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EMBRYO CULTURE

cDNA-AFLP analysis of differentially expressed genes during microspore embryogenesis in non-heading Chinese cabbage

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Abstract

Stress can induce microspores to change their developmental pathway from the gametophytic to the embryogenic pathway. To explore the molecular mechanisms of microspore embryogenesis, complement DNA-amplified fragment length polymorphism was used to isolate the transcript-derived fragments during microspore embryogenesis of the non-heading *Brassica campestris* L. ssp. *chinensis* 'Wuyueman'. With 256 primer combinations screened, a total of 94 transcript-derived fragments were identified, and 15 were successfully sequenced. Based on a BLAST search in the *Brassica* database, 12 of the 15 sequenced transcript-derived fragments were homologous to genes with annotations; the remaining three transcript-derived fragments did not match any sequences. Transcript-derived fragments with annotations were involved in cell wall formation, hormones, and resistance. Analysis of *cis*-elements indicated that there were heat shock-related and stress-related *cis*-elements in the promoter sequences of 12 transcript-derived fragments. *TDF1*(*Bra040720*), *TDF6*(*Bra013664*), and *TDF15*(*Bra022587*) were selected for validation of complement DNA-amplified fragment length polymorphism expression patterns by real-time quantitative PCR. Results confirmed the altered expression patterns of three genes revealed by the complement DNA-amplified fragment length polymorphism. This study provides novel information on the molecular mechanism of microspore embryogenesis in non-heading Chinese cabbage.

Keywords cDNA-AFLP · Microspore embryogenesis · Transcript-derived fragments (TDFs) · *Cis*-elements · Non-heading Chinese cabbage

Introduction

Isolated microspore culture (IMC) is important for practical breeding and molecular research in *Brassica* crops. Compared to traditional breeding, IMC is a more prominent approach to generate stable homozygous doubled-haploid parental lines for the production of F1 hybrids (Abercrombie *et al.* 2005), which dramatically accelerates the breeding process and facilitates the selection of fine recessive traits (Henderson and Pauls 1992; Chan 2010; Ferrie and Caswell 2011). The cultured double haploid population is an ideal material for the identification of molecular markers and construction of gene maps (Kitashiba *et al.* 2016; Liu *et al.* 2017; Valdés *et al.* 2018). IMC also contributes to mutation and selection. Lu *et al.* (2016) obtained 142 mutants of Chinese cabbage with distinct variations in phenotype and disease resistance. Liu *et al.* (2010) also obtained a semi-dwarf *B. napus* mutant known as *ds-1*, using IMC by EMS mutagenesis. Furthermore, researchers are attempting to establish an IMC-mediated genetic transformation system (Abdollahi *et al.* 2007; Cegielska-Taras *et al.* 2008; Maheshwari *et al.* 2011). In addition, the process of microspore embryogenesis *in vitro* can provide a better understanding of the process of zygotic embryogenesis. However, the molecular mechanism of microspore embryogenesis is still unclear.

Various techniques and strategies are being used to identify genes involved in microspore embryogenesis. Boutilier *et al.* (2002) isolated the *BABY BOOM (BBM)* gene from the microspore-derived embryo development of oilseed rape by the suppression subtractive hybridization (SSH) method, in which the gene expressed preferentially at different stages of

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embryogenesis and promoted somatic embryo formation after its ectopic expression. Malik *et al.* (2007) isolated a large number of ESTs (expressed sequence tags) of *Brassica* microspores, or microspore-derived embryos at different stages of development by constructing cDNA libraries, and molecular markers for microspore embryogenesis were identified. By segregation distortion analysis, Kitashiba *et al.* (2016) identified three physical positions associated with embryo yield in microspore-derived embryos of *B. rapa*. Valdés *et al.* (2018) identified 13 quantitative trait loci (QTL) on linkage groups of *B. napus*, which provided information for genetic variation and inheritance of the microspore embryogenic potential. Therefore, screening differentially expressed genes directly or indirectly related to microspore embryogenesis can reveal the molecular basis of microspore embryogenesis and provide new pathways to initiate embryogenesis.

Complement DNA-amplified fragment length polymorphism (cDNA-AFLP) is mainly based on restriction enzymes and PCR (Vos *et al.* 1995; Bachem *et al.* 1996) and has been successfully used to identify differentially expressed genes during plant growth and development, such as drought response, abscisic acid production, and plant-pathogen systems (Sarosh and Meijer 2007; Wang *et al.* 2009; Gong *et al.* 2014; Melloul *et al.* 2014; Xiao *et al.* 2016). The objectives of this study were to apply the cDNA-AFLP technique to isolated transcript-derived fragments (TDFs) during microspore embryogenesis, identify genes regulating microspore embryogenesis, analyze the *cis*-elements of identified genes, and validate the expression patterns for some of the regulated genes using real-time quantitative PCR (RT-qPCR).

