Effects of Genotype and Culture Conditions on Microspore Embryogenesis in Radish (*Raphanus sativus* L.)

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Research Article

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Abstract

Radish (*Raphanus sativus* L.), an important annual or biennial root vegetable crop, is widely cultivated in the world for its high nutritive value. Isolated microspore culture (IMC) is one of the most effective methods for rapid development of homozygous lines. Due to the imperfection of IMC technology system, it is particularly important to establish an efficient IMC system. In this study, the effects of different factors on radish microspore embryogenesis were investigated with 23 genotypes. Buds with the largest population of late-uninucleate stage microspores were most suitable for embryogenesis, with a ratio of petal length to anther length (P/A) in buds of about 3/4~1. Cold pre-treatment was found to be genotype specific, and the highest microspore-derived embryo (MDE) yield occurred for treatment of the heat shock of 48 h. In addition, the supplement of 0.75 g/L activated charcoal (AC) could increase the yield of embryoids. It was found that genotypes, bud size as well as temperature treatments had significant effects on microspore embryogenesis. Furthermore, *somatic embryogenesis-related kinase (SERK)* genes were profiled by quantitative real-time polymerase chain reaction (RT-qPCR) analysis, which indicated that they are involved in the process of MDE formation and plantlet regeneration. The ploidy of microspore-derived plants was identified by chromosome counting and flow cytometry, and the microspore derived plants were further proved as homozygous plants through expressed sequence tags-simple sequence repeats (EST-SSR) and genetic-SSR markers. The results would facilitate generating the large-scale double haploid (DH) from various genotypes, and promoting further highly efficient genetic improvement in radish.

Introduction

Radish (*Raphanus sativus* L.), a typical cross-pollinated crop, is one of the most important vegetable crops around the world, especially in East Asia with abundant nutrition and medicinal value. For vegetable crop genetic improvement, homozygotes with stable characters can be achieved through haploid induction, which significantly reduces the generations of parent selfing and accelerates the development process of elite cultivars. Haploid and double haploid (DH) plant induction technology provides an excellent system for accelerating plant breeding, genetic analysis, physiological investigation, genetic transformation, and QTL mapping (Brew-Appiah et al. 2013). It is well known that radish is difficult to obtain homozygous plants for advanced inbred lines due to its self-incompatibility. Conventional breeding methods take a long time, more evidences indicate that modern biotechnology including haploid induction could shorten the gap as soon as possible and promote the genetic gains in crop breeding programs. Throughout the practical technology of vegetable crop breeding in recent years, isolated microspore culture (IMC) technology has the advantages of the achievement of rapid homozygosity, providing a large number of homozygotes for breeding in relative short time (Zhang et al. 2013). Nowadays, regeneration of haploid or DH plants through IMC technology has become an advanced and useful method for haploid production in various species (Shariatpanahi et al. 2016). However, due to the unavailability of an efficient protocol for large scale induction of haploid plants through IMC, microspore culture technology is difficult to be widely applied in breeding programmes (Reeta et al. 2018).

Since Lichter (1982) firstly induced embryoid in *Brassica napus*, microspore culture has been widely employed to develop double haploid plants in many vegetable crops, including *B. napus* L (Ahmadi, et al.

2014a; Solís et al. 2016), *B. oleracea* var. *capitata* L (Reeta et al. 2018) and *B. oleracea* var. *acephala* (Chen et al. 2019). Radish is considered to be one of the most recalcitrant species in *Brassicaceae* for IMC (Takahata et al. 1996). It was reported that 13 out of 19 genotypes produced embryoids with the yield of embryos varied from 0.125 to 10 per bud (Zhou et al. 2007). Similarly, only three regenerated plants were obtained from 22 radish genotypes through IMC (Wang et al. 2013a). It was found that efficient induction of microspore-derived embryo (MDE) depends on various factors such as genotype-dependency, low embryoid yield and germination rate (Testillano 2019). Furthermore, embryogenesis induction is accompanied by many biochemical and morphological changes, which have been proved to be closely related to the alterations in gene expression pattern (Munoz-Amatriain et al. 2009). Therefore, it is particularly important to investigate the factors affecting microspore embryogenesis. Recently, a number of factors were reported to affect critical embryogenesis in microspore culture, which included the growth condition of donor plants, pre-treatment of microspores, pollen developmental stage, the growth regulators in induction medium, the concentration of microspores in culture media, and temperature shock (Wang et al. 2013b; Makowska et al. 2015; Bhatia et al. 2016, 2017). Meanwhile, the *somatic embryogenesis receptor-like kinase* (*SERK*) genes were found to be involved in embryogenesis during microspore culture (Podio et al. 2014).

In this study, to identify the critical factors for IMC in radish, the effects of several factors including genotype of donor plants, pre-treatment of the microspores, developmental stages of microspore, and the supplement of activated charcoal (AC) were investigated. Moreover, the expression profiles of *RsSERK* genes during the microspore development and plantlet regeneration was conducted in radish, as well as the ploidy levels and the homozygosity of microspore-derived plantlets were determined. The aim of this study was to provide valuable information for the establishment of microspore culture system of radish, and facilitate generating abundant DH lines for genetic improvement in radish.

