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ORIGINAL PAPER

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IDENTIFICATION AND EXPRESSION OF GA-RELATED GENES ASSOCIATED WITH *in vitro* MICRO-TUBERS FORMATION IN *Pinellia ternata*

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ABSTRACT

Pinellia ternata is one of the most important herbs in traditional Chinese medicine. Recently, its yield is hard to meet the market demand, therefore the system of *in vitro* micro-tubers was developed for its propagation and endogenous GA was revealed probably function negatively during micro-tubers induction. However, the mechanism is still unknown. In this study, 70 mg/L chlormequat chloride (CCC) was successfully used to inhibit the endogenous GA content and promote the micro-tubers induction. Subsequently, suppression subtractive hybridization (SSH) was performed to identify GA-related genes associated with *in vitro* micro-tubers formation in *P. ternata*. The cDNAs of micro-tubers induced with and without CCC were used as the "tester", and "driver", respectively. SSH library sequencing yielded 300 expressed sequence tags (ESTs). Finally, 226 ESTs were retained after screening, 84 of which had no significant homology to any of previously identified genes and 39 of the remaining 142 ESTs represented singletons. Real-time quantitative RT-PCR analysis of the expression patterns showed that all 5 transcripts showed signal alteration during the process of *in vitro* micro-tubers formation. The sequences appeared to be highly homologous with 60S ribosomal protein, 26S ribosomal RNA gene, zinc transporter protein, 12kD storage protein and malate dehydrogenase, respectively. These results would facilitate the functional characterization of the GA-related genes associated with *in vitro* micro-tubers development and subsequent *in vitro* manipulation.

Key words: *in vitro* micro-tubers, *Pinellia ternata*, suppression subtractive hybridization, GA

INTRODUCTION

Pinellia ternata Breit has been widely used for thousands of years in China as a most important herb in traditional Chinese medicine [Luo et al. 2000, Lu et al. 2013]. Its tuber is the harvestable organ that is recognized for the antiemetic, antitussive, analgesic, anxiolytic, anticancer and anti-inflammatory effects [Luo et al. 2000, Han et al. 2006, Wu et al. 2012, Gombodorj et al. 2017, Xu et al. 2018]. In addition, previous studies revealed that the extracts of *P. ternate* had a strong insecticidal activity against multiple species of pests, which provided a possibility to develop eco-friendly biopesticide [Xiao-Yan et al. 2008, Li et al. 2010, Zhang et al. 2017]. Based on these valuable effects, the market demand is growing.

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However, the low yield resulted from virus-induced variety degeneration and the vast consume of tubers for propagation together limited the market development of *P. ternate* [Cui et al. 2009].

Currently, the regeneration system of *P. ternate* has been established *via* undergoing callus induction and shoot regeneration [Zhang and Xie 1998, Cui et al. 2009]. Yet, it is noted that the variation is unavoidable during this process that probably affects the genuineness of *P. ternate*. Thereafter, our lab developed an easy micro-tuber induction system by inverting the petioles of *P. ternate* into MS medium and micro-tubers grew in the physiology upper ends of the petiole, which realizes stable micropropagation of *P. ternate* [Xue et al. 2004, Chang 2007]. Additionally, the endogenous hormone contents during micro-tuber development were analyzed and GA was proved as an important inhibitor during micro-tuber formation [Chang 2007, Sheng et al. 2010]. However, the molecular mechanism of GA regulating tuber development is unclear. Uncovering the GA-related genes regulating tuber of *P. ternate* formation probably facilitate their prebiotic application and molecular breeding.

In this study, the micro-tuber induction system was optimized *via* reducing the GA content by adding the chlormequat chloride (CCC). And suppression subtractive hybridization (SSH) was employed to identify the GA-related genes associated with micro-tubers formation in *P. ternate*. The putative functions of these genes were established by alignment with the sequences deposited in the GenBank. Finally, expression of some candidate genes was verified by quantitative RT-PCR. Therefore, the results will contribute to further studies towards ultimately decipher the tuber development mechanism.

MATERIAL AND METHODS

Plant material and growth conditions. The tubers of *P. ternata* purchased from Feilong Traditional Chinese Medicine Institute of Science and Technology (Huaibei City, Anhui Province, China) were sand-cultured at room temperature. Petiole were sheared from the seedlings, washed thoroughly with tap water, and surface-sterilized by immersion in 70% (v/v) ethanol for 30 s and 0.1% (w/v) sodium hypochlorite solution (NaOCl) for 10 min. Micro-tubers were induced from the inverted-petiole in MS medium followed the protocol described previously [Chang 2007].

