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RESEARCH ARTICLE

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Cytological observation of the microspore development of Chinese kale and false pakchoi

Abstract The development process and morphology of the microspores in Chinese kale and false pakchoi were observed by using the whole stain-clearing technique. The results showed that the morphological characteristics of microspores were not only extremely similar, but were also in their development processes. The microspores at tetrad stage showed an arrangement of tetrahedral type, and after being released and passing through two mitoses, they developed gradually to form rotundly-shaped mature pollen grains containing three nuclei, one bigger alimentation nucleus and two smaller similar-sized generative nuclei. Determination of bud sizes at four typical microspore developmental stages revealed that the bud size had a stable heredity at each development stage of the microspores. The ratio of the bud length at the late uninucleate stage to the largest bud length differed little between Chinese kale and false pakchoi, ranging from 0.37 to 0.45 with an average of 0.41, though there was significant difference among their cultivars. It was concluded that the length of buds at late uninucleate stage can be estimated for undetermined cultivars of these two *Brassica* crops by multiplying the largest bud length with the following coefficient or regression equation: $Y = 0.3898X + 0.1503$, where X is the length of the largest bud.

Keywords Chinese kale, false pakchoi, microspore, late uninucleate stage

1 Introduction

Chinese kale (*Brassica alboglabra* Bailey) and false pakchoi (*Brassica campestris* L. ssp. *chinensis* var. *utilis* Tsen et Lee), leafy vegetable crops, are native to Guangdong Province, China, and they play a quite important role in the year-round vegetable supply in the area. Over the past decades, however, the progress in the breeding of these two *Brassica* crops has been relatively slow due to their poor genetic backgrounds and less viable breeding approach (Zhang, 1997; Li et al., 2006). In recent years, the technique of isolated microspore culture has been widely applied in innovation of germplasm and in shortening breeding periods for many crops, therefore more attention has also been given to these two vegetable crops (Li et al., 1993; Cao et al., 1995; Zhu et al., 2003). Among the factors affecting microspore embryogenesis besides genotype, the developmental stage of the microspore has been considered as one of the determinants in successfully obtaining the pollen plants (Sato et al., 1989; Cao et al., 1993; Zhao et al., 2007b). Up to this date, however, no detailed report has been found on microspore development in either Chinese kale or false pakchoi. The objectives of this study were to observe the microspore development process and then perform analysis of size relationship between the buds at late uninucleate stage and the largest buds so as to provide a basis for establishing more efficient microspore culture systems for the crops.

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2 Materials and methods

2.1 Materials

Five Chinese kale cultivars, Cuibao, Lubao, Yuanyezhonghua, Cuiroudaxinjie and Cuitian No. 35, and seven false pakchoi cultivars, Sijiyouqing, Guangfuqing, Bilu, Hualuzaoshuyouqing, Hualuzhongshuyouqing, Youxuan No.1 and Sijiu, were used in this study. Their seeds were sown in nursery seedling plates (84 cells: $h \times \varphi = 7\,\text{cm} \times 3\,\text{cm}$) filled with a mixed medium of paddy soil and spent mushroom composts (3∶1, volume/volume, hereinafter the "v/v") on September 21, 2007. The 28-day-old seedlings

were transplanted into a green house in the Baiyun Experimental Base of Guangdong Academy of Agricultural Sciences, China. During the bolting or flowering stage, the flower buds were sampled and their microspore development status and bud sizes were observed and measured as described below.

2.2 Methods

2.2.1 Staining and observation of microspore

Cuibao and Sijiyouqing, as the respective representatives for Chinese kale and false pakchoi, were used for cytological observation. Microspores at different developmental stages were stained and optically cleared by using the whole stain-clearing technique with some modifications (Stelly et al., 1984; Zhang et al., 2007). The main inflorescences were taken to the laboratory and fixed in Carnoy's solution (ethanol∶acetic acid = 3∶1, v/v) for 48 h, and then all sepals and petals of each bud were removed, with all intact anthers put into weighing bottles (5 mL) according to their bud sizes. After being soaked in solutions of 50% and 25% ethanol and distilled water consecutively for 0.5 h each time, the anthers were dipped in 10 times-diluted Ehrlich's hematoxylin staining solution for 24 h and washed 4–5 times with distilled water and 3 times with tap water, with the last dipping done for 8–12 h till tissues were made full blue. All the blued tissues were then dehydrated in anhydrous ethanol for 2 h and passed, respectively, through three changes of 1∶1 anhydrous ethanol and wintergreen oil (v/v), and wintergreen oil, in which the tissues were cleared for more than 24 h at the last pass. Afterwards, microspores were extruded on glass slides and observed under a light microscope (Olympus BH22) at 15×20 times of amplification and photographed.