Materials And Methods

Plant materials

A total of 23 radish genotypes were used for microspore culture (Table 1). All plants were cultivated in the greenhouse. Regular watering, fertilization, insect pest and disease management were conducted.

Isolation of microspore

The microspore isolation was basicly followed the procedure of Bhatia et al. (2018) with a slight modification. Floral buds at 1.5~4.5 mm were collected from young inflorescences and then classified into three types by size. The developmental stages of the microspores were analyzed using 4', 6-diamidino-2-phenylindole (DAPI) (H-1200) solution and observed by fluorescence microscope (Olympus) (Winarto et al. 2011). The buds were put into beaker containing a few drops of water, kept at 4℃ for 0~4 d, and then surface-sterilized with 75% (v/v) ethanol for 30 s, 8% (w/v) sodium hypochlorite for 12 min, and rinsed with sterile distilled water for 3 times. Sterile buds were transferred to B5 medium supplied with 13% (w/v) sucrose at pH 5.8. The microspores were squeezed out and then filtered through a 40 μm mesh into a 10 ml centrifuge tube. The microspore suspension was centrifuged at 1200 rpm for 3 min. The pellet were re-suspended in B5-13 medium and centrifuged at 1200 rpm for 3 min, this procedure was repeated twice.

After decanting supernatant, the microspores were re-suspended in NLN-13 medium (pH 5.8) with 13% sucrose supplemented with AC to achieve the final suspension density of microspore at a concentration of 1~2 $\times 10^5$ microspore/ml. The microspore suspension was then poured into 35-mm Petri dishes (2 ml per Petri dish), and the cultures were incubated at 32.5℃ in the dark for 2 d, and then incubation continued at 25℃ in the dark for 20 d. The embryoid numbers were calculated and developmental stages of microspores were observed with an inverted microscope.

Temperature shock and AC treatment in radish genotypes

The selected buds were treated with cold treatment at 4℃ for 0, 12, 36, 48, 60, 72 and 96 h before the isolation of microspore, and the microspore suspension was treated with heat shock at 32.5℃ for 0, 24, 48, 72 and 96 h before culturing at 25℃, respectively. To investigate the effect of AC on microspore embryogenesis, different concentrations of AC was supplemented into the medium to maintain the concentration at 0.25, 0.50, 0.75, 1.0, 1.25 and 1.50 g/L, respectively.

Microspore embryoid germination and plant regeneration

The microspore embryoid germination was conducted based on the procedure of Takahata et al. (1996) with some modifications. When the embryoids were visible to the naked eyes, they were cultured at 25℃ under a 16-h photoperiod (4000 Lx) for 5~7 d. After the microspore embryoid turned green, the cotyledonary embryoids were transferred onto solid B5 medium with 3% sucrose, 0.8% agar, 0.2 mg/L 6-BA, 0.02 mg/L NAA and 0.1% AC (pH 5.8). The embryoid viability was evaluated after one week. After several subcultures, the plantlets were transferred onto solid B5 medium with 3% sucrose, 0.7% agar, 0.2 mg/L NAA for rooting.

The rooted plantlets were transplanted into a pot with sterilized mixture of peat soil, nutrient soil and vermiculite (2:1:1) and kept in growth chamber for one week with a day temperature of 25℃ (14 h) and night temperature of 16℃ (10 h) under high relative humidity (85%) during the first seven days, and then transplanted into an experimental plot in the greenhouse.

Ploidy level evaluation

Ploidy levels of the microspore-derived embryos (MDE) plantlets were determined by chromosome counting and flow cytometry analysis. For chromosome counting, root tips were pre-treated with ice water for 24 h and fixed in a mixture of absolute ethanol and glacial acetic acid (3:1, V/V) for 24 h at 4℃. Fixed root tips were hydrolyzed in 1 M hydrochloric acid in the water bath at 60℃ for 6 min and stained with a drop of 1% acetocarmine solution. The cells were then squashed and observed under a light microscope. The flow cytometry procedure was carried out as described by Zhang et al. (2007). The 100mg young leaves was chopped with sharp scalpel in 15 mM Tris-HCl (pH 7.5), 80 mM KCl, 20 mM NaCl, 20 mM EDTA-Na_2, 15 mM mercaptoethanol and 0.05% (V/V) Triton X 100. Nuclei isolated from young leaves of plants were then stained with RNase and PI (propidium iodide) for 2 h at 4℃ in the dark. The DNA contents were measured with flow cytometry (American Beckman Co.)

Homozygosity analysis of microspore-derived plantlets

The developed EST-SSR and genetic-SSR markers (Zhai et al. 2014) were employed to identify the homozygosity of microspore-derived plantlets. Genomic DNA was extracted from young leaves using a modified CTAB protocol (Liu et al. 2003). PCR amplification was performed on a Thermal Cycler (Senso Quest) with a 15 μl final reaction volume containing 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 U *Taq* DNA polymerase (TaKaRa Bio Inc.), 0.1 μM of each primer and 10 ng of template DNA. The PCR conditions comprised initial denaturation at 94℃ for 2 min, followed by 35 cycles of 94℃ for 40 s, 55~60℃ (varying with the T_m of the different primers) of for 45 s, 72℃ for 1 min, and a final extension of 72℃ for 7 min. PCR products were separated on 8% non-denaturing polyacrylamide gels at 120 V for 2~2.5 h and visualized with a silver staining method (Liu et al. 2008).

