0.0 c	0.0 e
In vitro plantlet	Colchicine	0	100±0.0 a	0.0 e	0.0 c	0.0 e
		50	52.2±1.6 f	3.9±2.8 d	0.0 c	3.9±2.8 d
		250	31.7±3.6 h	7.8±1.6 bc	0.0 c	7.8±1.6 c
		500	20±0.0 i	0.0 e	0.0 c	0.0 e
		1000	7.8±1.6 k	0.0 e	0.0 c	0.0 e
	Amiprophos-methyl	3	67.3±1.5 d	10.7±1.9 b	0.0 c	10.7±1.9 bc
		6	6.6±1.9 k	0.0 e	0.0 c	0.0 e
		9	0.0 l	0.0 e	0.0 c	0.0 e

Ploidy level was checked with new leaves emerged 3 months after chromosome doubling treatment. Rate of tetraploid, mixoploid was calculated as (total number of tetraploid or mixoploid plants / total number of treated seeds or plants) × 100. Number of seeds, 5 day-old seedlings and *in vitro* plantlets with 3 or 4 leaves used were 50, 25 and 10 for each treatment, respectively. Treatment was repeated three times. Different letters indicate significant differences at $p<0.05$, as determined by Tukey's HSD test.

Figure 2 Three types of kale seedlings classified according to the fresh weight 7 days after chromosome doubling treatment of seeds with 1000 mg l^{-1} colchicine for 24 hours. a) ≦ 1.0 g, b) 1.0 - 1.5 g and c) ≧ 1.5 g. Bar = 10 mm. They were confirmed as tetraploid, mixoploid and diploid, respectively, by flow cytometric analysis as shown in Figure. 1.

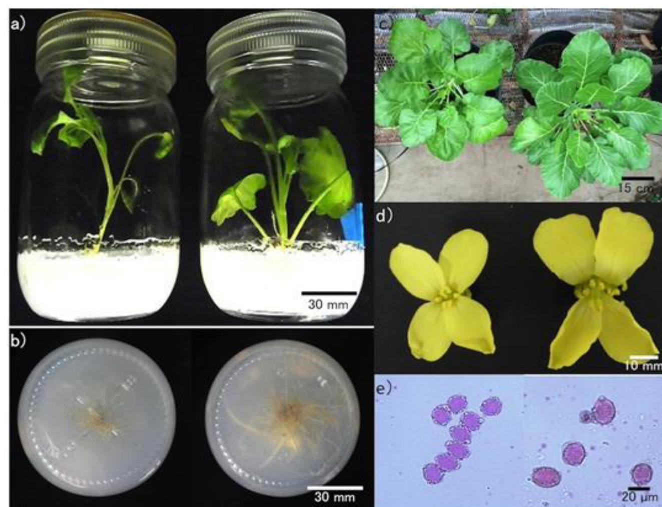

Figure 3 Characteristic of diploid and tetraploid plants of kale

a, b) shoot (a) and root (b) of *in vitro* cultured diploid (left) and tetraploid plants one month after subculture of stem node segment. c) diploid (left) and tetraploid (right) plants 3 months after transfer from *in vitro* to greenhouse condition. d) flowers of diploid (left) and tetraploid (right) produced 3 months after the culture in greenhouse. e) pollen of diploid (left) and tetraploid (right) stained with acetocarmine.

Table 2 Characterization of diploid and tetraploid in kale.

Ploidy level	Plant height (cm)	Maximum leaf length (cm)	Maximum leaf width (cm)	Petiole length (cm)	Number of leaves
Diploid	30.1±1.2 b	22.9±3.0 a	13.9±0.4 a	11.4±0.3 a	24.8±0.8 b
Tetraploid	37.2±1.0 a	26.8±1.9 a	12.4±0.7 a	11.1±0.7 a	30.3±1.5 a

Plant height, maximum length of leaf, maximum width of leaf, length of petiole and number of leaves were measured with 3 month-old plants in green house. Number of plants was 10 for a treatment. Different letters indicated significant differences at p<0.05, as determined by Tukey's HSD test after arc-sine transformation of the data.

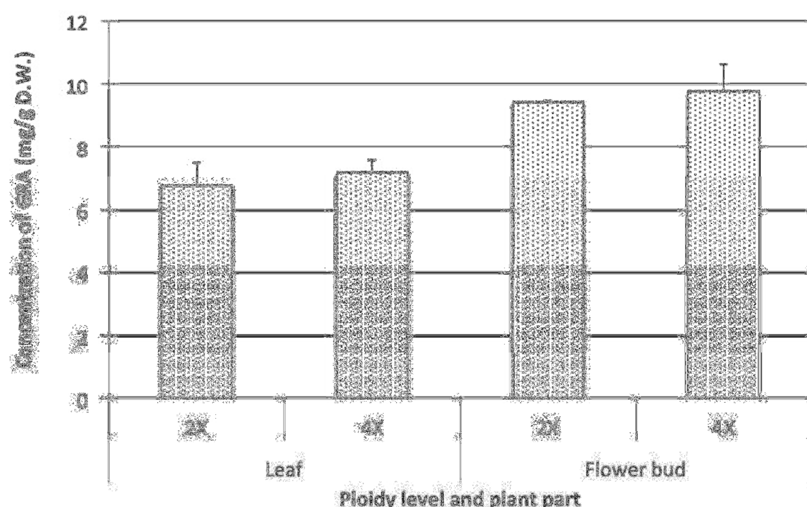

Figure 4 Glucoraphanin contents in leaf and flower bud of diploid and tetraploid in kale.