2.2.2 Determination of bud size at various microspore development stages

Because the two mitoses in the microspores were relatively difficult to distinguish, as a substitute, we determined the bud size in the two crops of Chinese kale (Chuibao and Lubao) and false pakchoi (Sijiyouqing and Hualuzaoshuyouqing) at the four typical microspore developmental stages of tetrad, mid uninucleate, late uninucleate, and binucleate stages. Since the development status of microspores in a given bud was somewhat unsynchronized, the length of buds seen in over 80% of the microspores that developed into the same stage was considered as the judging standard for bud sizes at that stage, as observed on four individual inflorescences in each cultivar. Microspore staining was performed as described above.

2.2.3 Size relationship between the bud at late uninucleate stage and the largest bud

In our experiment, microspores were dyed using the rapid staining method with acetic acid magenta as described by Hong (2007). To define the size relationship between the bud at the late uninucleate stage and the largest bud, the length of the bud at this stage and the largest bud for each cultivar was measured randomly from the inflorescences that blossomed out 0–15 buds. Their ratios were then calculated and assessed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS) software.

3 Results

3.1 Development of microspores

Results in Fig. 1 showed that the development process and morphology of microspores were extremely similar between Chinese kale and false pakchoi. In tetrad phase, the microspore cells were encapsulated by callose walls and arranged almost tetrahedrally. The microspores released from the tetrads were irregular and soon became subround, with less evident nuclei and cell walls (Fig. 1 A, J and K). With development, microspores entered the mid uninucleate stage, showing a typical trilobation shape with thickened walls (Fig. 1 B, C and L). During this stage,

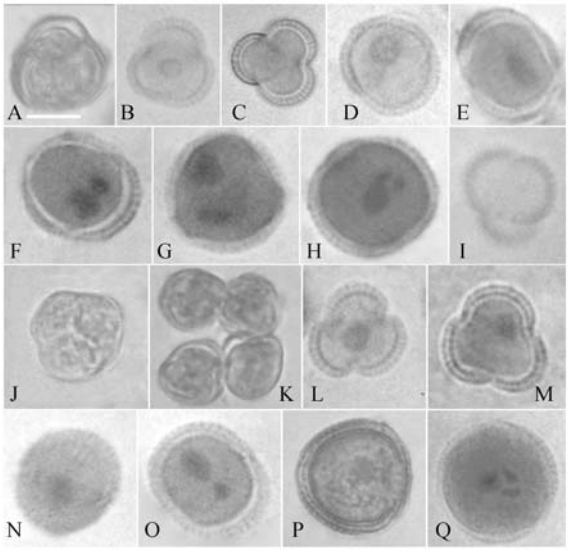

Fig. 1 Microspore developments of Chinese kale and false pakchoi

Note: A–I stand for Cuibao, a Chinese kale cultivar, and J–Q for Sijiyouqing, a false pakchoi cultivar; A, J and K are tetrads; B, C and L are at mid uninucleate stage; D and M are at late uninucleate stage; E and N are in uninucleate mitosis; F and O are at binucleate stage; G and P are in generative nucleus division; H and Q are trinucleate mature pollens; I is the abortive microspore; Bar = 5 μm.

abortive microspores without inclusion were observed at times (Fig. 1 I). With further development and until the late uninucleate stage, the number of vacuoles in the cytoplasm increased and became more obvious, and the nuclei moved, with some coming up close to the edge of the cells (Fig. 1 D and M). Afterwards, microspores continued expanding, the cytoplasm became denser, and trilobation disappeared progressively; subsequently, this was followed by one mitotic division which produced one bigger alimentation nucleus and one smaller generative one (Fig. 1 E, F, N and O). Then, as cells further enlarged, the microspores that developed more round and generative nuclei near the cell walls underwent the second mitosis (Fig. 1 G and P), producing two same-sized sperm nuclei. Finally, round-shaped mature pollen grains were formed with 3 nuclei and dense plasma, except that Chinese kale had less big ones than did false pakchoi (Fig. 1 H and Q).