Reverse transcription quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from MDE using RNAsimple total RNA kit (Tiangen). Then, the RNA was reversely transcribed into cDNA uisng PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. The RT-qPCR was conducted in a 20 μl reaction volume with 10 μl of 2 × SYBR Green PCR Master Mix (TaKaRa), 0.2 μM of each primer (Supplementary Table 2) and 2 μl diluted cDNA. The iCycler iQreal-time PCR detection system (BIO-RAD, USA) was used with the following conditions: 95℃ for 3 min, and 45 cycles of 95℃ for 5 s, 58℃ for 30 s, and 72℃ for 10 s, and the *RsActin* was used as the reference gene for normalization (Xu et al. 2012). The relative expression level of each gene was calculated by $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen. 2001).

Statistical analysis

The number of embryoids per petri dish was counted 40 days after microspore isolation. The data were analyzed and compared using SPSS 16.0 software by Duncan's multiple range test (p = 0.05) to determine the significance of differences among treatments.

Results

The effect of genotype on microspore embryogenesis

To explore the effect of genotype on microspore embryogenesis, 23 radish genotypes were used for IMC. The results showed that 16 of 23 radish genotypes could produce embryoids, while embryoid yield was significantly different. The maximum microspore embryogenesis rate of 20.00 (embryoids per dish) occurred in genotype L-22, while minimum rate of 0.12 was found in genotype L-8 (Table 1). However, MDE couldn't be obtained in seven genotypes through IMC, indicating that the difference of microspore embryogenesis in the responsiveness of individual genotypes was significantly in radish.

The effect of developmental stage on microspore embryogenesis

The optimal bud size for microspore embryogenesis in radish is different according to the genotypes (Table 2). Microspores from buds of 2.5~3.5 mm in length produced the highest ratio of embryoid per dish in genotype L-1, while buds with 1.5~2.5 mm long was optimal for IMC in genotype L-4, indicating that the developmental stage of microspores was critical for microspore embryogenesis. Cytological observation

indicated that microspores in the corresponding buds were in the late uni-nucleate to early bi-nucleate stage, and the ratio of petal/anther (P/A) was 0.75~1.0 (Fig. 1). As compared with the bud length, the ratio of P/A was relatively reliable for optimal anther selection due to the variation of environment factors and genotypes of donor plants.

The temperature treatment and microspore embryogenesis

Two genotypes, L-14 and L-18, were used to investigate the effect of cold treatment on microspore embryogenesis. The suitable cold pretreatment time between these two genotypes was different (Fig. 2). In genotype L-18, the highest yield of embryoid occurred in the control without cold treatment, and the embryoid rate decreased with the pretreatment duration. However, the genotype L-14 produced maximum embryoid when the buds were treated at 4℃ for 36 h, and then the rate of MDE decreased significantly.

No microspore embryogenesis was observed in the cultures of three genotypes (L-1, L-2, L-3) were treated with heat shock (32.5℃) for 96 h or maintained continuously at 25℃), indicating that the isolated microspores could not be further developed without heat shock pre-treatments. A appropriate heat shock pre-treatments time would help to improve the embryo yield. The highest microspore embryoid yield were obtained from the treatment of heat shock for 48 h, which were significantly different from other heat shock treatments. The embryoid yield of genotype L-3 from the treatment of heat shock for 48 h was higher than that for 24 h, while there was no significant difference between these two treatments (Table 3).

The effect of activated charcoal on microspore embryogenesis

The number of embryoid per dish was significantly enhanced with the increase of AC, and reached to the maximum at 0.75 g/L of AC concentration. After that the embryoid yield decreased (Fig. 3). With the supplement of AC, the embryoid yield increased 4.66-, 4.0- and 10.0-fold in the three genotypes, L-14, L-15 and L-18, respectively.

Expression analysis of *RsSERKs*

To explore the function of *RsSERK* genes, the gene expression patterns of the main stages of MDE including globular, heart-shaped, torpedo-shaped and cytoledonary embryoid in the process of MDE development and plantlet regeneration of radish (Fig. 4), six *SERK* genes were investigated with RT-qPCR analysis (Fig. 5; Fig. 6). Several genes including *RsSERK1A*, *RsSERK1B* and *RsSERK1C* were up-regulated during the development of MDE, while low expression level was observed in the *RsSERK3A*. A stable expression level in *RsSERK3B* was detected in MDE development and lower expression was found in the browning MDEs. Among *RsSERKs* except *SERK4*, the expressions in shoots was down-regulated during plant regeneration, and root and shoot developing. *RsSERK4* was highly expressed in torpedo-shaped embryoid and developing roots.

