In Vitro Segregation of Tetraploid and Octoploid Plantlets from Colchicine-induced Ploidy Chimeras in *Echinacea purpurea* L.

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Abstract. *Echinacea purpurea* L. is one of the important ornamental and medicinal plant species. Ploidy manipulation is a valuable tool for improving plant quality or production in *E. purpurea* as well as in many other plants. To study the segregation of pure ploidy plantlets from colchicine-induced ploidy chimeras in *E. purpurea*, we used a chimera plantlet that consisted of 1.93% diploid, 35.04% tetraploid, and 63.03% octoploid cells as the source material for experiments. The results showed that three factors significantly influenced the segregation, i.e., the component ratios of different ploidy cells in the chimera, the number of sequential passages, and the methods of segregation culture of the chimera plantlets. Other factors, such as explant types (i.e., leaf, petiole, or root) and 6-benzyladenine (BA) concentrations (i.e., 0.2, 0.4, 0.8, and 1.2 mg·L⁻¹) occasionally influenced the segregation. Pure chromosome-doubled polyploids are not easily obtained in various plant species, so segregation culture of ploidy chimeras may potentially be more effective. The morphological characteristic and content of cichoric acid were compared among diploid, tetraploid, and octoploid plants. Results indicated that tetraploid and octoploid plants had more stunted growth, larger stomata, lower stomata frequency, more chloroplast number in guard cells, and higher cichoric acid content than original diploid lines.

Echinacea purpurea is an important herbaceous plant primarily native to North America (McGregor, 1968) and has received considerable attention for its ornamental and medicinal value in recent years. *E. purpurea* has long been a popular ornamental garden plant and was widely cultivated in the world. The key ornamental attribute of this species is the flower head that has beautiful colors, long flowering period, and could attract honeybees and butterflies. The whole flowering head (containing disk and ligules) diameter ranges from 3.5 cm to nearly 18 cm (Ault, 2007). A number of ornamental cultivars have been

developed in *E. purpurea*, such as ‘Sparkler’ (Harini, 2006a), ‘Fancy Frills’ (Harini, 2006b), ‘Hope’ (Harini, 2006c), ‘Green Eyes’ (Harini, 2006d), ‘Raspberry Tart’ (Harini, 2008a), ‘Mars’ (Harini, 2008b), and so on. Except ornamental value, *E. purpurea* is one of the most well-known medicinal herbs in the world and has anti-inflammatory properties and immunoregulatory functions (Barrett, 2003). As its potential to replace antibiotics for treating human illness as well as livestock (Hudson, 2012; Landy et al., 2011; Pugh et al., 2008), the demand for *E. purpurea* products has increased vastly in recent years.

In breeding work, ploidy manipulation is a valuable tool for improving crop quality or production and polyploids promote evolution history significantly (Adams and Wendel, 2005; Leitch and Bennett, 1997; Otto and Whitton, 2000). Polyploidization often results in a wide range of changes on plants including morphology, physiology, cytology, and phytochemicals, and can also generate useful variants that provide innovative germplasm resources for breeding studies (Ramanna

and Jacobsen, 2003; Thao et al., 2003). Polyploidy induction has been adopted as an efficient breeding strategy in many plant species, such as *Astragalus membranaceus* (Chen and Gao, 2007), *Cymbopogon* (Lavania et al., 2012), *Dioscorea* (Zhang et al., 2010), *Humulus lupulus* (Roy et al., 2001), *Tanacetum parthenium* (Majdi et al., 2010), and *Zizyphus jujuba* ‘Zhanhua’ (Gu et al., 2005). Colchicine has been successfully and widely used in polyploidy induction process (Gu et al., 2005; Ning et al., 2009; Roy et al., 2001; Thao et al., 2003; Ye et al., 2010; Zhang et al., 2008). Colchicine is a compound that effectively arrests mitosis at the metaphase stage, so only the cells that are in metaphase stage could become polyploidized. However, the mitosis stages of the cells in meristematic tissues are not completely synchronous. Thus, we can find the areas where both original diploid and polyploidized cells are intermixed and are termed mixoploid or cytochimera (Dermen and Henry, 1944; Pryor and Frazier, 1968; Wan et al., 1989). This kind of chimera has frequently been observed in the process of chromosome doubling (Chen and Gao, 2007; Ning et al., 2009; Petersen et al., 2003; Roy et al., 2001; Thao et al., 2003).

In plant science, the term chimera refers to a plant that has at least two genetically different cell layers or groups as a result of mutation growing together in the apical meristem (Neilson-Jones, 1969; Tilney-Bassett, 1986). A chimera may develop in several ways: spontaneous mutation, chemical mutagenesis (such as colchicine treatment), grafting, or inheritance (Hartmann and Kester, 1990). Chimeras with different ploidy level cells are also called ploidy chimeras (i.e., mixoploid). Many previous studies used ploidy chimeras to trace the origination of each tissue (Dermen, 1953; Dermen and Stewart, 1973; Satina and Blakeslee, 1941; Satina et al., 1940) and investigate the fate of the apical or germ layers in the chimeras (Zonneveld, 2007). Chimeras can also be used to separate good variation for developing new cultivars in breeding work.