3.2 Difference and stability of bud size at different microspore development stages

The length of the largest bud at the four typical microspore development stages is shown in Table 1. It is obvious that the length of the largest bud remained unchanged during the early flowering period, and averaged 9.35 cm and 7.56 cm for the two Chinese kale cultivars of Cuibao and Lubao, and 4.56 cm and 4.06 cm for the two false pakchoi cultivars of Sijiyouqing and Guangfuqing, respectively, showing a considerable difference between these two crops and these cultivars. Additionally, the length of the buds at the four typical microspore developmental stages showed no variance during the early flowering period, but had a marked difference between species or cultivars. These results suggested that the size of the largest buds was dependent on crop species and cultivars at different development stages, and these parameters had good genetic stability during flowering.

3.3 Size relationship between buds at late uninucleate stage and the largest bud

Correlation analysis of twelve cultivars of five Chinese kale and seven false pakchoi plants indicated that the bud length at the late uninucleate stage was positively correlated with that of the largest bud (Fig. 2). The regression equation, $Y = 0.3898X + 0.1503$, was developed with a correlation coefficient of $r = 0.9774^{**}$ ($P \leqslant 0.01$).

Figure 3 shows the ratios between bud length at late uninucleate stage and the largest buds in the twelve cultivars. The statistical analysis indicated that there was no significant difference in the ratios between Chinese kale and false pakchoi ($P \leqslant 0.05$), with average values of 0.41

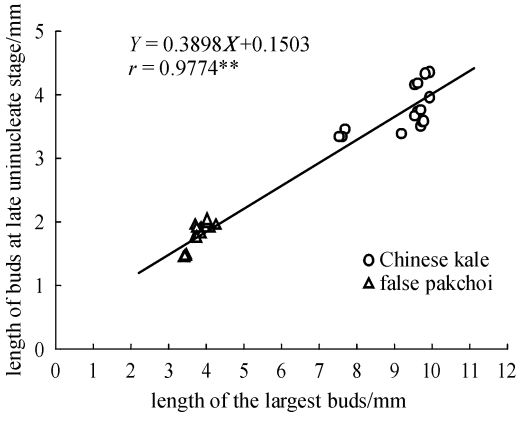

Fig. 2 Correlation between bud length at late uninucleate stage and the largest bud length
Note: ** means significantly correlated at $P \leqslant 0.01$.

Table 1 Size of the largest buds and buds at four typical microspore development stages

species	cultivar	blooming bud number	bud length/mm				
			the largest bud (trinucleate)	binucleate	late uninucleate	mid uninucleate	tetrad
Chinese kale	Cuibao	1	9.30 ± 0.24	6.58 ± 0.10	3.43 ± 0.43	2.70 ± 0.18	1.94 ± 0.11
		5	9.35 ± 0.24	6.50 ± 0.16	3.50 ± 0.41	2.73 ± 0.17	1.93 ± 0.10
		15	9.38 ± 0.25	6.65 ± 0.17	3.50 ± 0.41	2.78 ± 0.21	1.90 ± 0.08
	Lubao	1	7.63 ± 0.48	5.33 ± 0.25	3.38 ± 0.35	2.30 ± 0.14	1.43 ± 0.10
		5	7.55 ± 0.35	5.38 ± 0.17	3.28 ± 0.15	2.38 ± 0.13	1.48 ± 0.13
		15	7.50 ± 0.25	5.35 ± 0.21	3.28 ± 0.38	2.33 ± 0.24	1.50 ± 0.08
false pakchoi	Sijiyouqing	1	4.60 ± 0.24	2.98 ± 0.33	1.98 ± 0.13	1.35 ± 0.13	0.68 ± 0.10
		5	4.43 ± 0.30	2.90 ± 0.27	1.98 ± 0.17	1.38 ± 0.26	0.73 ± 0.17
		15	4.73 ± 0.22	2.95 ± 0.13	2.03 ± 0.13	1.40 ± 0.18	0.70 ± 0.18
	Guangfuqing	1	4.05 ± 0.21	2.38 ± 0.26	1.58 ± 0.22	1.00 ± 0.16	0.50 ± 0.08
		5	4.05 ± 0.10	2.58 ± 0.10	1.50 ± 0.08	0.95 ± 0.13	0.50 ± 0.08
		15	4.08 ± 0.10	2.60 ± 0.08	1.58 ± 0.10	0.98 ± 0.13	0.53 ± 0.10

Note: Data are mean ± *SE*, $n = 4$.

and 0.42, respectively, but a significant difference existed among their cultivars. The ratios of these twelve genotypes tested ranged from 0.37–0.47, with an average of 0.41, and a low variance of 7.05%.