Chromosome counting and ploidy level of microspore-derived plantlets

The chromosome number of microspore-devived plantlets was analyzed. It could be found that the regenerated plantlets from microspore embryoids consisted of haploid (n=x=9), diploid (2n=2x=18) and tetraploid plants (2n=4x=36) as compared with the diploid donor plant (2n=2x=18) (Fig. 7). At the same time,

ploidy level of regenerated plants was also determined by flow cytomatic analysis, and the results were in accordance with those from chromosome analysis of root-tip cell (Fig. 8).

Homozyosity identification of microspore-derived plantlets with molecular markers

A total of ten pairs of SSR primers which could generate co-dominant markers were selected for PCR amplification in microspore-derived plantlets and 11 accessions (Supplementary Table 1, Table 4). Microspore-derived plantlets (No. 1-13) showed a single band at an specific allele, while other materials show co-dominant bands (No. 14-24) (Supplementary Fig. S1), indicating that these microspore-derived plantlets were homozygous plants and could be further used for DH population construction.

Discussion

Radish is a typical cross-pollinated crop. Due to the self-incompatibility, it is difficult to obtain inbred lines with complete homozygosity by conventional breeding methods. The IMC technology is considered to be an effective method to obtain homozygous DH lines for successful hybrid breeding programs (Corral et al. 2012). However, the application of IMC technology is very limited due to unavailability of highly efficient protocol for the development of DHs. The ability of microspores to convent its gametophyte development pathway which leads to mature pollen grains into sporophyte pathway determines the success of IMC. This critical transition results in cell formation of embryoids (Bhatia et al. 2018). Many factors such as donor plant genotype, microspore developmental stage, temperature treatment, supplements of vancomycin, putrescine, abscisic acid etc was critical for IMC. (Ahmadi et al. 2014a, 2014b; Bhatia et al. 2018). In this study, totally 23 genotypes were used to optimize the protocol of microspore embryogenesis in radish and the critical factors including genotype, bud developmental stage, stress and AC treatment were investigated to optimize the technology of IMC in radish. Furthermore, the potential role of *RsSERK*s in the process of the development of MDE was explored.

The effectiveness of microspore embryogenesis is determined by a complex network of internal and environmental factors (Żur et al. 2021). Embryogenesis ability of microspore and embryoid yield might be controlled by genetically heredity (Zhang et al. 2020). It was found that genotypes play an important role in the microspore embryogenesis of Chinese cabbage (Shumilina et al. 2015). Different genotypes of the same species have various responses to microspore embryogenesis (Bhatia et al. 2017). Recently, it was reported that genotype was one of the most crucial factors for microspore embryogenesis, which significantly affected the embryoid yield among radish cultivars (Shumilina et al. 2020). In most cases, microspore embryogenesis tends to be high genotype specificity (Tuncer et al. 2016). In this study, the embryoid yield of different genotypes ranged from 0 to 17.03 per dish, and the embryogenesis rate of various genotypes was quite different, which was in well accordance with the finding of genotypic dependence. Therefore, it can be concluded that genotypes play a critical role in radish microspore embryogenesis.

Characterization of bud size and P/A ratio could reveal development process of microspores (Mao et al. 2012). The optimum bud size varied considerably among genotypes for microspore embryogenesis (Bhatia et al. 2018). In cabbage, bud size of 3.2~3.5 mm was most effective for microspore culture, at this time, the most microspores were found to be at uninucleate stage (Cristea, 2013). However, buds size between 4.0~5.0

mm were found to be most suitable for isolated microspore culture in cabbage (Bhatia et al. 2018). For a synchronous development within single bud, the bud length could not been regarded as the appropriate index for bud selection. The length of the optimum bud for microspore culture was 2.5~4.0 mm, and the ratio of petal to anther (P/A) was 2/3~7/6 in *B. oleracea* (Wang et al. 2013a). In this study, the optional developmental stages of microspores could be estimated by the P/A ratio of 0.75~1.0 since microspores with this ratio were at the stages of uni-nucleate and early binucleate. The anther selection with this P/A ratio is of great practical significance for microspore culture.

The success of IMC depends on the ability of microspores to transfer their gametophytic developmental pathway to sporophytic pathway, resulting in cell division at a haploid ploidy level and formation of embryoid (Bhatia et al. 2018). The yield of MDE production has several bottlenecks at various stages of the process, such as induction and initiation of embryogenesis. The induction of microspore embryogenesis is carried out by specific stress treatments (cold/heat) to the isolated microspores (Testillano, 2019). Heat treatment can trigger auxin polarization and induce microspores embryogenesis of *B. napus* (Dubas et al. 2014). It was found that the exine of heat-stressed microspores can be broken at one or more pollen germination furrows (Tang et al. 2013). For *Brassicaceae* crops, heat shock treatment at the beginning of the culture was essential to promote the microspore embryogenesis and enhance the embryoid quality (Testillano, 2019). Temperature exhibited a significant effect on embryogenesis, and treatment at 30.5℃ for 48 h is the best treatment to induce embryogenesis of microspores of all sizes (Winarto Budi and Teixeira da Silva, 2011). In cauliflower, the efficiency of microspore embryogenesis in each group could be optimized by cold treatment (Bhatia et al. 2017). In this study, it was evident that the effect is different from various genotypes. Genotype L-18 responded positively to cold pre-treatment, but genotype L-8 was not sensitive, indicating that the effect of cold treatment is genotype-dependent (Gu et al. 2014; Bhatia et al. 2017).