Polyploidization has also been confirmed to be highly effective in *E. purpurea*. In comparison with original diploid plants, *E. purpurea* tetraploid plants had a significantly higher biomass, higher contents of the main functional compounds, e.g., caffeic acid derivatives and alkamides, higher phenylalanine ammonia-lyase and cinnamate 4-hydroxylase activities, thicker roots that can facilitate harvesting and considerable larger stomatas, pollen grains, and seeds (Abdoli et al., 2013; Xu et al., 2013). Additionally, flower diameter of tetraploid plants was significantly larger than that in diploid plants (Abdoli et al., 2013). As we all know that flower is the key ornamental attribute of *E. purpurea*, so the ornamental value of tetraploid plants should be improved as compared with diploid lines.

During tetraploid induction in *E. purpurea* (Nilanthi et al., 2009a), chimeras were

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observed in almost all the treatments. This was similar to the reports in many other plant species, e.g., *Alocasia* (Thao et al., 2003), *Dioscorea zingiberensis* (Huang et al., 2010), *Miscanthus sinensis* (Petersen et al., 2003), *Petunia hybrida* (Ning et al., 2009), *Punica granatum* (Shao et al., 2003), and *Rosa rugosa* (Allum et al., 2007). In our study, we found more chimeras were produced than pure chromosome-doubled plants, and obtained a chimera plantlet simultaneously composed of diploid, tetraploid, and octoploid cells. This kind of chimera was observed for the first time in *E. purpurea*. Additionally, octoploid cells are only produced when diploid cell chromosomes double two times continuously, so we can infer that pure octoploid plantlets cannot be easily obtained in chromosome-doubling process. Furthermore, there are still no relative reports with respect to pure octoploid plantlets produced from chromosome doubling of diploid cells in *E. purpurea*. So this chimera plantlet is precious and segregating octoploid plantlets from it is important and compulsory. Using tissue culture method to separate chimeras into their consistent genotypes has been mostly accepted (Canli and Skirvin, 2003, 2008; Chen and Gao, 2007; Ning et al., 2009; Roy et al., 2001). The tissue culture system has been well established in *E. purpurea* (Choffe et al., 2000a, 2000b; Harbage, 2001; Koroch et al., 2002; Lakshmanan et al., 2002; Mechanda et al., 2003; Pan et al., 2004; Zobayed and Saxena, 2003). Adventitious shoot formation and shoot tip multiplication in vitro are the most common used methods for propagation in *E. purpurea* as well as in other plants (Manjula et al., 2015; Mazri, 2015; Nagar et al., 2015). Therefore, we used these two methods to segregate pure octoploid and tetraploid plantlets from the ploidy chimeras with the purpose of contributing to the segregation studies of other plant species.

Relative studies on segregation of ploidy chimeras were found in several plants (Huang et al., 2010; Ning et al., 2009; Roy et al., 2001; Shao et al., 2003). However, they just only separated chimeras into pure ploidy plants and did not describe the segregating process in detail. So the overall objectives of this research were: 1) to investigate the quantity changes of different ploidy level cells in the chimera plantlet; 2) to determine the effects of chimera type, explant type, sequential passage number, and BA concentration in segregation process; 3) to estimate the segregation efficiency of two methods; and 4) to compare the morphological characteristics and the important medicinal compound contents of diploid, tetraploid, and octoploid plants obtained in this study.

Materials and Methods

Plant source. The original diploid seeds were purchased from the Company of Plantation Products (Norton, MA) and were cultivated in the Garden of Chinese Medicinal Plants of South China Agricultural University.

The offspring seeds were collected and surface sterilized by immersion in 70% (v/v) ethanol for 30 s and 1% sodium hypochlorite water solution for 10 min. The sterile seeds were sown on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and cultured for 2 months. Then the in vitro plantlets were prepared for the following colchicine treatment.

Colchicine treatment and ploidy level determination. The leaf, petiole, and root explants (leaf explants: ≈ 0.6 cm², petiole and root explants: ≈ 0.8 cm in length) of in vitro diploid plantlets above were inoculated on MS medium containing 0.4 mg·L⁻¹ BA and 0.01 mg·L⁻¹ naphthaleneacetic acid (NAA) for 3-d pretreatment and then transferred to the fresh medium of same composition supplemented with 100 mg·L⁻¹ colchicine for different treatment periods (15, 22, 30, 37, and 47 d). After colchicine treatment, all the treated explants were transferred to the same and fresh medium with pretreatment and cultured for 45 d. Then the regenerated shoots from treated explants were cut and cultured on MS rooting medium containing 0.01 mg·L⁻¹ NAA to become intact plantlets. The ploidy level determination was based on the observation of chromosome number. The detailed procedures are described in our previous research (Nilanthi et al., 2009a). At least three actively growing root tips were sampled for each plantlet. More than 50 metaphase cells that had clear chromosomes and could judge ploidy level were observed for each root tip. The plantlets were considered as diploids when all cells contained 22 chromosomes, as tetraploids when all cells contained 44 chromosomes, and as octoploids when all cells contained 88 chromosomes. The plantlets that contained

two or more kinds of ploidy level cells were considered as chimeras.