Measurement of GA content. The morphological upper end of the petiole where micro-tubers generated, were collected with an interval time of five days during the induction process of micro-tubers. Endogenous GA of the above materials were extracted and measured with the method as described [Chang 2007].

RNA extraction and subtractive cDNA library construction. A complementary deoxyribonucleic acid (cDNA) forward subtraction library was constructed, in which the "driver" cDNA was subtracted from "tester" cDNA, resulting in a subtraction library consisting of genes that were upregulated in "tester" cDNA. Total RNA was extracted from the morphological upper end of the petiole with and without CCC treatment using a protocol described previously [Lu et al. 2013]. Double-stranded "tester" cDNA was prepared from 0.4 mg total RNA extracted from CCC-treated materials, and the "driver" cDNA was prepared from untreated materials, using a SMART^{TM} polymerase chain reaction (PCR) cDNA synthesis kit (Clontech Laboratries, Mountain View, CA, USA) in accordance with the manufacturer's protocol. The forward subtraction library was constructed using the PCR-select cDNA subtraction kit (Clontech Laboratories). The amplified cDNA fragments were cloned into PMD-19 vector (Takara Bio, Japan). A total of 300 white clones were selected and cultured for plasmid extraction.

Sequencing and sequence analysis. Recombinant plasmids containing cDNA fragments were extracted from *E. coli* and sequenced with the M13F sequencing primer. All the inserted sequences were verified for homologies in the GeneBank database using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST).

Relative quantification of 5 ESTs. Total RNA was isolated from micro-tubers induced with and without CCC at days 5, 10 and 15, respectively. The cDNA synthesis and transcript abundances analysis was performed using the protocol described previously [Xue et al. 2017]. The reference sequence was *PtTUB*. The relevant primer sequences are given in Tab. 1. Each assay was run in triplicate.

Table 1. Primers used for real-time quantitative RT-PCR

Gene name		Sequence
PtRSP60	-F	5'- TAGATTTGGCCCTCACTTCG -3'
	-R	5'- GGGAACTTTCGGTTTCATCA -3'
PtRSR26	-F	5'- TGCGTCGGATTATGACTGAA -3'
	-R	5'- CCGACTGGAATAACACTCCAA -3'
PtZT	-F	5'- CCATTCGGTTTTTGAGGGTAT -3'
	-R	5'- GGCCTCTTCGGTATCAATCTC -3'
PtMDH	-F	5'- CATCCTGCGTCCTCTTTGTAA -3'
	-R	5'- AAGAGCCAAGACCTTCTACGC -3'
PtSP12	-F	5'- GACGACGACTTCAAGAGCATC -3'
	-R	5'- ATTCACCTTCTCCGTCACCAT -3'
TUB	-F	5'- AACCTACACGAACCTCAACCG -3'
	-R	5'- ATGGATGAAGGCTCAAACGCA -3'

Table 2. GA-related SSH cDNA library associated with induction of micro-tubers in *P. ternata*