AC has strong adsorption properties and is usually used to absorb gases and dissolved solids (Cheng et al. 2013). The supplement of AC to the medium was reported to modify medium composition *in vitro*. It was found that the *in vitro* androgenesis rate of pepper on 1% AC medium was the highest, regardless of the low-response or high-response genotypes of microspore culture (Supena et al. 2006). Among the tested genotypes, the yield of embryoid-like structure increased significantly after adding 0.1 ml AC to microspore medium (Cheng et al. 2013). The addition of 1% AC promoted the development of a large number of double haploid plants (Shumilina et al. 2015). In this study, embryoid yield and quality were significantly increased in genotype L-14, L-15 and L-18 with the supplement of 0.75 g/L AC, resulting in up to 4.66-, 4.00- and 10.0-fold of embryoid yield, respectively. While the concentration of AC was above 0.75 g/L, embryoid yield began to decrease. It was found that 2% active charcoal impaired embryonic shoot development (Supena et al. 2006). Therefore, the suitable concentration should be ascertained for certain species. It could be found that the supplement of AC at 0.75 g/L had a great effect on embryogenesis, which played a certain role in the optimization of effective microspore embryogenesis in radish.

The *SERK* family of receptor kinases is involved in cell-to-embryo transition and controlling a number of other fundamental aspects of plant development (Ahmadi et al. 2016). Over-expression of *AtSERK1* was proved to enhance embryogenic (Hecht et al. 2001). The *TnSERK* was up-regulated during the development of embryoids (Pilarska et al. 2016). Besides, a steady increase in *BnSERK2* expression was detected during MDE

development, indicating that these genes were involved in the process of MDE development in *B. napus*. Our results showed that the expression of *RsSERK1A*, *RsSERK1B* and *RsSERK1C* increased steadily during MDE development, implying that these up-regulated genes might play roles in this procedure. Intriguingly, the expression of *RsSERK* genes except *RsSERK4* in primary shoots and roots was higher than that in developed shoots and roots, indicating their role (s) in the early stage of organ formation, rather than being specific to the embryogenesis. Furthermore, the expression of *RsSERK*s was distinct. For example, the *RsSERK3A* had a low expression level only at the beginning of MDE development, and its expression was not detected during the later MDE development, but the high expression of the *RsSERK3A* was detected in primary shoots and roots. Meanwhile, a high expression level of *RsSERK4* was found in torpedo-shaped embryoid and developed roots. Overall, *RsSERKs* genes might be involved in the process of MDE development and plantlet regeneration in radish.

Conclusions

In this study, the significant influence of important factors including genotype, bud size, heat shock treatment and AC, are very critical for the success of embryogenesis in radish. Furthermore, the expression of *RsSERKs* in MDE development and planet regeneration would benefit the process of microspore embryogenesis, which may facilitate overcoming the challenges related to MDE production and plant regeneration in recalcitrant species. The haploid plants could be obtained successfully as well as the double haploids and tetraploid from IMC. Plants produced by microspore culture were identified as homozygous through the developed EST-SSR and genetic SSR markers. Homozygous plants obtained from IMC can be used directly for hybrid breeding based on the specific breeding objectives, and their offspring were highly uniform. This newly established IMC system could be employed for high efficient haploid induction and further application in radish heterosis breeding.

Declarations

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Data availability All data generated or analyzed in this study are included in this published article and its supplementary information files.

Code availability Not applicable.

Ethics approval Not applicable.

Conflict of interest The authors declare no competing interests.

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Tables

Table 1 The effect of genotype on embryoid production in radish

(Code)/ Genotype	No. of embryoid/dish	(Code)/ Genotype	No. of embryoid/dish	(Code)/ Genotype	No. of embryoid/dish
L-1/05-1262×ZW	3.48±0.95b	L-9/CWDG	0.13±0.11e	L-17/127	2.17±0.54b
L-2/05-Y-23×05-1259	2.15±0.64bcd	L-10/TDSGQ	1.37±0.14cde	L-18/XLM	15±3.54a
L-3/05-1551×05-1034	2.64±0.95bc	L-11/YDZBT	0.25±0.07e	L-19/UJBY	0±0e
L-4/SPZ×HP	17.03±3.36a	L-12/WYQ	0.76±0.11de	L-20/CXMM	0±0e
L-5/MTHS×XWFDH	0±0e	L-13/NPZWLB	0±0e	L-21/JLCX	0±0e
L-6/SHYDG	0±0e	L-14/JPCXL	3.33±0.41b	L-22/wh	20±2.12a
L-7/LF	0±0e	L-15/RG	1.67±0.41cde	L-23/sjmsh	15±4.00a
L-8/ZSDG	0.12±0.13e	L-16/DJ	1.33±0.41cde		

Note: [a] Means of columns followed by the same letters are not significantly different at P= 0.05