Materials and methods

Plant materials and isolated microspore culture Two cultivars of *Brassica campestris* L. ssp. c*hinensis*, including 'Wuyueman' and 'Kuaicai', were grown in 20 cm pots in the greenhouse in September in Nanjing, Jiangsu, P.R. China. When the plants flowered, 2.0 to 3.0 mm buds were selected for IMC. IMC was performed according to Sato *et al.* (1989), with minor modifications. The harvested buds were surface sterilized by 1% (*v/v*) sodium hypochlorite with gentle shaking for 20 min and washed three times with sterile distilled water. Next, the buds were ground in 1/2 NLN-13 medium using a grinder (IKA, Staufen, Germany). The crude suspension was filtered through a 40 μm cell filter screen, and the filtrate was centrifuged at 150×g for 4 min. The supernatant was decanted, and the pellet was rinsed in fresh 1/2 NLN-13 medium. The procedure was repeated twice. The final pellet was resuspended in NLN-13 medium. The density of the microspores was adjusted to approximately 1×10^5 microspores mL^{-1}. Finally, 4.5 mL of the microspore suspension was dispensed into sterilized Petri dishes (diameter, 60 mm; height, 15 mm). Since 33°C is commonly used to induce *Brassica* microspore embryogenesis and the effect of 33°C temperature on microspore embryogenesis was identified from a pre-experiment, the cultures of the two cultivars were divided into two groups. One group was incubated in the dark at 33°C for 24 h and then transferred to 25°C in the dark for 2 wk., and the other group was only incubated at 25°C without the 33°C heat shock treatment. After 2–3 wk., the number of microspore-derived embryos was counted (the embryogenesis frequency = microspore-derived embryos number/bud number). There were three replicates, and each replicate consisted of five Petri dishes. Statistical analyses were compared using Duncan's multiple-range test ($P < 0.05$). The cultures were monitored under an inverted microscope (Olympus IX73, Scientifica, Tokyo, Japan) and stereomicroscope (Leica M165 FC, Leica Microsystems, Wetzar, Germany).

RNA isolation and cDNA-AFLP analysis Because 'Wuyueman' is an important cultivar with the characteristic of late bolting, which is important for the breeding of non-heading Chinese cabbage, cDNA-AFLP analysis was first conducted using 'Wuyueman.' The microspore suspensions of different culture methods and different culture times, including freshly isolated microspores (0 h), microspores that were cultured for 7 d without heat shock treatment, and microspore-derived embryos cultured for 7 d and 20 d under normal microspore embryogenesis inducing conditions were collected and centrifuged at 100×g for 3 min. Next, the supernatant was discarded, and the microspores were quickly frozen in liquid nitrogen. Total RNA was extracted using the RNAiso Plus Kit (Takara Biomedical Technology, Beijing, China). The integrity and quantity of total RNA were detected by 1% (*w/v*) agarose gel electrophoresis and spectrophotometer at 260 nm, respectively. Double-stranded cDNA was synthesized using M-MLV RTase cDNA Synthesis Kit (Takara Biomedical Technology, Beijing, China) and purified with the DNA Fragment Purification Kit ver.2.0 (Takara Biomedical Technology, Beijing, China).

The cDNA-AFLP and silver-staining processes were performed as described by Xiao *et al.* (2016) with minor modifications. Double-strand cDNA was digested with *Taq I* and *Vsp I* restriction enzymes, respectively, and then ligated to the *Taq I* and *Vsp I* double-strand anchor. After ligation to the adaptor, preamplification and selective amplification were conducted on the PCR products (Anchor, preamplification primer, and selective primer sequences are shown in Table 1). The initial small-scale screen using 256 AFLP primer combinations was performed using 16 *Taq I* forward selective amplification primers (extension NN), in combination with 16 *Vsp I* reverse selective amplification primers (extension NN), respectively. The preamplification PCR was carried out as follows: 94°C for 30 s; 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. The selective amplification

Table 1 Adaptors and primers pairs used for complement DNA-amplified fragment length polymorphism (cDNA-AFLP) analysis

Adaptors and primers	The sequence of nucleotides (5′-3′)
Taq I adaptors	GACGATGAGTCCTGAC
	CGGTCAGGACTCAT
Vsp I adaptors	GCGTAGACTGCGTACC
	TAGGTACGCAGTC
Taq I primer for pre-amplification	GACGATGAGTCCTGACCGA
Vsp I primer for pre-amplification	CTCGTAGACTGCGTACCTAAT
Taq I primer for selective amplification	GATGAGTCCAGACCGANN
Vsp I primer for selective amplification	GACTGCGTACCTAATNN

Note: N stands for A, T, C, and G

PCR was carried out as follows: 94°C for 30 s; 11 cycles of 94°C for 30 s, 65°C for 30 s (−0.7°C/cycle), and 72°C for 1 min; 24 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. Selective amplification products were separated on polyacrylamide gels and visualized by silver staining. A total of 94 different TDFs were obtained, and bands were photographed and analyzed. According to the analysis, 15 bands were excised from polyacrylamide gels and were boiled in 20 to 30 μL ddH_2O at 95°C for 30 min. The TDFs were reamplified as described previously. The amplified fragments were characterized by separation on a 1.5% (*w/v*) agarose gel, cloned into the pMD18-T vector (Takara Biomedical Technology, Beijing, China), and sequenced (Invitrogen Biotechnology, Shanghai, China).

Sequence analysis A homology search of the TDF sequences was performed using the BLAST program with *E* value $<10^{-5}$ in the *Brassica* database (http://brassicadb.org/brad/). The 12 selected sequences were annotated by TAIR (http://www.arabidopsis.org). To interpret the function of the homologous genes, gene ontology (GO) analysis was completed using WEGO (http://wego.genomics.org.cn). Putative *cis*-elements of TDFs were predicted using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). A 2-kb sequence upstream of the ATG initiation codon was selected as the promoter region for this analysis.