Sequence name	Homology	Length (bp)	*E* value	Identity
05210760(1)-212-M13-_G05	udp-glucose 4-epimerase	309	8.16E-17	91.61%
07020830(PCR)-79-M13-_B12	tumor-related protein	581	4.37E-15	55.40%
05211760(1)-185-M13-_H08	zinc transporter	335	4.03E-33	92.70%
05211760(1)-93-M13-_G07	usp family protein	577	2.44E-48	85.75%
05211760(1)-195-M13-_B10	vacuolar sorting protein 4b	410	2.49E-26	95.50%
05211760(1)-26-M13-_F10	transitional endoplasmic reticulum	571	3.46E-98	96.40%
05211760(1)-106-M13-_D09	syntaxin 1b 2 3	1070	6.99E-95	91.35%
05210760(1)-221-M13-_H06	senescence-associated protein	317	2.22E-18	81.00%
07020830(PCR)-55-M13-_B09	tumor-related protein	581	4.37E-15	55.40%
05211760(1)-350-M13-_E08	protein vernalization insensitive 3-like	300	5.78E-27	81.30%
05211760(1)-111-M13-_A10	poly-binding protein	1020	3.83E-39	92.15%
05210760(1)-226-M13-_E07	plastid lipid-associated protein chloroplast	371	9.27E-22	84.15%
05211760(1)-292-M13-_C01	metallothionein-like protein	642	2.02E-13	80.15%
05210760(1)-251-M13-_F10	vacuolar membrane protein	584	6.32E-33	64.20%
05211760(1)-8-M13-_B07	mannose-binding lectin	389	2.24E-09	78.41%
05211760(1)-54-M13-_H02	malate dehydrogenase	461	3.20E-84	94.15%
05211760(1)-16-M13-_D09	fiber protein fb11	344	1.11E-29	88.50%
05211760(1)-39-M13-_A01	glucose acyltransferase	544	6.82E-16	60.60%
07020830(PCR)-72-M13-_C11	long chain acyl-synthetase 9	635	5.10E-32	83.35%
07020830(PCR)-82-M13-_E12	glycyl-trna synthetase mitochondrial-like	310	2.29E-30	91.65%
07020830(PCR)-2-M13-_E02	transport protein	350	8.56E-21	89.45%
05211760(1)-10-M13-_F08	exocyst complex component 2	627	7.10E-44	70.20%
05211760(1)-25-M13-_E10	enolase	196	7.52E-11	89.05%

07020830(PCR)-29-M13-_H05	metal ion binding protein	574	3.96E-63	80.15%
05211760(1)-66-M13-_D04	membrane transport family protein	480	8.68E-69	92.05%
05210760(1)-228-M13-_G07	elongation factor 1-gamma 3	386	3.73E-18	96.75%
07020830(PCR)-97-M13-_D02	elongation factor 1-alpha	290	3.28E-49	95.60%
05210760(1)-205-M13-_H04	diguanylate cyclase	1085	1.28E-158	99.80%
05211760(1)-59-M13-_E03	coatomer subunit beta -2-like	655	7.87E-64	91.40%
05211760(1)-290-M13-_A01	beta-lactamase tem	1082	1.59E-49	89.45%
05211760(1)-102-M13-_H08	beta-galactosidase alpha fragment	1079	1.86E-67	96.50%
07020830(PCR)-58-M13-_E09	cytochrome p450 monooxygenase cyp704g7	255	1.93E-20	85.70%
05210760(1)-214-M13-_A06	ankyrin repeat-containing protein at3g12360-like	801	6.56E-114	93.35%
05211760(1)-97-M13-_C08	60s ribosomal protein l18a	206	2.85E-24	95.55%
05211760(1)-69-M13-_G04	alpha a53d	1090	2.76E-57	92.80%
05211760(1)-320-M13-_G04	gamma-tocopherol methyltransferase	1127	2.12E-78	68.15%
05211760(1)-32-M13-_D11	transitional endoplasmic reticulum	571	3.46E-98	96.40%
05211760(1)-171-M13-_B07	probable 3-beta-hydroxysteroid-delta-isomerase-like	379	5.27E-32	80.15%
05211760(1)-79-M13-_A06	upf0051protein chloroplastic-like	236	1.00E-21	92.10%
05211760(1)-88-M13-_B07	unknown	416	3.29E-06	57.50%
05210760(1)-235-M13-_F08	unknown	417	3.36E-06	57.50%
05210760(1)-219-M13-_F06	unknown	584	6.32E-33	64.20%
05211760(1)-127-M13-_F01	unknown	513	9.55E-19	66.15%

RESULTS

Screening of optimal concentration of CCC for *in vitro* micro-tubers induction. Our previous study revealed that GA probably inhibited the micro-tubers induction [Chang 2007], therefore, the function of its inhibitor CCC in micro-tubers induction was explored *via* adding different concentrations of CCC into the medium. Compared with the CK, the induction rate of micro-tubers increased, while the concentration changed from 0 to 70 mg/L, however it decreased along with further increase of concentration (Fig. 1 A-F). Then the average weight of micro-tubers induced with different concentrations of CCC was analyzed and the result showed that 50 or 70 mg/L concentration of CCC largely promoted micro-tubers development, while 110 mg/L concentration of CCC severely inhibited its growth (Fig. 1 G).

Inhibition of CCC on the contents of GA in micro-tubers. Based on previous experiment, 50 or 70 mg/L concentration of CCC was proved to promote the induction rate of micro-tuber, then the effect of 70 mg/L concentration of CCC on the content of endogenous GA during micro-tuber induction needed to be explored. Compared with CK, it was dramatically decreased during micro-tuber induction process (Fig. 2).