Table 2 The effect of microspore developmental stage on microspore embryogenesis

Genotype Code	The ratio of petal/anther	The ratio of petal/anther	The developmental stage of microspore	The ratio of embryoids/dish
L-1	1.5~2.5	1/4~3/4	middle-uninuclear stage	0.36±0.13b
	2.5~3.5	3/4~1/1	late uni-nucleate stage	3.48±0.95a
	3.5~4.5	1/1~4/3	binucleate stage	0±0b
L-2	1.5~2.5	1/5~3/5	middle-uninuclear stage	0.30±0.48b
	2.5~3.5	3/5~1/1	late uni-nucleate stage	2.15±0.64a
	3.5~4.5	1/1~5/4	binucleate stage	0.75±1.5ab
L-3	1.5~2.5	2/5~4/5	early-mononuclear stage	0.73±0.20b
	2.5~3.5	4/5~1/1	late uni-nucleate stage	2.64±0.95a
	3.5~4.5	1/1~5/4	binucleate stage	0.83±1.45b
L-4	1.5~2.5	4/6~3/5	late uni-nucleate stage	17.03±3.36a
	2.5~3.5	3/5~1/1	binucleate stage	1.57±0.30b
	3.5~4.5	1/1~4/3	trinucleate stage	0±0b

Note: [a] Means of columns followed by the same letters are not significantly different at P= 0.05

Table 3 The effect of heat shock (32.5℃) treatment on embryoid yield per dish

Genotype	Temperature treatments (h)	No. of Embryoid/dish
L-1	25 °C Continuous	0±0c
	32.5℃ (24h)	2.72±0.83ab
	32.5 (48h)	3.52±0.82a
	32.5 (72h)	1.86±0.38b
	32.5 (96h)	0±0c
L-2	25 °C Continuous	0±0c
	32.5℃ (24h)	1.86±0.70ab
	32.5 (48h)	2.13±0.64a
	32.5 (72h)	1.04±0.32b
	32.5 (96h)	0±0c
L-3	25 °C Continuous	0±0c
	32.5℃ (24h)	2.21±0.37a
	32.5 (48h)	2.62±0.89a
	32.5 (72h)	1.32±0.40b
	32.5 (96h)	0±0c

Table 4 The materials of microspore-derived plantlets and other occerssions in radish

Code	Genotype	Description	Code	Genotype	Description	Code	Genotype	Description
1	L1-1	microspore-derived plantlet	9	L4-2	microspore-derived plantlet	17	BX	F_1 hybrid
2	L1-2	microspore-derived plantlet	10	L4-3	microspore-derived plantlet	18	CL-7	F_1 hybrid
3	L1-3	microspore-derived plantlet	11	L4-4	microspore-derived plantlet	19	XXSR	F_1 hybrid
4	L1-4	microspore-derived plantlet	12	L4-5	microspore-derived plantlet	20	XXS	F_1 hybrid
5	L3-1	microspore-derived plantlet	13	L4-6	microspore-derived plantlet	21	NCB-9	F_1 hybrid
6	L3-2	microspore-derived plantlet	14	Lu127	advanced inbred line	22	NCB-8	F_1 hybrid
7	L3-3	microspore-derived plantlet	15	JJUN	F_1 hybrid	23	Lw23	F_1 hybrid
8	L4-1	microspore-derived plantlet	16	XMN	advanced inbred line	24	XBZ	F_1 hybrid

Figures

Figure 1

The developmental stages of microspores. **a** middle-uninucleate (nucleus located in the center of the cell). **b** late-uninucleate (nucleus displaced to the periphery of the cell). **c** binucleate (nucleus is clearly divided into two nuclei, vegetative (less bright) and generative (bright) nuclei). **d** trinuclear (three distinct nucleus, one vegetative nucleus and two sperm nuclei)