Source chimera material and in vitro propagation. A chimera plantlet that simultaneously composed of diploid, tetraploid, and octoploid cells (Fig. 1A) arose in the treatment of petiole explant for 37-d colchicine duration. The ratios of diploid, tetraploid, and octoploid cells in this chimera plantlet were 1.93%, 35.04%, and 63.03%, respectively. The ratios were calculated as follows. Five actively growing root tips were sampled, and more than 50 clear metaphase cells were observed for each root tip. After counting the number of different ploidy level cells, the proportion percentages of three different ploidy level cells in each root tip was obtained. Then, the weighted average of the proportion percentages from five root tips was as the final percentage data. The clear chromosome pictures of diploid, tetraploid, and octoploid cell were presented in Fig. 1B–D.

The leaf, petiole, and root explants (leaf explants: ≈ 0.6 cm², petiole and root explants: ≈ 0.8 cm in length) were excised from the source chimera plantlet, and inoculated on MS medium containing 0.4 mg·L⁻¹ BA and 0.01 mg·L⁻¹ NAA for 45 d. Then the regenerated shoots of all explants were cut and cultured on the same and fresh medium for shoot tip multiplication culture. After 45 d, the multiplied shoots were cut and transferred to rooting medium containing 0.1 mg·L⁻¹ NAA to become intact plantlets. After 45 d, all the intact plantlets were examined the ploidy states. At least three actively growing root tips for each plantlet were sampled and the proportion percentages of diploid, tetraploid, and octoploid cells for each plantlet were calculated using the method described above.

Fig. 1. Root tip chromosomes of the source chimera material plantlet in *Echinacea purpurea*. (A) Diploid, tetraploid, and octoploid cells in the same microscopic view. (B–D) Chromosomes of diploid cell (2x = 22), tetraploid cell (4x = 44), and octoploid cell (8x = 88).

Classification of the chimera plantlets. Among all the plantlets propagated above, as diploid cells occurred in only a few plantlets with diploid cell ratios less than 9%, the plantlets with diploid cells were abandoned and only the plantlets with tetraploid and octoploid cells were counted. According to the proportion percentages of tetraploid and octoploid cells, the chimera plantlets propagated above were classified as below:

- Grade-1 chimera (chimeras with at least 1.5-fold more octoploid cells than tetraploid ones);
- Grade-2 chimera (chimeras with about equal numbers of octoploid and tetraploid cells); and
- Grade-3 chimera (chimeras with at least 1.5-fold more tetraploid cells than octoploid cells).

Segregation of tetraploid and octoploid plantlets from different chimera type plantlets

Adventitious shoot formation culture. Leaf, petiole, and root explants were excised from three kinds of chimera types, respectively, and inoculated onto MS medium containing $0.01 \text{ mg}\cdot\text{L}^{-1}$ NAA and different BA concentrations ($0.2, 0.4, 0.8,$ and $1.2 \text{ mg}\cdot\text{L}^{-1}$) to induce adventitious shoots (Fig. 2A–C). Each treatment consisted of six bottles and five explants in each bottle. After 45 d, the regenerated shoots were transferred onto MS rooting medium containing $0.1 \text{ mg}\cdot\text{L}^{-1}$ NAA. Then their ploidy states were confirmed using the methods described above. The remaining part of the original explants was subcultured to the same and fresh shoot formation medium to induce adventitious shoots for the second passage. The culture periods for adventitious shoot formation and rooting were both 45 d. This was repeated twice (a total of three sequential passages). All the shoots propagated in three sequential passages were examined the ploidy states.

Shoot tip multiplication culture. Shoot tips of Grade-1 and Grade-2 chimeras were inoculated onto the same medium with adventitious shoot formation culture to stimulate the growth of the axillary shoots (Fig. 2D). Each treatment consisted of six bottles and three shoot tips were inoculated in each bottle. After 45 d culture, the multiplied axillary shoots were isolated and divided into two groups. A group was inoculated for root induction and later ploidy state confirmation, and the other group was inoculated to the same and fresh shoot multiplication medium for the second passage. The culture periods for multiplication and rooting were both 45 d. This was repeated twice (a total of three sequential passages). All the shoots propagated in three sequential passages were examined the ploidy states.

Medium preparation and culture conditions. All of media contained MS basal medium elements, 3% sucrose and 0.5% agar (Company of Jiafeng Horticultural Products, Shanghai, China). The media was adjusted to

a PH of 5.8 and autoclaved at 104 kPa at 121°C for 15 min. Each 220-mL bottle contained 35 mL medium. All the cultures in this study were maintained under lighted conditions with a 12-h photoperiod under cool-white light ($\approx 50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) in a room with $25\text{--}27^\circ\text{C}$.

Cichoric acid determination. The above-ground and underground parts of 2-month-old diploid, tetraploid, and octoploid plantlets were dried with hot air at 50°C for 72 h and ground to a fine powder using a porcelain mortar. The powder was sieved using a 200 mesh sieve. Accurately weighed

Fig. 2. Adventitious shoot formation and shoot tip multiplication culture of Grade-2 chimera in *Echinacea purpurea*. (A–C) Leaf, petiole, and root explant in adventitious shoot formation culture. (D) Shoot tip explant in shoot tip multiplication culture. Bar = 1 cm.

Fig. 3. Morphological comparison of in vitro growing plantlets of various ploidy level states in *Echinacea purpurea*. (A) Grade-1 chimera, (B) Grade-2 chimera, (C) Grade-3 chimera, (D) octoploid, (E) tetraploid, (F) diploid. Bar = 1 cm.