Single-pass sequencing and classification of SSH library. The cDNA inserts varied in size from 200 to 800 bp (Fig. 3). Out of 300 sequencing reactions attempted, the vector leader and poor quality sequences were trimmed. 3'-vetor and linker sequences were removed if poly (A+) were included in the results. Sequences of bacterial origin were also excluded based on the BLASTn search results. Finally, 226 ESTs were retained after screening, of which 84 ESTs had no significant homology to any previously identified genes. Of the remaining 142 ESTs, 39 represented singletons, and these were grouped as listed in Fig. 4. The EST sequences obtained were identified by homology searches in BLASTn and BLASTx databases. The partial results are summarized in Tab. 2.

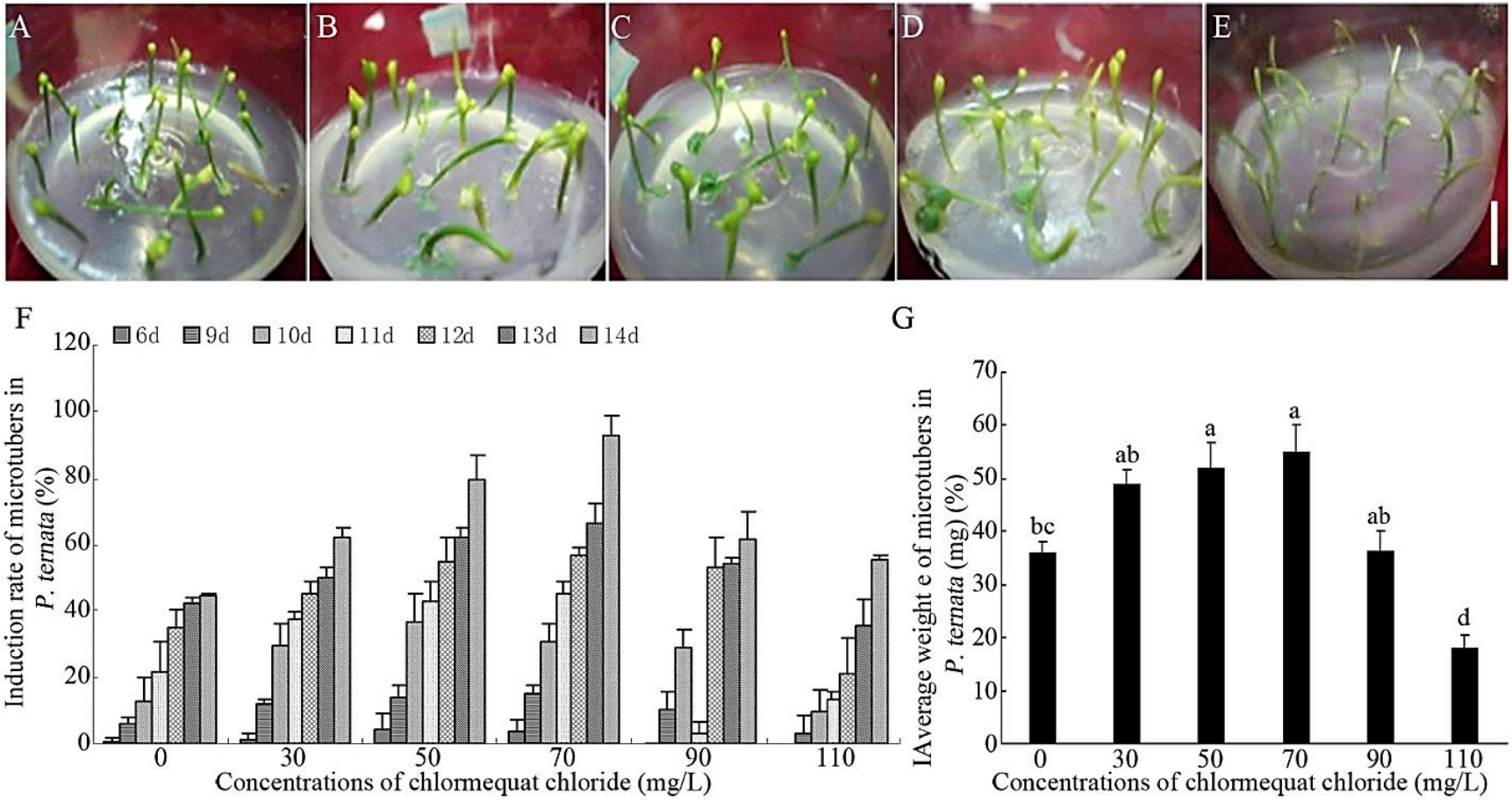