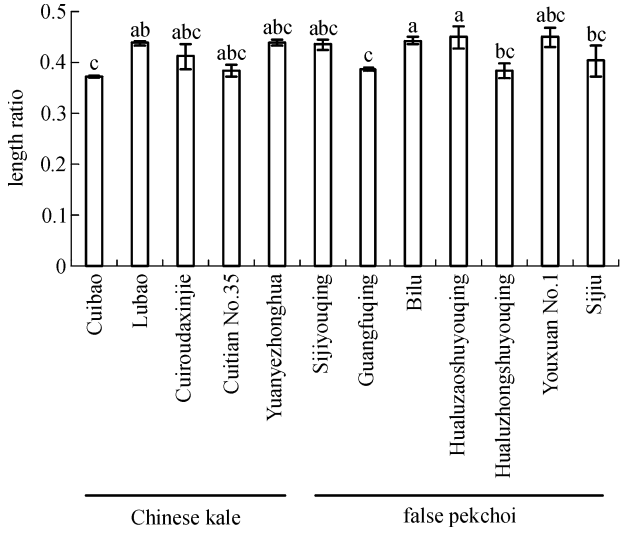

Fig. 3 Difference in ratios of bud length at late uninucleate stage to the largest bud length among Chinese kale and false pakchoi cultivars
Note: Different letters show significant differences at $P \leqslant 0.05$.

4 Discussion

Although Chinese kale and false pakchoi differed greatly in the characteristics of vegetative organs such as stem diameter, leaf type and color as well as bud size etc., their development processes and microspore morphologies were extremely similar. In these two crops, the tetrads were irregularly shaped, and showed the tetrahedral arrangement in most cases. After being released, accompanied by cell wall thickening, the microspores underwent two mitoses and developed gradually into rotundly-shaped mature pollen grains, which is similar to that of some other cruciferae crops (Zhao et al., 2007a; Zhang et al., 2007).

Inflorescences of Chinese kale and false pakchoi are racemes, and their flowering habits are characterized by blossoming progressively in order from the base to the top. According to our observations, the largest buds of these plants would blossom the next day in normal cases, and they contain almost 100% of mature pollen grains and hence could be considered as a representative of the trinucleate stage. In the present study, the size of the largest buds had significant differences between Chinese kale and false pakchoi or among their cultivars, but for the buds at four typical microspore development stages, there appeared no variation during the early period of flowering, indicating an inheritance stability. Therefore, those buds suitable to microspore culture in Chinese kale and false pakchoi can be selected without care for flowering at their early blossoming stages.

In many cruciferae crops, it has been well documented that a higher frequency of embryogenesis can be obtained from late uninucleate microspores (Kott et al., 1988; Sato et al., 1989; Hansen and Svinnset, 1993; Cao et al., 1995; Chen et al., 1995; Prem et al., 2005). It has been reported that bud length (Thurling and Chay, 1984; Hansen and Svinnset, 1993), anther length and color (Li et al., 2003) and ratio of petal length to anther length (Sato et al., 1989; Zhao et al., 2007b) could be used as the indexes to judge the microspore development stage. Our results, using five and seven cultivars of Chinese kale and false pakchoi respectively, indicated that the size of buds at late uninucleate stage was positively correlated with that of the largest buds, and the regression equation had a high correlation coefficient of 0.9774. As to the ratio of the bud length at late uninucleate stage to the largest bud length, although there was a significant difference among the cultivars tested, variance was at a relatively narrow range, from 0.37 to 0.45, with an average of 0.41, and the variation coefficient was low, at 7.04%. According to our findings, it seems possible that the size of buds at late uninucleate stage can be calculated directly for an undetermined cultivar by the largest buds. To validate if so, we speculated for several other *Brassica* crops such as broccoli (*Brassica oleracea* var. *italica* Planch) and rape (*Brassica napus* L.) on the sizes of the late uninucleate stage by this value or the regression equation, and the results revealed that all the expected buds contained at least 75% of the microspores at the late uninucleate stage (data not shown). Thus, these parameters are probably common or have little difference in *Brassica* crops, but will need further studies.