0.1 g of the sample powder was extracted with 10 mL of 70% ethanol for 30 min assisted by ultrasonic treatment (40 kHz). The extracted solution was filtered with filter paper. The supernatant was collected and diluted with 70% ethanol to 10 mL. The solution was filtered again through a 0.45 µm microporous membrane, and the filtrate was used as the sample solution for the determination of cichoric acid. Reference standard of cichoric acid was dissolved in appropriate volume of 70% ethanol and diluted to obtain solutions of 0.02, 0.04, 0.06, 0.08, and 0.1 mg·mL⁻¹.

The amounts of cichoric acid were analyzed by high-performance liquid chromatography (HPLC). Each sample was conducted in triplicate. Equal amounts (10 µL) of each replication were automatically injected into the HPLC system (1260 Infinity; Agilent) equipped with a Inertsil/WondaSil C18 column (250 × 4.6 mm, pore size 5 µm; Shimadzu). The mobile phase was an isocratic 25% (v/v) acetonitrile:75% water containing 1.8% (v/v) acetic acid at rate of 1 mL·min⁻¹ for 20 min. The ultraviolet detector G1314F was set at 330 nm. Cichoric acid standard was purchased from Sigma. The cichoric acid concentrations were measured on a dry weight basis (mg·g⁻¹).

Stomata and chloroplast measurement. Length, width, frequency of stomata, and chloroplast number in guard cells were measured in the abaxial side of mature leaves. Stomatal length and width and number of chloroplasts per stomatal guard cell (stained with a drop of distilled water) were measured under magnification of ×400 with Axio Observer A1 microscope (Zeiss). The stomatal density was estimated by the mean number of stomata counted from five different microscopic fields of view at ×200 magnification.

Statistical analysis. All the data were statistically analyzed using SPSS 17.0 software. Significant differences between means were determined using Duncan's multiple range test at $P \leq 0.05$.

Results

Morphology of different chimera types. The ploidy level noticeably influenced the morphology of the chimeras (Fig. 3). Grade-1 chimera (Fig. 3A) presented more stunted growth, darker green pigmentation, and thicker and rough textured leaves as compared with Grade-2 (Fig. 3B) and Grade-3 chimeras (Fig. 3C). Grade-1 chimera with higher ratio of octoploid cells had thicker roots, hairier leaves, and were closer in shape to pure octoploid plantlets (Fig. 3D). Grade-3 chimera with higher ratio of tetraploid cells had thinner roots, hairless leaves, and were closer in shape to pure tetraploid plantlets (Fig. 3E). The morphology of Grade-2 chimera was between Grade-1 and Grade-3 chimeras.

Segregation of tetraploid and octoploid plantlets from Grade-1, -2, and -3 chimeras in adventitious shoot formation culture. The segregation results are presented in Table 1. Grade-1 chimera segregated a high proportion

Table 1. Effect of chimera types, sequential passage numbers, and 6-benzyladenine (BA) concentrations on the ploidy state of shoots regenerated from petiole explants of Grade-1/-2/-3 chimera in adventitious shoot formation culture in *Echinacea purpurea*.

Chimera type	Passage no.	BA (mg·L ⁻¹)	No. of plantlets examined	Ploidy state		
				Tetraploid	Octoploid	Chimera
Grade-1	1	0.2	19	0 (0)	19 (100)	0 (0)
		0.4	9	0 (0)	7 (77.78)	2 (22.22)
		0.8	12	0 (0)	8 (66.67)	4 (33.33)
		1.2	6	0 (0)	5 (83.33)	1 (16.67)
	2	0.2	11	0 (0)	10 (90.91)	1 (9.09)
		0.4	26	0 (0)	13 (50.00)	13 (50)
		0.8	22	0 (0)	14 (63.64)	8 (36.36)
		1.2	29	0 (0)	21 (72.41)	8 (27.59)
	3	0.2	18	0 (0)	8 (44.44)	10 (55.56)
		0.4	14	0 (0)	0 (0)	14 (100)
		0.8	17	4 (23.53)	0 (0)	13 (76.47)
		1.2	29	0 (0)	7 (24.14)	22 (75.86)
	Total		212	4 (1.89)	112 (52.83)	96 (45.28)
Grade-2	1	0.2	53	0 (0)	0 (0)	53 (100)
		0.4	38	0 (0)	0 (0)	38 (100)
		0.8	71	0 (0)	0 (0)	71 (100)
		1.2	70	0 (0)	0 (0)	70 (100)
	2	0.2	5	0 (0)	4 (80.00)	1 (20)
		0.4	21	0 (0)	2 (9.52)	19 (90.48)
		0.8	17	0 (0)	3 (17.65)	14 (82.35)
		1.2	54	0 (0)	1 (1.85)	53 (98.15)
	3	0.2	6	0 (0)	0 (0)	6 (100)
		0.4	110	0 (0)	6 (5.45)	104 (94.55)
		0.8	149	17 (11.41)	2 (1.34)	130 (87.25)
		1.2	80	0 (0)	0 (0)	80 (100)
	Total		674	17 (2.52)	18 (2.67)	639 (94.81)
Grade-3	1	0.2	19	0 (0)	0 (0)	19 (100)
		0.4	38	7 (18.42)	0 (0)	31 (81.58)
		0.8	28	6 (21.43)	0 (0)	22 (78.57)
		1.2	24	16 (66.67)	0 (0)	8 (33.34)
	2	0.2	21	16 (76.19)	0 (0)	5 (23.81)
		0.4	9	5 (55.56)	0 (0)	4 (44.44)
		0.8	55	33 (60)	0 (0)	22 (40)
		1.2	19	8 (42.11)	0 (0)	11 (57.89)
	3	0.2	6	4 (66.67)	0 (0)	2 (33.33)
		0.4	7	5 (71.43)	0 (0)	2 (28.57)
		0.8	60	50 (83.33)	0 (0)	10 (16.67)
		1.2	80	65 (81.25)	0 (0)	15 (18.75)
	Total		366	215 (58.74)	0 (0)	151 (41.26)