Fig. 1. Effects of CCC on micro-tubers induction in *P. ternata*. Formation of micro-tubers from petiole in MS medium supplemented with 30 mg/L (A), 50 mg/L (B), 70 mg/L (C), 90 mg/L (D) and 110 mg/L (E) CCC, respectively at day 14. Scale bars in A-E: 1 cm. F: comparison of the induction rate during the process of micro-tubers formation when present of different concentrations of CCC. G: effects of CCC on the average weight of micro-tubers at day 14. Different lowercases indicate statistically significant difference ($P < 0.05$)

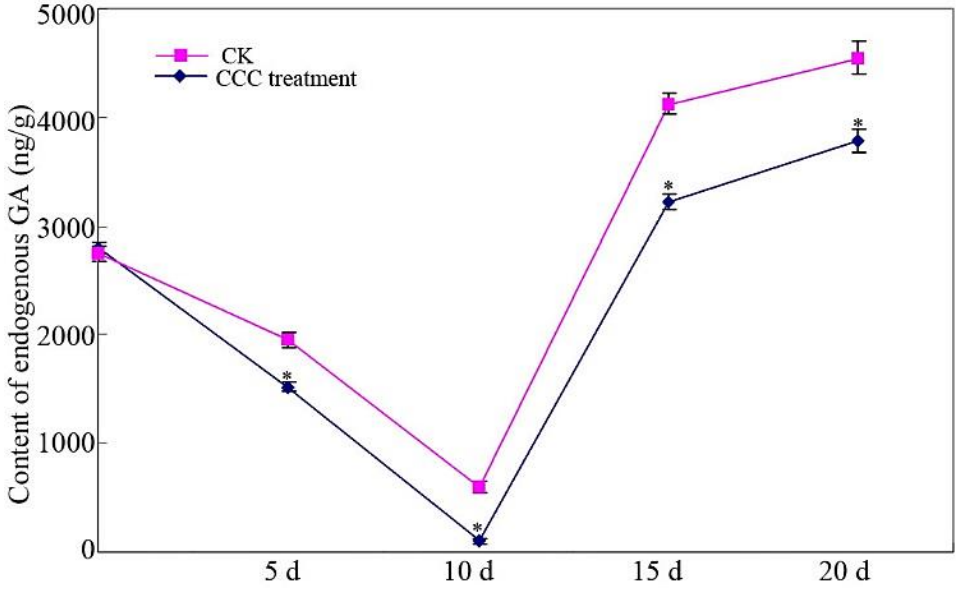


Fig. 2. Effects of CCC on the content of endogenous GA during the induction process of micro-tubers in *P. ternate*

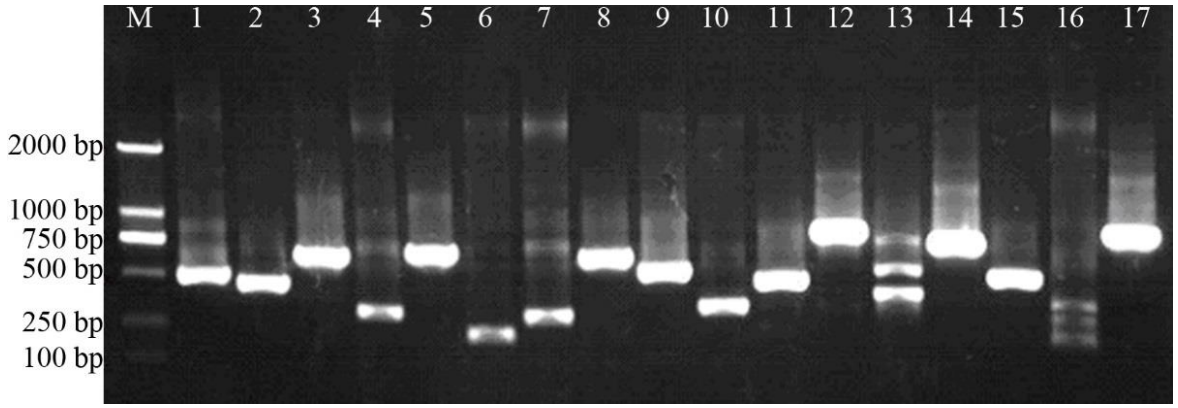


Fig. 3. Length of clones in SSH library. M, DL2000 marker (Tiangen, China); 1-17, PCR products of 17 clones