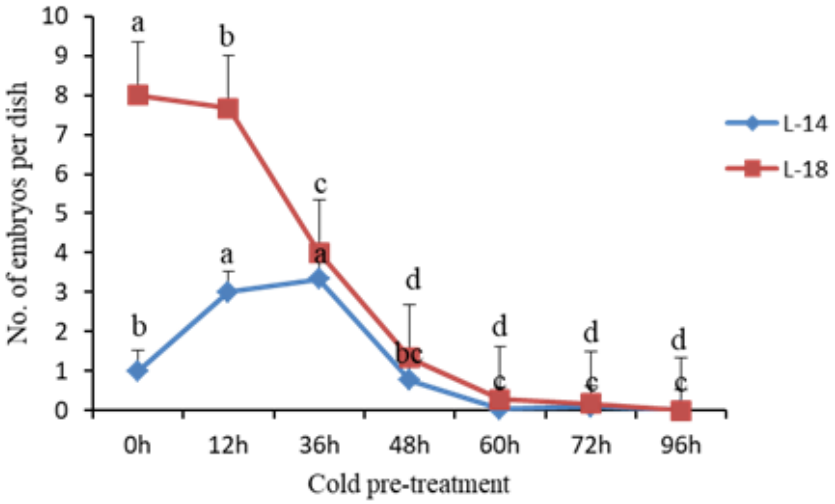

Figure 2

The effect of cold pre-treatment on embryoid yield in IMC

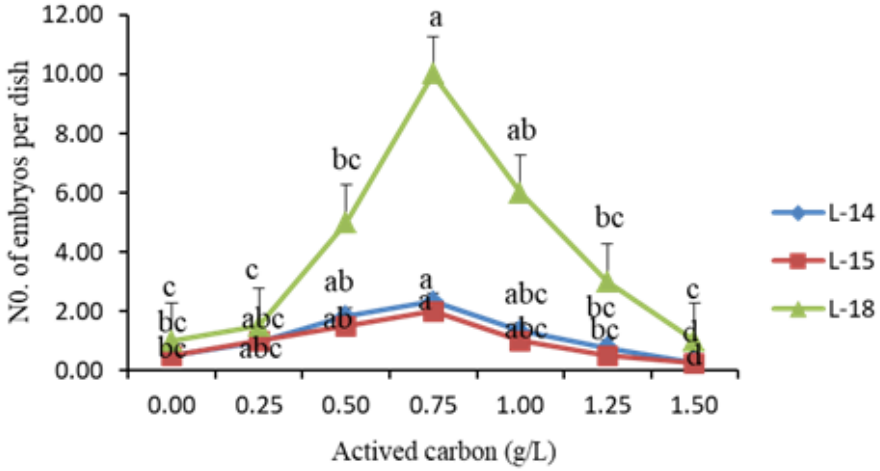

Figure 3

The effect of activated charcoal on embryoid yield

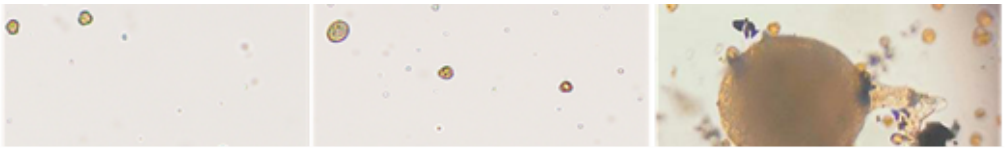

Figure 4

Microspore embryogenesis and plant regeneration of radish geneotype ‘XLM’. **a** isolated microspores from buds. **b** growing microspores at 7~10 days after culture. **c** gobular embryoid. **d** heart-shaped embryoid. **e** torpedo-shaped embryoid. **f** cotyledonary embryoid. **g** germinated microspores embryoids (35 days after culture). **h** cotyledonary embryoid transferred to subculture. **i** plantlet regeneration on root-inducing medium. **j** plants developed through microspore embryogenesis

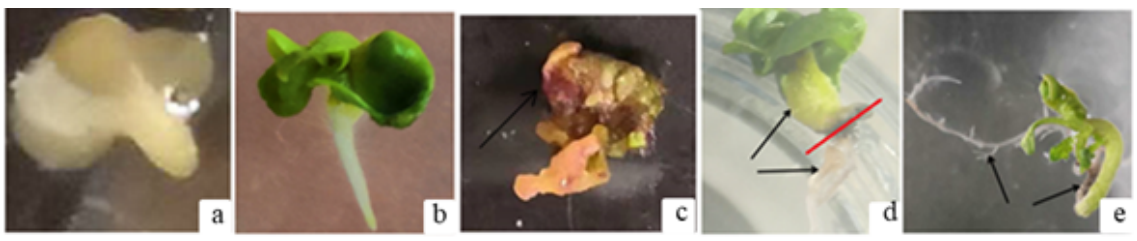

Figure 5

Microspore-derived embryo (MDE) regeneration in *Raphanus sativus* L. **a** Early cotyledonary MDE. **b** mature MDE. **c** Browning MDE. **d** primary shoot and root. **e** developing shoot and root