of octoploid plantlets and a little number of chimeras in the first passage. In the second and third passages, proportion of octoploid plantlets decreased and proportion of chimeras increased continuously. A little proportion of tetraploid plantlets was segregated in the third passage. The majority of the recovered plantlets were octoploid plantlets (52.83%) and chimeras (45.28%) in three sequential passages.

Grade-2 chimera did not segregate tetraploid and octoploid plantlets in the first passage, all the recovered plantlets were chimeras. A few octoploid plantlets were segregated in the second passage and all of the rest were chimeras. In the third passage, proportion of octoploid plantlets decreased and a little proportion of tetraploid plantlets was segregated. The majority of the recovered plantlets were chimeras (94.81%) in three sequential passages.

Grade-3 chimera had a high potential to segregate tetraploid plantlets, and the segregation efficiency for tetraploid plantlets increased in successive passages. Octoploid plantlets were not recovered in any passage. The majority of the recovered plantlets were tetraploid plantlets (58.74%) in three sequential passages.

The segregation results of leaf (Table 2) and root explants (Table 3) were similar to those of petiole explants (Table 1). The majority of the recovered plantlets from leaf explants of Grade-1 chimera were octoploid plantlets (42.2%) and chimeras (54.99%); Grade-2 chimera segregated 88.41% chimeras in three sequential passages (Table 2). The majority of the recovered plantlets from root explants of Grade-1 chimera were octoploid plantlets (26.24%) and chimeras (73.05%); Grade-2 chimera segregated 87.71% chimeras in three sequential passages (Table 3). Additionally, the segregation efficiency of tetraploid and octoploid plantlets did not change apparently following BA concentrations either (Tables 1–3). So we deduced that explant types and BA concentrations had no significant effect on the segregation efficiency of tetraploid and octoploid plantlets in adventitious shoot formation culture.

Segregation of tetraploid and octoploid plantlets from Grade-1/-2 chimera in shoot tip multiplication culture. The segregation results are presented in Table 4. Grade-1 chimera segregated a high proportion of chimera plantlets, only a low percentage of octoploid plantlets and no tetraploid plantlets

Table 2. Effect of chimera types, sequential passage numbers, and 6-benzyladenine (BA) concentrations on the ploidy state of shoots regenerated from leaf explants of Grade-1/-2 chimera in adventitious shoot formation culture in *Echinacea purpurea*.

Chimera type	Passage no.	BA (mg·L ⁻¹)	No. of plantlets examined	Ploidy state		
				Tetraploid	Octoploid	Chimera
Grade-1	1	0.2	12	0 (0)	7 (58.33)	5 (41.57)
		0.4	9	0 (0)	7 (77.78)	2 (22.22)
		0.8	25	0 (0)	19 (76)	6 (24)
		1.2	24	0 (0)	22 (91.67)	2 (8.33)
	2	0.2	15	0 (0)	5 (33.33)	10 (66.67)
		0.4	4	0 (0)	4 (100)	0 (0)
		0.8	47	0 (0)	32 (68.09)	15 (31.91)
		1.2	35	0 (0)	21 (60)	14 (40)
	3	0.2	5	0 (0)	1 (20)	4 (80)
		0.4	5	0 (0)	2 (40)	3 (60)
		0.8	167	11 (6.59)	28 (16.77)	128 (76.64)
		1.2	43	0 (0)	17 (39.53)	26 (60.47)
	Total		391	11 (2.81)	165 (42.2)	215 (54.99)
Grade-2	1	0.2	10	0 (0)	1 (10)	9 (90)
		0.4	47	0 (0)	0 (0)	47 (100)
		0.8	58	0 (0)	0 (0)	58 (100)
		1.2	7	0 (0)	0 (0)	7 (100)
	2	0.2	— ^z	—	—	—
		0.4	41	0 (0)	9 (21.95)	32 (78.05)
		0.8	32	0 (0)	4 (12.5)	28 (77.5)
		1.2	4	0 (0)	0 (0)	4 (100)
	3	0.2	—	—	—	—
		0.4	95	11 (11.58)	8 (8.42)	76 (80)
		0.8	13	1 (7.69)	1 (7.69)	11 (84.62)
		1.2	21	0 (0)	3 (14.29)	18 (85.71)
	Total		328	12 (3.66)	26 (7.93)	290 (88.41)

^zExperiments were not performed as materials were not enough or contamination.

Table 3. Effect of chimera types, sequential passage numbers, and 6-benzyladenine (BA) concentrations on the ploidy state of shoots regenerated from root explants of Grade-1/-2 chimera in adventitious shoot formation culture in *Echinacea purpurea*.