Relative expression patterns of 5 micro-tubers development-related candidate genes. Relative expression of 5 interested candidate genes in the micro-tuber induced at different times were compared between CK and the 70 mg/L concentration of CCC treatment. Of these, transcripts of *26S Ribosomal RNA* (*RAR26*) largely accumulated in the micro-tubers induced with 70 mg/L concentration of CCC treatment, while transcripts of *60S Ribosomal Protein* (*SPR60*) severely decreased in it during micro-tuber development (Fig. 5 A, C). The expression of *Storage Protein12* (*SP12*) decreased in micro-tuber treated with CCC at days five and ten, while no changes at day 15 (Fig. 5 B). Additionally, compared with CK, the expression of *Zinc Transport Protein* (*ZT*) in the micro-tuber treated with CCC showed a trend of rise-reduce-rise at days five, ten and fifteen, respectively (Fig. 5 E). However, the mRNA of *Malic Dehydrogenase* (*MDH*) in micro-tuber treated with CCC decreased at day five and increased at day fifteen (Fig. 5 D).

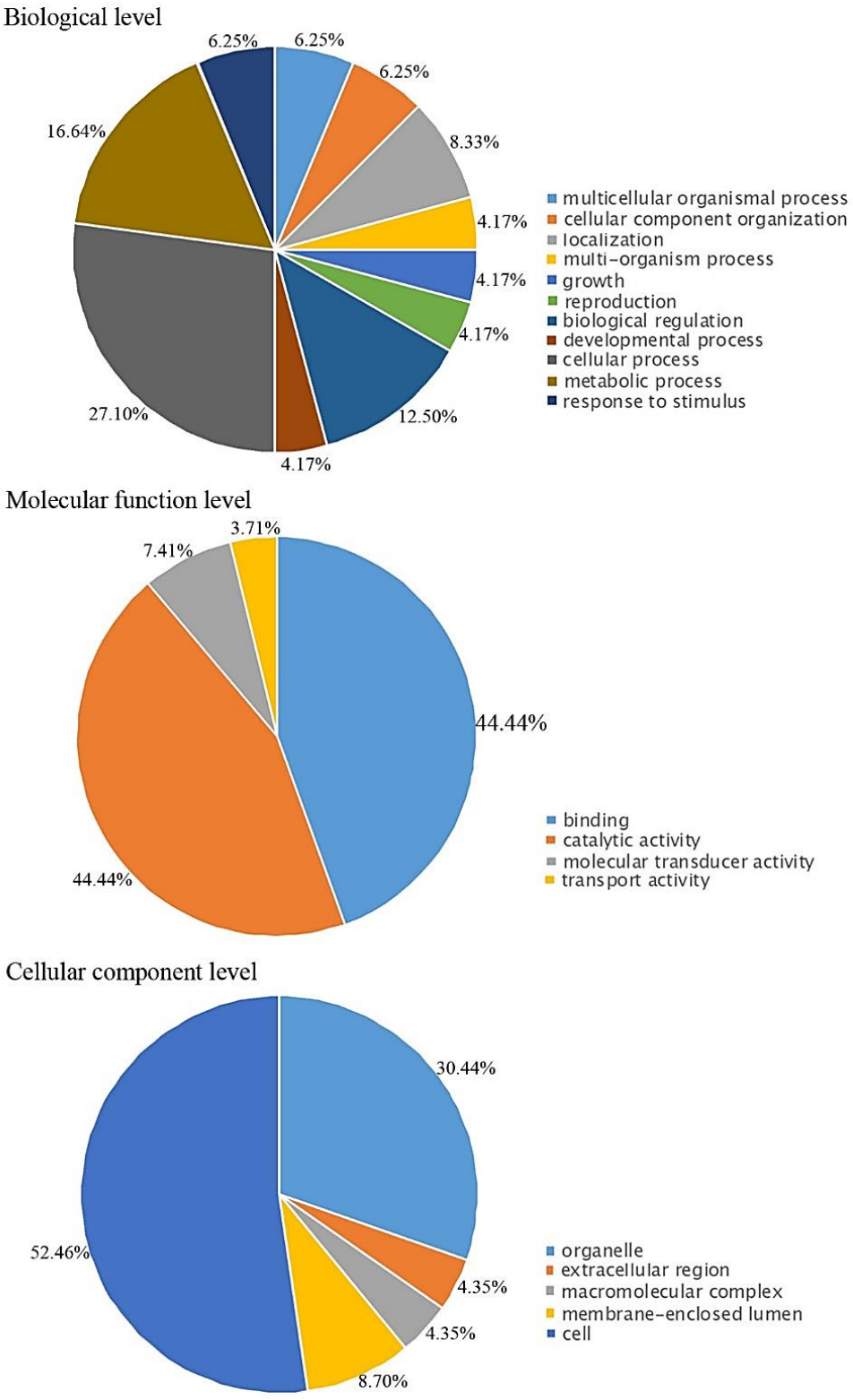
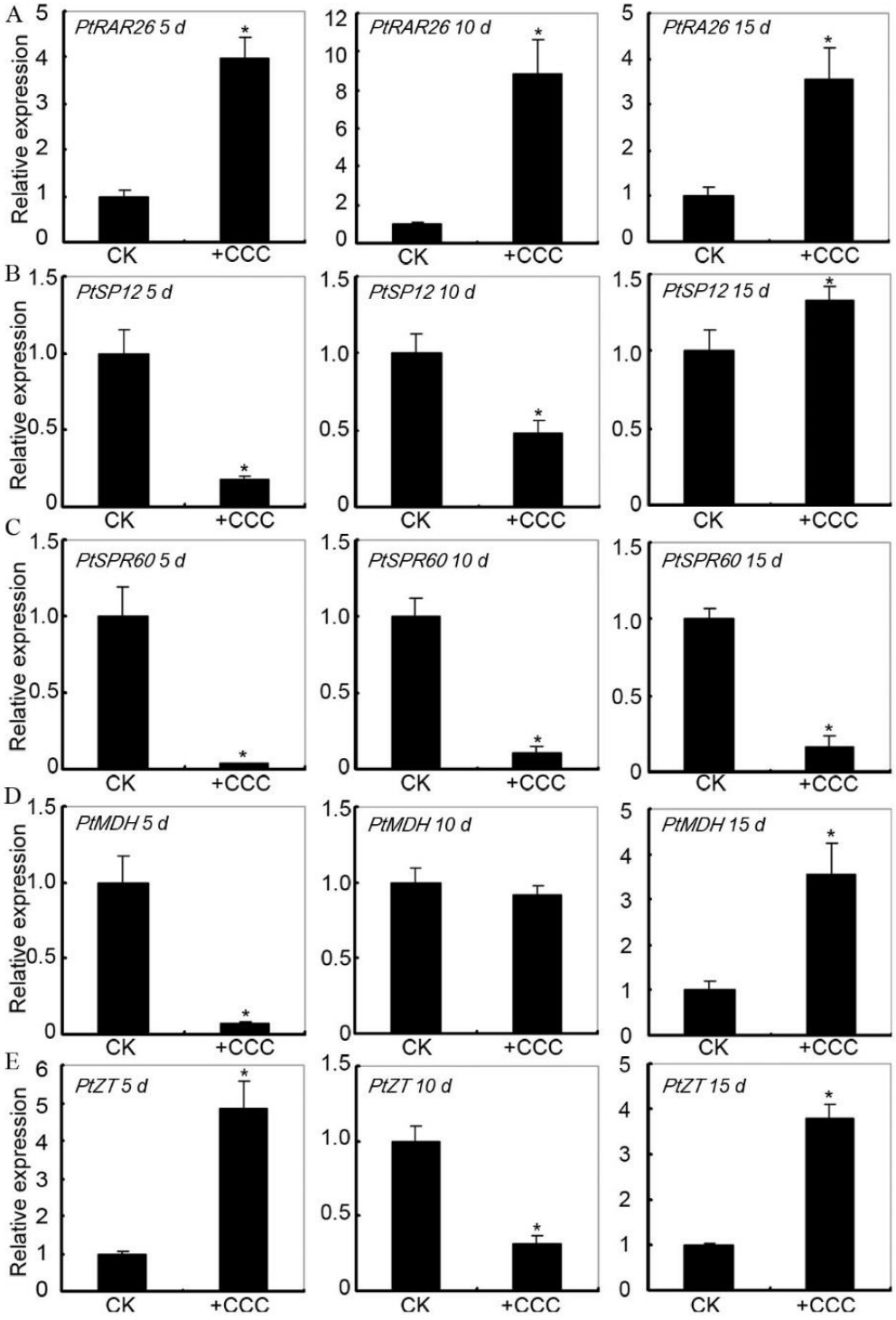