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References

Cao M Q, Li Y, Liu F (1993). Influence of genotype and donor plant growth environment on embryo genesis from isolated microspores in Chinese cabbage (*Brassica campestris* ssp. *pekinensis*). Acta Agriculturae Boreali-Sinica, 8(4): 1–6 (in Chinese)

Cao M Q, Li Y, Liu F, Jiang T, Liu G S (1995). Application of anther culture and isolated microspore culture to vegetable crop improvement. Acta Horticulture, 392: 27–38

Chen J, Chen Z H, Liu C Q, Yak Y G, Zhang L H, Guan Y L (1995). Embryogenesis of isolated microspores of *Brassica napus* in culture. Acta Genetica Sinica, 22(4): 307–315 (in Chinese)

Hansen M, Svinnset K (1993). Microspore culture of Swede (*Brassica napus* ssp. *rapifera*) and the effects of fresh and conditioned media. Plant Cell Reports, 12: 496–500

Kott L S, Plosoni L, Beversdorf W D (1988). Cytological aspects of isolated microspore culture of *Brassica napu*. Canadian Journal of Botany, 66: 1658–16641

Li X, Guan C Y, Chen S Y, Wang G H, Liu Z S (2003). Studies on microspore culture and double haploid breeding on rape. II. Influencing factors on microspore culture of F_1 hybrids between *Brassica napus* and *Brassica juncea*. Acta Agronomica Sinica, 29(5): 744–749 (in Chinese)

Li Y, Liu F, Cao M Q (1993). Embryogenesis and plant regeneration from isolated microspore cultures of *Brassica campestris* ssp. *chinensis*. Acta Agriculturae Boreali-Sinica, 8(3): 92–97 (in Chinese)

Li Z L, Chen Q H, Luo S B, He Y Z, Guo J X (2006). Collection, conservation, evaluation and utilization of vegetable germplasm in Guangdong. Journal of Plant Genetic Resources, 7(1): 111–117 (in Chinese)

Prem D, Gupta K, Agnihotri A (2005). Effect of various exogenous and endogenous factors on microspore embryogenesis in Indian mustard (*Brassica juncea* (L.) Czern and Coss). In Vitro Cellular and Developmental Biology-Plant, 41(3): 266–273

Sato T, Nishio T, Hirai M (1989). Plant regeneration from isolated microspore culture of Chinese cabbage (*Brassica campestris* ssp. *pekinensis*). Plant Cell Reports, 8: 486–488

Stelly D M, Peloquin S J, Palmer R G, Crane C F (1984). Mayer's hemalum-methyl salicylate: a stain-clearing technique for observations within whole ovules. Stain Technology, 59: 155–161

Thurling N, Chay P M (1984). The influence of donor plant genotype and environment on production of multicellular microspores in cultured anthers of *Brassica napus* ssp. *oleifera*. Annals of Botany, 54: 681–693

Zhang W, Zhang S N, Zhang H L (2007). Pollen mother cell mitosis and male gametophyte development of *Raphanus sativus* L. Journal of Nanjing Agricultural University, 30(3): 38–41 (in Chinese)

Zhang Y (1997). Current situation and prospects of breeding in false pakchoi. Guangdong Agricultural Sciences, (3): 14–16 (in Chinese)

Zhao D Q, Zhang A P, Wu J, Zhang C J, Tao L (2007a). Observation on cell morphology of F_1 pollens of Qianbai (a hybrid Chinese cabbage variety). Guizhou Agricultural Sciences, 35(4): 9–11 (in Chinese)

Zhao Q C, Li S W, Wen Z H, Sun D L (2007b). Isolated microspore culture and plant regeneration of Chinese kale. Northern Horticulture, 9: 4–6 (in Chinese)

Zhu Y H, Liu M Y, Wu C L (2003). Factors to affect isolated microspore culture in *Brassica Parachinensis* Bailey. Journal of Changjiang Vegetables, 9: 46–47 (in Chinese)