Chimera type	Passage no.	BA (mg·L ⁻¹)	No. of plantlets examined	Ploidy state		
				Tetraploid	Octoploid	Chimera
Grade-1	1	0.2	5	0 (0)	5 (100)	0 (0)
		0.4	4	0 (0)	2 (50)	2 (50)
		0.8	6	0 (0)	4 (66.67)	2 (33.33)
		1.2	2	0 (0)	2 (100)	0 (0)
	2	0.2	— ^z	—	—	—
		0.4	4	0 (0)	2 (50)	2 (50)
		0.8	15	0 (0)	4 (26.67)	11 (73.33)
		1.2	—	—	—	—
	3	0.2	—	—	—	—
		0.4	8	1 (12.5)	1 (12.5)	6 (75)
		0.8	79	0 (0)	10 (12.66)	69 (87.34)
		1.2	18	0 (0)	7 (38.89)	11 (61.11)
	Total		141	1 (0.71)	37 (26.24)	103 (73.05)
Grade-2	1	0.2	38	0 (0)	0 (0)	38 (100)
		0.4	19	0 (0)	0 (0)	19 (100)
		0.8	20	0 (0)	0 (0)	20 (100)
		1.2	8	0 (0)	0 (0)	8 (100)
	2	0.2	25	0 (0)	2 (8)	23 (92)
		0.4	42	0 (0)	15 (35.71)	27 (64.29)
		0.8	6	0 (0)	1 (16.67)	5 (83.33)
		1.2	5	0 (0)	0 (0)	5 (100)
	3	0.2	56	23 (41.07)	0 (0)	33 (58.93)
		0.4	48	0 (0)	0 (0)	48 (100)
		0.8	96	7 (7.29)	1 (1.04)	88 (91.67)
		1.2	44	0 (0)	1 (2.27)	43 (97.73)
	Total		407	30 (7.37)	20 (4.91)	357 (87.71)

^zExperiments were not performed as materials were not enough or contamination.

in the first passage. The percentage of chimera plantlets was reduced and a low percentage of tetraploid plantlets was found in the second passage. In the third passage, the percentage of chimera plantlets was further reduced and a higher number of tetraploid plantlets was segregated compared with the second passage. In three sequential passages,

the majority of the recovered plantlets were chimera plantlets (54.79%) and octoploid plantlets (33.6%).

Grade-2 chimera segregated a very high proportion of chimera plantlets and only a few octoploid plantlets in the first passage. No tetraploid plants were found in the first passage. The percentage of tetraploid plantlets

increased sharply in the second and third passages. In three sequential passages, the majority of the recovered plantlets were chimera plantlets (74.75%) and tetraploid plantlets (24.24%).

The results in Table 4 indicated that the occurrence efficiency of tetraploid and octoploid plantlets did not change apparently following BA concentrations either. So BA concentrations had no significant effect on the segregation efficiency of tetraploid and octoploid plantlets in shoot tip multiplication culture.

Morphology and cichoric acid content differences among diploid, tetraploid, and octoploid plants. Stomata size and frequency differed significantly among diploid, tetraploid, and octoploid plants (Fig. 4; Table 5). Stomata length and width in the abaxial side of octoploid (58.01 and 48.52 μm) leaves were larger than those of tetraploid (49.72 and 41.33 μm) and diploid (37.26 and 32.23 μm) leaves. However, the stomata frequency of octoploid (17.83) was less than that of tetraploid (31.7) and diploid (49.2) leaves. The mean chloroplast number in guard cells of diploid, tetraploid, and octoploid plants was significantly different from each other. The number in octoploid plants (55.33) was more than that in tetraploids (28.43) and diploids (16.68).

The tetraploid and octoploid plants presented obvious delayed growth compared with diploids, especially octoploids (Fig. 4A3, B3, and C3). The original diploid plants had more flower stalks and earlier flowering period than tetraploids. The tetraploids were only in early blooming when the diploids had been in full bloom. However, the tetraploid plants presented stronger growth and darker green pigmentation than diploids. The octoploid plants had dramatically more stunted growth than tetraploids and diploids. They cannot even bloom in the first year.

Results in Table 5 showed significant differences among diploid, tetraploid, and octoploid plants regarding the content of cichoric acid. Cichoric acid content in both the aboveground and underground parts of octoploid plantlets (13.76 and 29.84 mg·g⁻¹) were higher than that of tetraploid plantlets (10.38 and 22.32 mg·g⁻¹), and the content in tetraploids were higher than that in diploid plantlets (8.21 and 18.42 mg·g⁻¹). The results indicated that induction of polyploidy in *E. purpurea* significantly increased content of cichoric acid.

Discussion

Segregation of chimeras into pure plants containing only one type of component cells is a long-established topic (Canli, 2003; Canli and Skirvin, 2008; Chua et al., 1981; Hall et al., 1986; Marcotrigiano et al., 1987; McPheeters and Skirvin, 1983, 1989; Ning et al., 2009; Peary et al., 1988; Rosu et al., 1995; Roy et al., 2001). The mechanism was primarily explained using the theory of the tunica-carpus pattern of the meristematic

Table 4. Effect of chimera types, sequential passage numbers, and 6-benzyladenine (BA) concentrations on the ploidy state of shoots proliferated from shoot tip explants of Grade-1/-2 chimera in shoot tip multiplication culture in *Echinacea Purpurea*.