RT-qPCR analysis Quantification of the gene expression levels of TDF1, TDF6, and TDF15 was performed by RT-qPCR with *in vitro* culture samples at different stages of microspore embryogenesis of 'Kuaicai'. RT-qPCR assays were performed in an ABI StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) using the Takara SYBR Premix Ex TaqTM kit with amplification conditions as recommended. Primers for RT-qPCR were designed using the PrimerQuest Tool (https://sg.idtdna.com/Primerquest/Home/Index) (Table 2). The PCR program included an initial denaturation at 94°C for 30 s; 45 cycles of 5 s at 94°C, 30 s at 60°C, and 30 s at 72°C; and a final gradient of 60–95°C to determine melting curves. For the normalization of gene expression, the actin gene was used as the internal control for each RT-qPCR. Samples for RT-qPCR were run in three biological replicates with three technical replicates. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and analyzed using Rotor Gene 6.0 and Excel Software.

Results

The effect of heat shock treatment on the embryogenesis frequency of two cultivars of non-heading Chinese cabbage, 'Wuyueman' and 'Kuaicai' The effect of heat shock treatment on the embryogenesis rate was explored in two cultivars of non-heading Chinese cabbage. The results showed that microspore embryogenesis was not observed in the two cultivars when there was no heat shock treatment applied. When the heat shock treatment was applied, the embryo yields of the two cultivars significantly increased, and there were significant differences in the two cultivars (Table 3). The rate of embryogenesis of 'Wuyueman' and 'Kuaicai' was 6.00 and 13.62 embryos per bud, respectively. These results suggest that heat shock was an essential factor to induce microspore embryogenesis.

Table 2 Primer pairs used for real-time quantitative PCR (RT-qPCR)

TDF No.	Primer sequences
TDF1	F 5'-CGGACACAGTTGCGATTCTA-3'
	R 5'-CTACAGCTTGAAGGTACGGAAG-3'
TDF6	F 5'-CCTGGAAGTGCTAATACCTGTC-3'
	R 5'-CTTCCGCCACATCAAACTTATTC-3'
TDF15	F 5'-CGTCTTGGAGCAGAGCATTA-3'
	R 5'-TGTGACCTTGTCTGCTTCTATC-3'
Actin	F 5'-CTCAGTCCAAAAGAGGTATTCT-3'
	R 5'-GTAGAATGTGTGATGCCAGATC-3'

Table 3 Effect of 33°C heat shock treatment on embryogenesis frequency of *Brassica campestris* L. ssp. *chinensis* 'Wuyueman' and 'Kuaicai'

Culture conditions	Embryogenesis frequency (No. of embryos per bud, means ± SD)	
	Wuyueman	Kuaicai
25°C, 14 d	0 ± 0a	0 ± 0c
33°C, 24 h; 25°C, 13 d	6.00 ± 0.51b	13.62 ± 1.24a

Note: Embryogenesis was assessed as the average frequency of embryos per bud from three replicates, and each replicate consisted of five Petri dishes. *Lowercase letters* indicate significant differences at $P < 0.05$ according to Duncan's multiple-range test

Isolation of differentially expressed TDFs To isolate differentially expressed TDFs, cDNA-AFLP analysis was completed on total RNA samples from microspore-derived embryos induced by different culture methods and culture times. Using 256 combinations of selective primers, a total of 94 TDFs were isolated. According to the results, the TDFs were divided into the following three types (Fig. 1): the first type was only expressed in microspores that were cultured for 7 d with heat shock treatment (Fig. 1*a*); the second type was only expressed in microspores that were cultured for 20 d (cotyledon embryo) with heat shock treatment (Fig. 1*b*); and the third type was expressed in both microspores that were cultured for 7 d and 20 d with heat shock treatment (Fig. 1*c*). The results showed that 36 TDFs belonged to the first type, 23 TDFs belonged to the second type, and 35 TDFs belonged to the third type. A total of 15 samples with distinct differences and good reproducibility were used for reamplification, cloning, and sequencing. Among them, TDF3, TDF4, TDF7, TDF10, and TDF15 belonged to the first type; TDF2, TDF6, TDF11, TDF12, and TDF14 belonged to the second type; TDF1, TDF5, TDF8, TDF9, and TDF13 belonged to the third type.

Homology analysis and functional classification of differentially expressed TDFs The sequence comparison of 15 TDFs with the *Brassica* database revealed that a total of 12 TDFs had high similarity to genes with annotations, and 3 TDFs, including TDF11, TDF13, and TDF14, did not share similarity with any sequence (Table 4). Based on gene annotations in the *Arabidopsis* database, four TDFs were associated with cell wall formation, including plant invertase/pectin methylesterase inhibitor superfamily (TDF7), Kelch repeat-containing F-box family protein (TDF8), phenylalanine ammonia-lyase 1 (TDF9), and hydroxyproline-rich glycoprotein family protein (TDF10), which affects the *PAL* in the phenylpropanoid metabolism. Three TDFs were related to hormones, cytochrome P450 family protein (TDF1), 14–3-3-like protein (TDF2), and basic helix-loop-helix (bHLH) DNA-binding superfamily protein (TDF3). Four TDFs were related to resistance, response to low sulfur 3 protein (TDF4), desiccation-induced 1VOC superfamily protein (TDF5), NB-ARC domain-containing disease resistance protein (TDF12), and actin-depolymerizing factor 10 (TDF15); TDF6 encoding FAM63A-like protein (DUF544) may play an important role in the regulation of protein function.