Leaves and flower buds were sampled from each ploidy plant. GSL contents were measured by LC-MS/MS with leaves of plants at 3 months and with flower buds at 3.5 months after acclimatization, respectively. The data were analyzed from at least 8 samples of each ploidy plant. Different letters indicate significant differences at p<0.05, as determined by Tukey's HSD test.

the greenhouse, they initiated to bloom and were subjected to the characterization of morphological traits. As the results, maximum leaf length, width and petiole length did not differ between diploid and tetraploid plants, but tetraploid had larger plant height and number of leaves (Table 2, Figure 3c), and larger flower and pollen sizes than diploid (Figure 3d,e). Although flower buds showed higher concentration of GRA (ca. 9.5-9.8 mg g^{-1} dry weight) than leaves (6.9-7.3 mg g^{-1} dry weight), no significant differences were found in the concentration of GRA between diploid and tetraploid plants in both organs (Figure 4).

DISCUSSION

It was reported that *in vitro* propagated plants have great potential for improving the efficiency of chromosome doubling, because *in vitro* cultures offer a more controlled and standardized environment than greenhouse and field cultivation for the treatment (Murashige and Nakano, 1966). Different explant types have successfully been used for the chromosome doubling: i.e., plantlets, shoots, buds, shoot tips, callus, somatic and zygotic embryos, seeds, seedlings, nodal segments and tuber segments (Dhooghe *et al*., 2011). Very recently, Kumar and Dwivedi(2014) succeeded to obtain tetraploids at the frequency of 10% by treating 7 day-old seedlings with colchicine, but they could not obtain any tetraploid plants from the seed treatment although the details on the method for the treatment was not described. In the present study, tetraploid plants were successfully produced through the treatments with colchicine and APM in *in vitro* condition by targeting kale at three different stages of development, which include seeds, 5 day-old seedlings and plantlets having 3 or 4 leaves obtained after repeated subcultures of a seedling. Especially APM was more effective to induce tetraploid than colchicine for all of these plant materials and seed treatment with APM gave the highest frequency of tetraploids (24%), which is more than twice the value reported by Kumar and Dwivedi(2014), suggesting that in vitro seed treatment with APM may also be effective for producing tetraploids in other Brassicaceae plants.

In crape myrtle (*Lagerstroemia indica*), tetraploids were selected based on morphological characters such as thicker stems, increased ratios of widthto length of leaf, larger stomata and higher number of chloroplast per guard cell (Zhang *et al*., 2010). However, only 50% of the plants selected as putative tetraploids by morphological screening were confirmed to be true tetraploid and the remaining half was the mixoploids of diploid and tetraploid, as shown in other studies (Allum *et al*., 2007; Dhooghe *et al*., 2009). In the present study, tetraploid of kale could be selected by morphology and fresh weight of seedlings 7 days after the treatment. These results indicate that APM treatment of seeds at 9 mg l^{-1} followed by selection with morphology and fresh weight is the most effective way for tetraploid production in kale.

It has been well known that polyploids generally show the alterations in plant morphology from its diploid counterpart (Otto and Whitton, 2000). Since both stomata and pollen sizes usually differ according to the ploidy level and tetraploid tends to have larger sizes compared to diploid (Hodgson *et al*., 2010), they have been used as simple markers for detecting tetraploid after chromosome doubling treatments (Cohen and Yao, 1996; Kadota and Niimi, 2002; Yang *et al*., 2013). Although in some species such as tobacco (Murashige and Nakano, 1966) and *Dendranthemanankingense*(Liu et al., 2011), induced tetraploids were smaller than the original diploids, tetraploid of kale produced in the present study became larger than diploid like as *Artemisia annua*(Banyai *et al*., 2010)and *Citrus*(Saleh et al., 2008). In the present study, morphological characters were also compared between diploid and tetraploid plants both originated from one seedling of kale. In both *in vitro* and greenhouse conditions, tetraploid showed bigger plant size than diploid. Although leaf size of tetraploid plants was not so differ compared to diploid plants, number of leaves was

increased in tetraploid. Therefore, total yield of biomass was increased in tetraploid.

In lemon grass (*Cymbopogonflexuosus*), a positive correlation was reported between the content of essential oil and the three different ploidy levels of plants including diploid, tetraploid and hexaploid(Janaki Ammal and Gupta, 1966). It has also been reported that tetraploids showed higher colonizing ability and greater diversity in flavonoid expression in *Lotus corniculatus* and *L. albinus*(Reynaud *et al.*, 1991) and enhanced nitrogen fixing ability in *Medicago sativa*(Shui *et al.*, 2010). In our study, concentration of GRA (mg g^{-1}) in tetraploid plants did not differ from that of diploid plants. Although the concentration of GSL such as GRA did not increase, tetraploid kale might be useful as a functional vegetable crop, because total yield per plant is higher than that of diploid due to the increase in plant size.

CONCLUSION

In the present study, highly efficient method of chromosome doubling of kale was established, which might also be applied to the other *Brassica* species and cruciferous plants. For further evaluation of tetraploids of kale obtained in this study, it is necessary to confirm the stability of the morphological characters and GSL contents in the successive generations in addition to the seed productivity at the tetraploid level.

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