Chimera type	Passage no.	BA (mg·L ⁻¹)	No. of plantlets examined	Ploidy state		
				Tetraploid	Octoploid	Chimera
Grade-1	1	0.2	22	0 (0)	5 (22.72)	17 (77.78)
		0.4	23	0 (0)	3 (13.04)	20 (86.96)
		0.8	25	0 (0)	5 (20)	20 (80)
		1.2	16	0 (0)	4 (25)	12 (75)
	2	0.2	22	4 (18.18)	4 (18.18)	14 (63.64)
		0.4	45	8 (17.78)	10 (22.22)	27 (60)
		0.8	66	4 (6.06)	24 (36.36)	38 (57.58)
		1.2	76	6 (7.89)	38 (50)	32 (42.11)
	3	0.2	21	0 (0)	6 (28.57)	15 (71.43)
		0.4	38	12 (31.58)	8 (21.05)	18 (47.37)
		0.8	47	9 (19.15)	18 (38.30)	20 (76.6)
		1.2	90	14 (15.56)	40 (44.44)	36 (40)
	Total		491	57 (11.61)	165 (33.6)	269 (54.79)
Grade-2	1	0.2	70	0 (0)	3 (4.29)	67 (95.71)
		0.4	36	0 (0)	0 (0)	36 (100)
		0.8	29	0 (0)	0 (0)	29 (100)
		1.2	29	0 (0)	0 (0)	29 (100)
	2	0.2	35	8 (22.86)	0 (0)	27 (77.14)
		0.4	35	10 (28.57)	0 (0)	25 (71.43)
		0.8	32	16 (50)	2 (6.25)	14 (43.75)
		1.2	23	5 (21.74)	0 (0)	18 (78.26)
	3	0.2	77	29 (37.66)	0 (0)	48 (62.34)
		0.4	50	20 (40)	0 (0)	30 (60)
		0.8	49	19 (38.78)	0 (0)	30 (61.22)
		1.2	26	12 (46.2)	0 (0)	14 (53.8)
	Total		491	119 (24.24)	5 (1.02)	367 (74.75)

ploidy chimeras were unstable and this was reported in *Alocasia* (Thao et al., 2003).

In this article, we first classified the chimeras into three types according to the proportion of tetraploid and octoploid cells and described the morphology of three types of chimeras. After segregation culture, various proportions of tetraploid and octoploid plantlets were segregated from three kinds of chimera types. At last, we described the major phenotypic characteristics (including stomata size and frequency, chloroplast numbers in guard cells, and the morphology differences of mature plants) and determined cichoric acid content among diploid, tetraploid, and octoploid lines. This study emphasized several crucial factors that notably influenced the segregation process and provided some evidence regarding the correlation between ploidy level and phenotypic and phytochemical traits.

The results in segregation culture indicated that in vitro segregation of ploidy chimeras was mainly influenced by three factors. 1) The component ratios of different ploidy cells (i.e., the chimera types): pure ploidy plantlets were more easily to be segregated when this ploidy cells hold a comparative high proportion in the chimeric plantlets, e.g., Grade-1 chimera and Grade-3 chimera could segregate high proportions of octoploid and tetraploid plantlets, respectively (Table 1). There was a low segregation efficiency of pure ploidy plantlets when the component ratios of different ploidy cells were similar, e.g., Grade-2 chimera segregated higher percentage of chimeras than pure ploidy plantlets (Tables 1–4). Thus, we concluded that pure ploidy plantlets were easily segregated only when the quantity of this ploidy cells was dominant in the chimeras. 2) The number of sequential passages: Grade-1 chimera segregated a high proportion of octoploid plantlets in the first passage, but the proportion of octoploid plantlets decreased continuously in the second and third passages (Tables 1–4). Grade-2 chimera did not segregate pure ploidy plantlets in the first passage, but obtained tetraploid plantlets with increasing efficiency in the second and third passages (Tables 1–4). 3) The methods of segregation culture: shoot tip multiplication was more effective than adventitious shoot formation when the component ratios of different ploidy cells were similar, e.g., Grade-2 chimera segregated more tetraploid plantlets in shoot tip multiplication than that in adventitious shoot formation culture (Tables 1–4). The differences between the two methods may result from the differences between the explants used (i.e., leaf, petiole, and root explants vs. shoot tip explants), and differences in the two methods themselves. Other factors, e.g., explant types or BA concentrations occasionally influenced the segregation, and just only resulted in some propagation rate (i.e., no. of plantlets examined in Tables 1–4) differences. This was different from previous studies on other chimeras that BA concentration was the main factor influencing the separation of a rose

Fig. 4. Comparison of stomata characteristics and morphology differences among diploid, tetraploid, and octoploid plants in *Echinacea purpurea*. (A1, B1, C1) Stomata size and frequency of diploid, tetraploid, and octoploid plants. Bar = 50 µm. (A2, B2, C2) Chloroplast number in guard cells of diploid, tetraploid, and octoploid plants. Bar = 10 µm. (A3, B3, C3) Mature plants of diploid, tetraploid, and octoploid. Bar in A3 and B3 = 10 cm. Bar in C3 = 2 cm.

region described by Schmidt (1924) and later refined by Tilney-Bassett (1986, 1991). In ploidy chimeras, as the cell mitotic division

cycle of the different ploidy level cells were different, the proportion of different ploidy cells were renewed persistently. Thus, the

Table 5. Comparison of cichoric acid content and stomata characteristics among diploid, tetraploid, and octoploid plants in *Echinacea purpurea*.