To further investigate the potential functions of the 15 TDFs, a GO analysis was performed. The GO analysis of seven TDFs showed that TDF15 was involved in cellular components; TDF3, TDF9, and TDF12 were involved in regulation of transcription, biosynthetic processes, and the apoptotic process in the biological processes, respectively. Seven TDFs were involved in 11 different molecular functions including 4 kinds of bindings, 4 kinds of enzyme catalytic, 1 obsolete transcription regulator activity, and 1 electron transfer activity (Table 5).

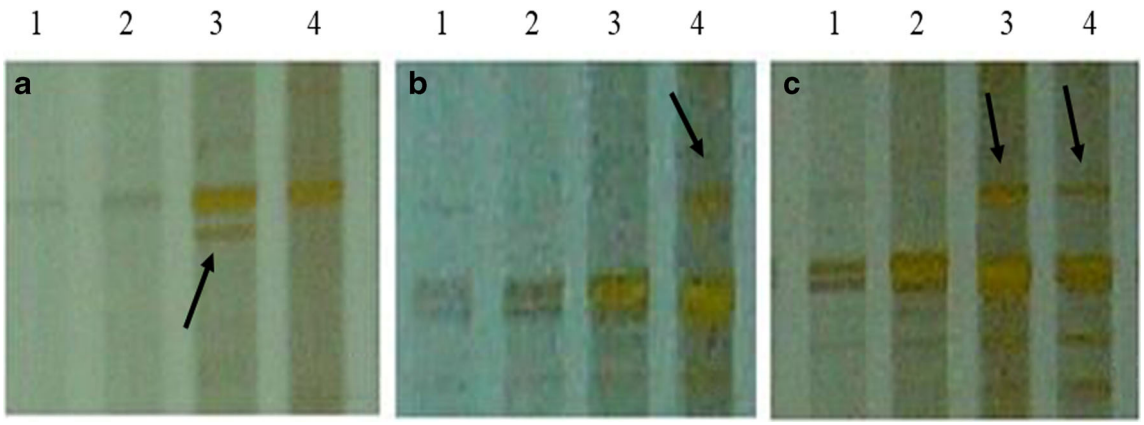

Fig. 1 Expression of *Brassica campestris L. ssp. chinensis* (non-heading Chinese cabbage) 'Wuyueman' genes in microspore embryogenesis determined by complement DNA-amplified fragment length polymorphism (cDNA-AFLP) analysis. *a*, the first type; *b*, the second type; and *c*, the third type. (*1*) 0 h (freshly isolated microspores); (*2*) microspores that were cultured for 7 d without heat shock treatment; (*3*) microspores that were cultured for 7 d with heat shock treatment; and (*4*) microspores that were cultured for 20 d with heat shock treatment. Arrow: differential bands.

Table 4 Homology analysis of the complement DNA-amplified fragment length polymorphism (cDNA-AFLP) fragment compared to sequences in the Brassica databases and their putative function

TDF No.	Primer combinations	Length (bp)	Gene_ ID	E-value	TAIR_ID	Annotation
TDF 1	T6A6	168	Bra040720	3e-57	AT1G13710	Cytochrome P450 family protein
TDF 2	T9A4	227	Bra037818	2e-87	AT5G65430	14–3-3 like protein
TDF 3	T5A7	194	Bra007358	1e-87	AT3G57800	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein
TDF 4	T5A7	136	Bra017975	4e-31	AT3G49570	Response to low sulfur 3 protein
TDF 5	T5A7	230	Bra031589	7e-59	AT1G07645	Desiccation-induced 1VOC superfamily protein
TDF 6	T9A7	124	Bra013664	7e-42	AT4G11860	FAM63A-like protein (DUF544)
FDF 7	T4A8	185	Bra018122	4e-69	AT3G47670	Plant invertase/pectin methylesterase inhibitor superfamily protein
TDF 8	T5A8	241	Bra026116	7e-93	AT1G15670	Kelch repeat-containing F-box family protein
TDF 9	T5A8	183	Bra005221	2e-74	AT2G37040	Phenylalanine ammonia-lyase 1
TDF 10	T5A8	139	Bra024560	2e-51	AT1G23040	Hydroxyproline-rich glycoprotein family protein
TDF 11	T5A8	254	No hits found	–	–	–
TDF 12	T5A8	227	Bra037453	1e-33	AT1G10920	NB-ARC domain-containing disease resistance protein
TDF 13	T9A8	244	No hits found	–	–	–
TDF 14	T9A8	240	No hits found	–	–	–
TDF 15	T16A8	220	Bra022587	1e-94	AT5G52360	Actin-depolymerizing factor 10

Regulatory *cis*-element analysis *Cis*-element analysis of promoters was performed for 12 TDFs. The sequences upstream of the ATG initiation codon of the TDFs were extracted and searched against the PlantCARE database. Interestingly, regulatory element analysis showed that the promoters of 12 TDFs possessed at least one heat shock-related *cis*-element among STRE, AT-rich, CCAAT-box, and ABRE (Table 6). Analysis showed that 8 of 12 TDFs had a STRE element. The AT-rich element was found in the promoter sequences of TDF1 (*Bra040720*), *TDF10* (*Bra024560*), and TDF12 (*Bra037453*). The CCAAT-box was found in the promoter sequences of TDF4 (*Bra017975*), TDF8 (*Bra026116*), and TDF15 (*Bra022587*). Except for TDF3 (*Bra07358*), *TDF6* (*Bra013664*), and TDF12 (*Bra037453*), the other TDFs possessed ABRE. Moreover, some *cis*-elements that are related to other stress conditions were identified. ARE, which is