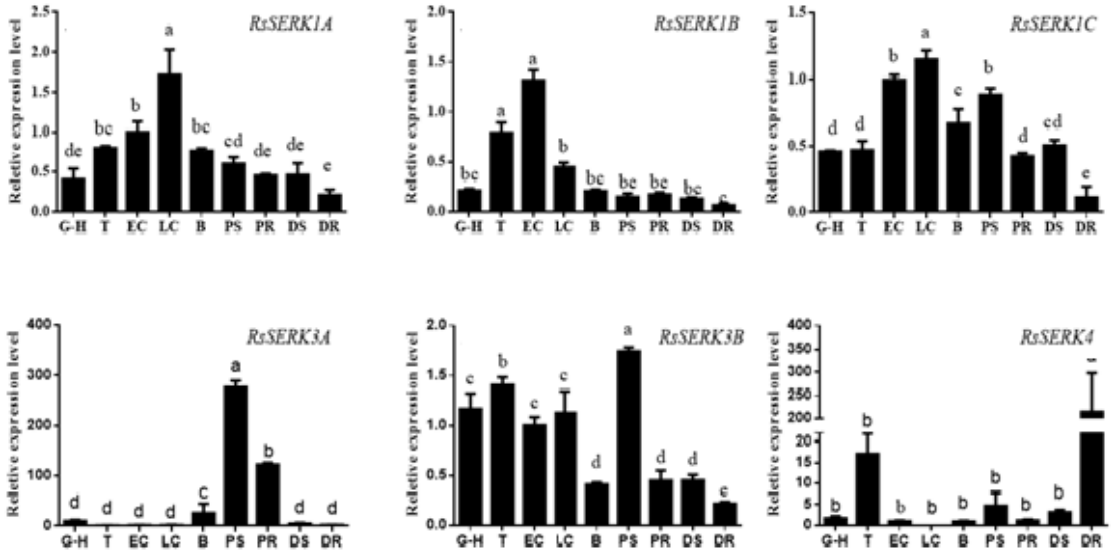

Figure 6

Expression profiles of *RsSERKs* during the microspore embryogenesis and plantlet regeneration in *Raphanus sativus* L. **G-H** globular-heart staged MDEs. **T** torpedo-staged MDEs. **EC** early cotyledonary MDEs. **LC** late cotyledonary MDEs. **B** browning MDEs. **PS** primary shoots. **PR** primary roots. **DS** developing shoots. **DR** developing roots. The error bar represents mean ± SD of three biological replicates

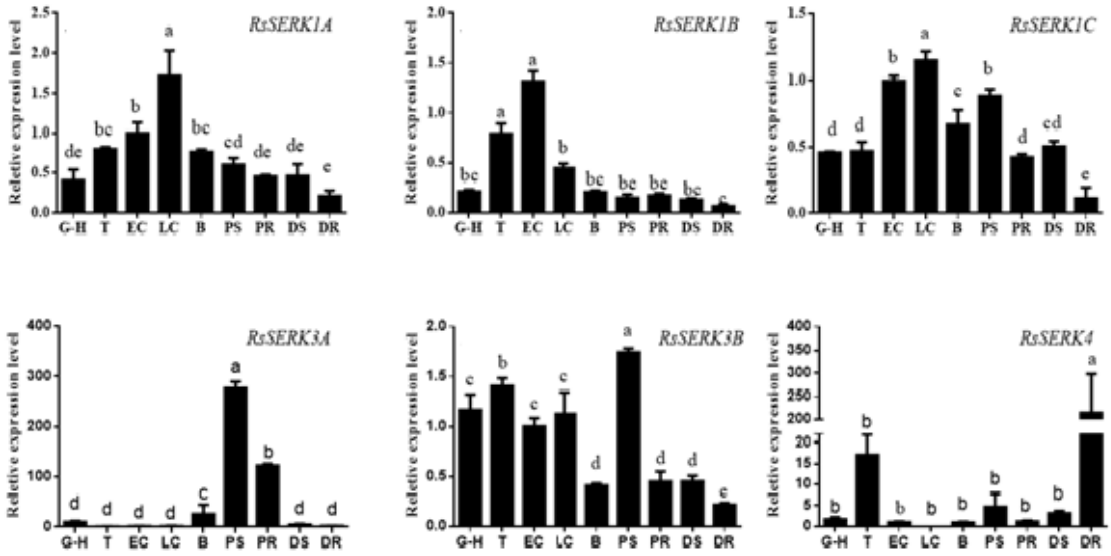

Figure 7

Ploidy level analysis on regeneration plants in radish with chromosome counting. **a** chromosomes (n=x=9) of a root-tip cell. **b** chromosomes (2n=2x=18) of a root-tip cell. **c** chromosomes (4n=4x=36) of a root-tip cell

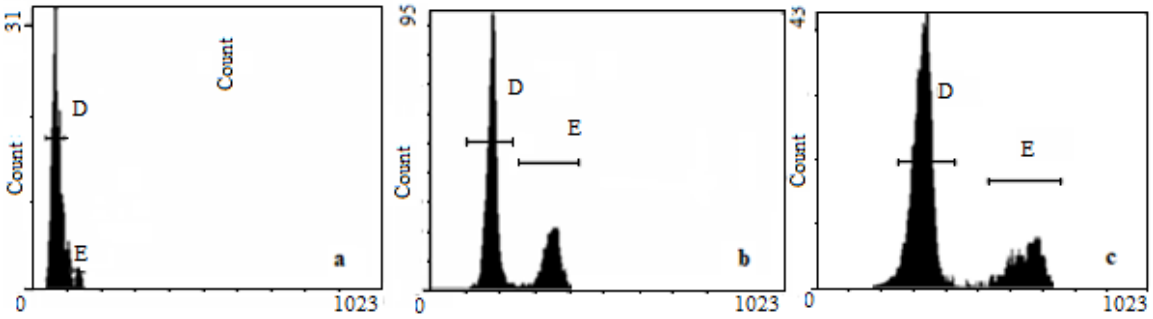

Figure 8

Analysis of ploidy level of plants by flow cytometric determination of leaf nuclear DNA contents. **a** haploid plant. **b** diploid plant. **c** tetraploid plant. The abscissa represents DNA content, the ordinate represents relative cell numbers, and **D** and **E** refer to the G1 and G2 phases, respectively

Supplementary Files

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