Ploidy level	Stomata frequency	Stomata length (μm)	Stomata width (μm)	Chloroplast/guard cells	Cichoric acid (mg·g ⁻¹)	
					Aboveground part	Underground part
Diploid	49.2 ± 4.53 a ²	37.26 ± 0.43 c	32.23 ± 0.41 c	16.68 ± 0.50 c	8.21 ± 0.85 b	18.42 ± 1.73 c
Tetraploid	31.7 ± 5.56 b	49.72 ± 1.08 b	41.33 ± 0.44 b	28.43 ± 0.87 b	10.38 ± 0.65 b	22.32 ± 4.27 b
Octoploid	17.83 ± 2.4 c	58.01 ± 1.63 a	48.52 ± 0.96 a	55.33 ± 1.52 a	13.76 ± 1.33 a	29.84 ± 4.72 a

²Each value represents the mean ±SE. Values in each vertical column followed by different letters are significantly different ($P \leq 0.05$).

chimera (Canli and Skirvin, 2008). It has been reported that higher ploidy level plants need higher BA concentrations than diploid plants in in vitro proliferation (Chen et al., 2012; Nilanthi et al., 2009b). However, BA did not notably influence the occurrence efficiency of octoploid and tetraploid plantlets. Maybe this was the difference between pure ploidies and ploidy chimeras.

The segregation efficiency of pure ploidy plantlets from three kinds of chimeras through two methods and three sequential passage numbers presented some regular change patterns. We deduced that the growth cycle differences of different ploidy level cells were the key factor to these changes. It was assumed that the mitotic cycle of polyploidy lasted longer than diploid. This was reported in *Antirrhinum majus* (Bennett, 1972) and *Hordeum vulgare* (Skult, 1969). From interkinesis to division phase, the duration time of polyploidy was longer than diploid. Based on the mitotic duration time differences of different ploidy level plants, the chimeric plants have poor ploidy stability. Furthermore, Stewart et al. (1972) reported that there was quantitative competition between mutational cells and original cells in the chimera. The competition led to two results: 1) mutational cells or original cells were substituted by each other and 2) mutational cells and original cells were kept in a dynamic balance. The pure ploidy segregation in this study might be a process of quantitative competition between tetraploid cells and octoploid cells. For instance, in Grade-1 chimera, octoploid cells were dominant in quantity, so a high proportion of octoploid plantlets were segregated in the first passage. As mitotic duration time of tetraploid cells was shorter than that of octoploid cells, the component ratio of tetraploid cells increased and the ratio of octoploid cells decreased, so a lower proportion of octoploid plantlets were segregated in the second passage than that in the first passage. In the third passage, the component ratio of tetraploid cells increased and the ratio of octoploid cells decreased continuously, so the proportion of octoploid plantlets segregated was still lower than that in the second passage, and even a little proportion of tetraploid plantlets was segregated in the third passage. The segregation process of Grade-2 and Grade-3 chimeras through two methods and three sequential passages could also be interpreted like this.

The octoploid and tetraploid plants obtained in the segregation culture displayed several morphological differences compared with the original diploid line (Fig. 4; Table 5). These changes were similar to those in some

other plant species (Gu et al., 2005; Majdi et al., 2010; Omidbaigi et al., 2010). Many reports found that morphological traits can be effective initial screen to discriminate polyploid plants from diploids, especially stomata characteristics observing, as a simple and efficient method, has been widely used in many plant species, such as *Acacia mearnsii* (Beck et al., 2003), *Aegilops neglecta* (Aryavand et al., 2003), *Alocasia* (Thao et al., 2003), *Beta vulgaris* (Yudanov et al., 2002), *E. purpurea* (Abdoli et al., 2013), *Lagerstroemia indica* (Ye et al., 2010), *Morus alba* (Chakraborti et al., 1998), *P. hybrida* (Ning et al., 2009), and *Pyrus pyrifolia* (Kadota and Niimi, 2002). Octoploid and tetraploid plants presented darker green than diploids. This was probably due to the increased chloroplast number, which is most likely accompanied by enhanced chlorophyll content (Xu et al., 2013). Additionally, polyploidy is also associated with an increase in the number of petals (Kermani et al., 2003), improved resistance to pests, and tolerance to stress (Kehr, 1996; Zhang et al., 2008).

The increasing trend of the cichoric acid content in tetraploid and octoploid plantlets (Table 5) indicated a positive correlation between genomic DNA amount and levels of secondary metabolites. This phenomenon has been previously reported in many other works, e.g., *Artemisia annua* (Gonzalez and Weathers, 2003; Lin et al., 2011), *Cymbopogon* (Lavania et al., 2012), *Datura stramonium* (Berkov and Philipov, 2002), *Dracocephalum kotschy* (Zahedi et al., 2014), *T. parthenium* (Majdi et al., 2010), *Thymus persicus* (Tavan et al., 2015), and *Zinger officinalis* (Adaniya and Shira, 2001). Tetraploids were often used to identify differential gene expressions regarding cell growth and functional secondary metabolites (Lin et al., 2011; Zhou et al., 2015). Octoploids had larger organs and more enhanced contents of secondary metabolites than tetraploids, so octoploids could also be attractive for breeding and genetic mechanisms studying as the novel germplasm in other medicinal, ornamental, or agricultural plants.

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