Table 5 Pathway and categorization of 7 transcript-derived fragments (TDFs) isolated from different developmental stages of embryos

TDF No.	Gene number	GO ID	Pathway
TDF 1	Bra040720	GO:0020037	Heme binding (M)
		GO:0009055	Electron transfer activity (M)
		GO:0005506	Iron ion binding (M)
		GO:0004497	Monooxygenase activity (M)
TDF 2	Bra037818	GO:0019904	Protein domain specific binding(M)
TDF 3	Bra007358	GO:0045449	Regulation of transcription(B)
		GO:0030528	Obsolete transcription regulator activity (M)
TDF 7	Bra018122	GO:0030599	Pectinesterase activity (M)
		GO:0004857	Enzyme inhibitor activity (M)
TDF 9	Bra005221	GO:0016211	Ammonia ligase activity (M)
		GO:0009058	Biosynthetic process (B)
TDF 12	Bra037453	GO:0006915	Apoptotic process (B)
		GO:0005524	ATP binding (M)
TDF 15	Bra022587	GO:0005622	Intracellular (C)
		GO:0003779	Actin binding (M)

Note: These transcript-derived fragments (TDFs) were categorized based on gene ontology annotation. C: cellular component; M: molecular function; B: biological process

Table 6 *Cis*-elements associated with promoters of transcript-derived fragments (TDFs)

TDF No.	Gene number	Number of *Cis*-elements						
		STRE	AT-rich	CCAAT-box	ABRE	TC-rich	ARE	WUN-motif
TDF 1	Bra040720		1		1	1	4	
TDF 2	Bra037818	1			1	3		
TDF 3	Bra007358	1					1	2
TDF 4	Bra017975	1		1	3	1	3	1
TDF 5	Bra031589	1			10		3	
TDF 6	Bra013664	1					2	1
TDF 7	Bra018122				1		1	
TDF 8	Bra026116	1		1	6		2	
TDF 9	Bra005221	1			1		5	
TDF 10	Bra024560	1	1		2		1	
TDF 12	Bra037453		1				1	
TDF 15	Bra022587			1	4	1	2	1

responsive to anaerobic stress, was identified in promoter sequences of most of the TDFs, except for TDF2 (*Bra073818*). The TC-rich element was related to defense and responsiveness and was identified in the promoter sequences of TDF1 (*Bra040720*), TDF2 (*Bra073818*), TDF4 (*Bra017975*), and TDF15 (*Bra022587*). The WUN-motif, which is a wound-responsive element, was identified in the promoter sequences of TDF3 (*Bra007358*), TDF4 (*Bra017975*), TDF6 (*Bra013664*), and TDF15 (*Bra022587*).

Expression pattern of isolated TDFs and impact on microspore embryogenesis To evaluate the reliability of cDNA-AFLP, the expression level of three TDFs [TDF1 (*Bra040720*), TDF6 (*Bra013664*), and TDF15 (*Bra022587*)], at different stages of microspore development was investigated in another cultivar of non-heading Chinese cabbage, 'Kuaicai'. Microspore-derived embryos that were freshly isolated (0 h), or cultured under normal conditions for 1 d, 7 d, 14 d, and 20 d, were collected. To ensure that the collected microspores were effective, the development of microspores was observed before the samples were collected. Microspores at the late uninucleate stage (Fig. 2*a*), which is the most responsive stage for the induction of microspores embryogenesis, were freshly isolated and subjected to inductive culture. Microspores that were heat shocked at 33°C for 1 d (Fig. 2*b*) were transferred to 25°C. After 7 d of culture,

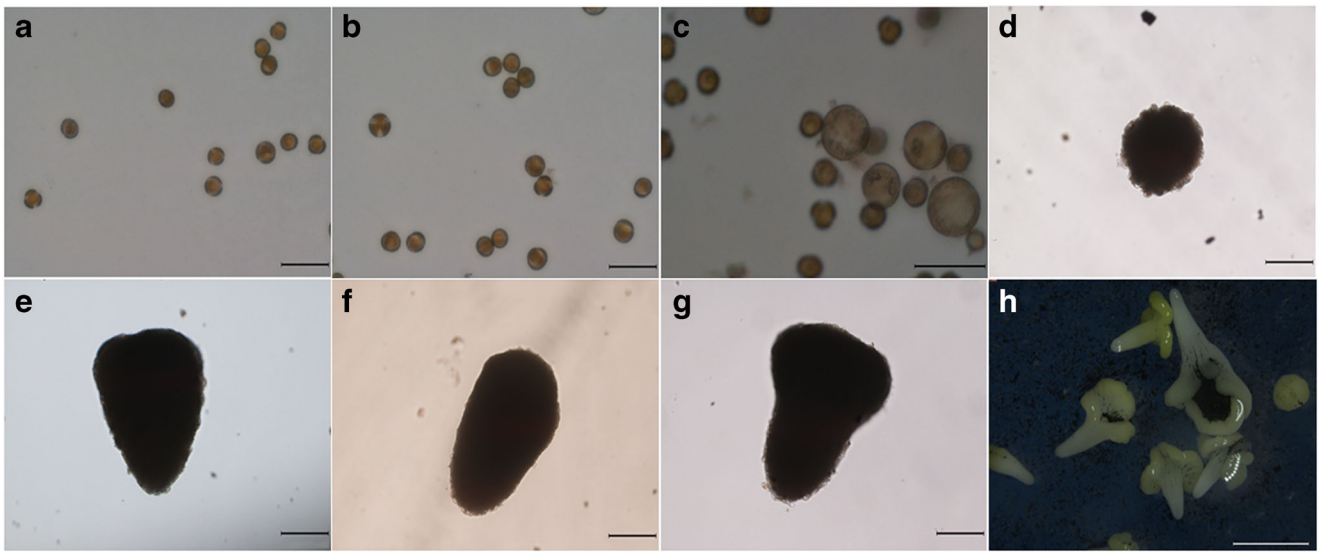

Fig. 2 The different development of embryogenesis in *Brassica campestris* L.*ssp. chinensis* 'Kuaicai'. *a*, the fresh isolated microspores (0 h). *b*, microspores after heat shock at 33°C for 1 d. *c*, microspores after 7 d of culture. *d*, *e*, *f*, and *g*, globular embryos, hearted-shaped embryos, torpedo embryos, and early cotyledon embryos, respectively, after 14 d of culture. *h*, green cotyledon embryos after 20 d of culture. *bars*: abc = 50 μm, defg = 200 μm, h = 2 mm.

multicellular structures (Fig. 2*c*) accompanied by nonresponsive microspores were formed. After 14 d of culture, globular embryos (Fig. 2*d*), hearted-shaped embryos (Fig. 2*e*), torpedo embryos (Fig. 2*f*), and early cotyledon embryos (Fig. 2*g*) were visible to the naked eye and were transferred to the shaker to allow maturation into green cotyledon embryos (Fig. 2*h*).

The expression patterns of the 3 TDFs at different stages of microspore development are shown in Fig. 3. By RT-qPCR analysis, it was found that TDF1 (*Bra040720*) and TDF15 (*Bra022587*) had similar expression patterns as observed in the cDNA-AFLP test, and the expression pattern of TDF6 (*Bra013664*) was different from that in the cDNA-AFLP tests. As shown in Fig. 3, after heat shock at 33°C for 1 d, the relative expression levels of TDF6 (*Bra013664*) and TDF15 (*Bra022587*) (Fig. 3*b*, *c*) both increased compared with freshly isolated microspores (0 h), whereas expression levels of TDF1 (*Bra040720*) (Fig. 3*a*) slightly decreased. Afterwards, TDF1 (*Bra040720*) was gradually upregulated and reached its highest level after induction culture for 14 d. Next, expression was gradually downregulated and remained at a relatively high expression level in microspores that were cultured for 20 d. The expression levels of TDF1 (*Bra040720*) in microspores that were cultured for 7 d and 20 d were relatively high, which is consistent with cDNA-AFLP tests that expressed in microspores cultured for both 7 d and 20 d. The relative expression of TDF15 (*Bra022587*) reached the highest in microspores that were cultured for 7 d, then was downregulated and nearly disappeared in microspores that were cultured for 14 d and 20 d, which was consistent with the cDNA-AFLP test that only had a high expression in microspores that were cultured for 7 d. In the cDNA-AFLP tests, TDF6 had a high expression level in microspores that were cultured for both 7 d and 20 d. The relative expression level of TDF6 (*Bra013664*) was consistent with that of TDF15 (*Bra022587*), which was not apparent in microspores that were cultured for 20 d.

Discussion

To gain insight into the molecular mechanism of microspore embryogenesis, genes were isolated that were differentially expressed during microspore embryogenesis. As a result of cDNA-AFLP analysis and differential screening, 94 TDFs associated with microspore embryogenesis were isolated, and 15 TDFs were used for additional research and analysis. Using the *Brassica* database, it was observed that 12 of 15 TDFs were homologous to genes with annotations, and 3 of 15 TDFs did not match any sequence in the database. Through the functional annotations conducted using the *Arabidopsis* database, it was found that TDFs can be classified into genes related to cell wall formation, hormones, resistance, and others. This study may provide a foundation to better understand mechanisms of microspore embryogenesis in the non-heading Chinese cabbage.

This study showed that four transcripts encoded proteins involved in cell wall formation (Table 4). The main components of dicotyledonous cell walls are pectins, and Rodríguez-Sanz *et al.* (2014) reported that cell wall remodeling by pectin esterification was involved in early *in vitro* embryogenesis. Pectinesterases have been found in abundance in the cDNA libraries of *B. napus* 'Topas' DH4079 microspore cultures that were induced for 3 d and 5 d (Malik *et al.* 2007). Pectin methylesterases were induced after microspore reprogramming and played a role in pectin de-esterification (Solís *et al.* 2016). Malik *et al.* (2007) also reported that invertase/pectin methylesterase inhibitor was highly expressed in induced microspores. In this study, TDF7 (*Bra018122*) was isolated, which encodes a plant invertase/pectin methylesterase inhibitor superfamily protein, and may play a role in regulating the activities of pectin methylesterases. TDF10 (*Bra024560*) encodes

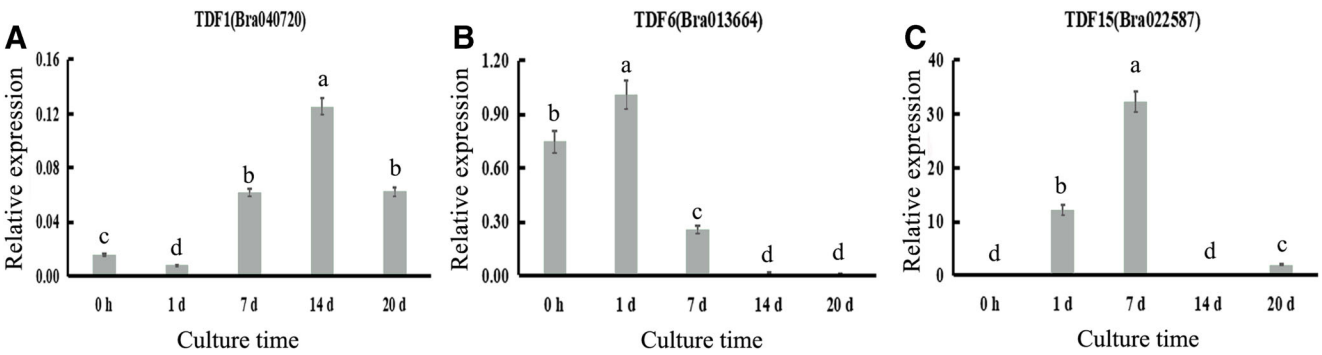

Fig. 3 Relative expression patterns of *TDF1*(*Bra040720*), *TDF6*(*Bra013664*), and *TDF15*(*Bra022587*). Eight Petri dishes of samples were collected at 0 h (fresh isolated microspores), 1 d (inflated microspores), 7 d (multicellular structure), 14 d (embryos visible to the naked eye), and 20 d after culture (green cotyledon embryo). Bars represent standard deviation among three independent biological replicates. Lowercase letters indicate significant differences at $P < 0.05$ according to Duncan's multiple-range test.

a hydroxyproline-rich glycoprotein family protein (HRGP), which are wall structural proteins that play important roles in plant growth and development. HRGPs are commonly divided into three major families: arabinogalactan proteins (AGPs), extensins (EXTs), and proline-rich proteins (PRPs). Malik *et al.* (2007) found numerous transcripts for AGPs during microspore embryogenesis. El-Tantawy *et al.* (2013) reported that AGPs associated with the newly formed walls of embryos of *Brassica* microspores at the two-four cell stage of embryogenesis and identified AGPs as early molecular markers of microspore embryogenesis. TDF8 (*Bra026116*) and TDF9 (*Bra05221*) were involved in the metabolism of phenylpropanoid, which is capable of producing lignin. Lignin is one of the components that make up the cell wall of plants. TDF9 (*Bra05221*), which encodes a phenylalanine ammonia-lyase 1 (PAL1), was detected. PAL is known as the first enzyme involved in the synthesis of phenylpropanoid (Kumar and Ellis 2001). Genes encoding PAL were also reported to be involved in the phenylpropanoid pathway in cultures of barley anthers (Jacquard *et al.* 2009; Bélanger *et al.* 2018). TDF8 (*Bra026116*) encodes Kelch repeat F-box (KFB) proteins that can regulate the proteolysis of PALs by mediating protein ubiquitination and degradation, and its expression level affects the stability of PAL enzymes, which changes the levels of phenylpropanoids (Zhang *et al.* 2013). Therefore, it is possible that TDF8 (*Bra026116*) and TDF9 (*Bra05221*) affected the formation of cell walls by directly or indirectly affecting *PAL* in the phenylpropanoid metabolism. These results may suggest that some cell wall formation related genes are involved in microspore embryogenesis.

Studies suggest that many hormones are closely related to microspore embryogenesis (Żur *et al.* 2015). In this study, three TDFs were identified that are related to hormones including TDF1 (*Bra040720*), TDF2 (*Bra037818*), and TDF3 (*Bra007358*). TDF1 (*Bra040720*) is homologous to *CYP78A5*, which is a member of the *CYP78A* family that encodes *Arabidopsis* cytochrome P450 monooxygenase. Malik *et al.* (2007) also found large EST clusters that encoded a cytochrome P450 family protein (CYP78A) and identified *BnCYP78A5* as a molecular marker gene for gametic embryogenesis (Malik *et al.* 2007). Cytochrome P450s are involved in the biosynthesis of cytokinins, IAA, gibberellins (GA), and brassinosteroids (BR) and in response to biotic and abiotic stressors (Kreps *et al.* 2002; Werck-Reichhart *et al.* 2002; Narusaka *et al.* 2004; Ehlting *et al.* 2008). TDF2 (*Bra037818*) encodes 14–3-3 proteins. Under BR stimulating conditions, 14–3-3 s and BKI1 can antagonize each other, and they play an important role in the BR signaling pathway (Wang *et al.* 2011). Malik *et al.* (2008) reported that when BR is added to the inducing medium, poorly induced embryogenic cultivars produce rare embryos, and Belmonte *et al.* (2010) also reported that BR contributed to enhanced embryogenesis. Maraschin Sde *et al.* (2003) reported that 14–3-3 isoforms were differentially regulated and processed during barley microspore embryogenesis. TDF3 (Bra007358) encodes the basic helix-loop-helix (bHLH) DNA-binding superfamily protein. Two bHLH proteins, which included bHLH48 and bHLH60, were found to be downstream components of the GA signaling pathway (Li *et al.* 2017); therefore, it is possible that TDF3 (*Bra007358*) also played an important role in GA regulation. In addition, Jacquard *et al.* (2009) and Bélanger *et al.* (2018) reported that the enzymes that they isolated are involved in the upregulation of jasmonic acid synthesis; however, genes related to jasmonic acid synthesis were not isolated in this study.

Microspore embryogenesis is a process of response to stress. In this study, four stress-related genes were isolated, including TDF4 (*Bra017975*), TDF5 (*Bra031589*), TDF12 (*Bra037453*), and TDF15 (*Bra022587*). TDF4 (*Bra017975*) is homologous to *AT3G49570*, which is involved in cellular oxidant detoxification. Antioxidants promote microspore embryogenesis (Hoseini *et al.* 2014; Zeng *et al.* 2015; Heidari-Zefreh *et al.* 2019) and may also play an important role in microspore embryogenesis. TDF5 (*Bra031589*) encodes the desiccation-induced 1VOC superfamily protein (dsi-1^{VOC}), which is a seed-specific gene that plays an important role in desiccation-sensitive plants (Mulako *et al.* 2008). TDF12 (*Bra037453*) is homologous to the NB-ARC domain-containing disease resistance protein, which is related to plant defense (Chandra *et al.* 2017). TDF15 (*Bra022587*) is homologous to actin-depolymerizing factor 10 (*ADF10*) and is expressed at the polarized microspore stage; it can protect actin in pollen grains from denaturation under stress conditions (Bou Daher *et al.* 2011). Although the functions of these genes in microspore embryogenesis are not completely clear, it is suggested that they may play a role to protect microspores against harm during acquisition of embryogenic potential. In this study, genes were not detected that encode glutathione S-transferases (GSTs), heat shock proteins (HSPs), or other genes that are known to be induced in response to stress and in the microspore response to stress (Maraschin Sde *et al.* 2006; Bélanger *et al.* 2018).

In addition, TDF6 (*Bra0136640*) is homologous to *AT4G11860*, which is involved in protein K48-linked deubiquitination. TDF6 (*Bra0136640*) may play an important role in the regulation of protein function. In this study, gene expression patterns during microspore embryogenesis were also validated by RT-qPCR in another cultivar of non-heading Chinese cabbage, 'Kuaicai'.

There are many studies on the effects of temperature on microspore embryogenesis. Some studies showed that low-temperature pretreatment on buds or inflorescence had positive effects on microspore embryogenesis (Sato *et al.* 2002), while this pre-experiment study showed its effect was not significant. Heat shock treatment is important for the induction microspore embryogenesis of *Brassica* (Custers *et al.* 1994; Yuan *et al.* 2011). This study showed that no embryos

were produced in two cultivars without heat shock treatment. When the heat shock treatment was performed, microspore embryogenesis was successfully induced (Table 1). Many researchers have previously focused on the relationship between heat shock and microspore embryogenesis. It has been postulated that heat shock induces the activities of heat shock proteins leading microspores embryogenesis. While microspore embryogenesis was successfully induced with colchicine instead of heat shock treatment, it did not induce the activity of HSPs, which indicates that HSPs are not required to trigger microspore embryogenesis (Zhao *et al.* 2003). Dubas *et al.* (2014) reported that heat shock caused auxin to be located in microspores in a polar manner, which promoted the initiation of microspore embryogenesis. Li *et al.* (2016) suggested that heat shock triggered global DNA hypomethylation and increased the differential methylation of transposons at CHG sites, but the relationship between DNA methylation and embryogenesis is not clear. To explore the relationship between heat shock and microspore embryogenesis, *cis*-elements in the promoter sequences of 12 TDFs that were isolated from different stages of microspore embryogenesis were analyzed in this study. The results showed that the promoter sequences of TDFs possessed at least one *cis*-element among STRE, AT-rich, CCAAT-box, and ABRE, which were reported to be related to heat shock (Rieping and Schöffl 1992; Haralampidis *et al.* 2002; Guo *et al.* 2008; Yi and Liu 2009; Dong *et al.* 2015) and at least one stress-related *cis*-element (Table 6). It is possible that these *cis*-elements are directly influenced by heat shock treatment and initiate the expression of these TDFs. In addition, ABRE is an ABA-inducible *cis*-element that plays an important role in ABA transcription. ABA was reported to play an important role in the initiation of embryogenesis (Wang *et al.* 2000; Żur *et al.* 2009). Various stressors, such as wounding, heat stress, and osmotic stress, can induce ABA synthesis. These results indicated that heat shock- and stress-related *cis*-elements could induce ABA synthesis, which leads to microspore embryogenesis.

Conclusions

cDNA-AFLP was successfully used to isolate the differential expression of microspore embryogenesis-related genes in non-heading Chinese cabbage. A total of 94 transcript-derived fragments were identified, and 15 were successfully sequenced. Sequence analysis of 12 differentially expressed TDFs was isolated and grouped into three categories: cell wall formation, hormones, and resistance. The promoter sequences of TDFs contained at least one heat shock-related *cis*-element, such as STER, AT-rich, CCAAT-box, or ABRE and at least one stress-related *cis*-element, such as TC-rich, ARE, or WUN-motif. These results facilitate the understanding of microspore embryogenesis. Additional studies are required to elucidate the functional characterization of these genes and their promoters.

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