Fig. 4. Biological process classification analysis of the ESTs screened out with CCC treatment



* means differed significantly ($P < 0.05$)

Fig. 5. Expression of 5 candidate genes tested during the process of micro-tubers induction. A. *PtRAR26*; B. *PtSP12*; C. *PtSPR60*; D. *PtMDH*; E. *PtZT*

DISCUSSION

Tubers and tuberous roots are a kind of storage organs that enlarge to store nutrients serving as energy for regeneration needed in the subsequent growing season [You et al. 2003, Sun et al. 2010, Carpenter et al. 2015]. Plants with tubers are usually valuable, for example, potato and Chinese yam are the most notable plants with tubers, which are abundant in starch and other types of carbohydrates and both are the world important food crops [Elrys et al. 2017, Shi et al. 2017]. As one of Chinese herbal medicine, tuber of *P. ternate* is an important organ for medicinal herbs resource [Gombodorj et al. 2017]. Recently, attempts have been made to isolate and identify genes and proteins relevant to tuber formation in potato [Agrawal et al. 2008, Shan et al. 2013]. However, the underlying genetic mechanism that manipulates tuber development is still obscure. In the case of *P. ternate*, *in vitro* micro-tubers were successfully induced, which provided a stable model for studying the mechanism of tuber formation [Xue et al. 2004, Chang 2007]. Endogenous GA was indicated as a mayor inhibitor for micro-tubers formation in *P. ternate*, which could probably regulate other endogenous hormones during the process [Chang 2007, Sheng et al. 2010]. Thereby, identification of GA-related genes in the biological process will be conductive to reveal the mechanism of tuber development. In this manuscript, it was verified that CCC could promote micro-tubers formation by inhibiting the endogenous GA.

Based on the results, a subtracted cDNA library was constructed by performing SSH. Of the 5 genes selected for expression analysis, *PtRAR26* and *PtSPR60* are particularly interesting, for transcripts of *PtRAR26* signally increased in micro-tubers, while *PtSPR60* dramatically decreased with CCC treatment. Gene *PtRAR26* is supposed to encode a protein highly similar to 26S ribosomal RNA, which is one of ribosomes. Ribosomes are generally accepted to be key regulators of gene transcription and cell cycle, which is involved in cell growth response to environment and developmental stage [Lucchini and Sogo 1992, Dammann et al. 1993, Tsai et al. 2008]. One of the most important characters for developing tubers is rapid cell proliferation and expansion. It is not strange that 26S ribosomal RNA will play an important role in the process of tuber formation. On the contrary, *PtSPR60* is homologous to 60S ribosomal protein that shows a negative effect in tubers formation, which is in line with the function in stem development of *Zizania latifolia*. It is indicated that the antagonism of these two ribosome subunits is probably responsible for accurately regulating developmental process, thus to accommodate complicated environment. Of these genes, *PtSP12* and *PtMDH* are putative to encode 12kD storage protein and malate dehydrogenase, respectively, which are included in plant growth [Wang 2006, Duan et al. 2015]. Their transcripts both decreased at the initial stage and increased at the later stage of micro-tubers formation in the presence of CCC treatment. Coincided with a previous study, it was explained that the initial GA probably accelerated the petiole growth thus to promote micro-tubers formation at earlier stage, while high concentration of GA impeded micro-tubers development at later period. *PtZT* encoded protein is homogeneous of zinc transporter protein that is widely revealed in arabidopsis and rice [Qı 2012]. The variation of *PtZT* transcripts in the presence of CCC hinted its new function in regulating the tuber development.

In conclusion, the SSH technology allowed to generate a subtractive cDNA library enriched for GA-related genes associated with *in vitro* micro-tubers formation, from which a set of interesting genes were identified. It constitutes the first genome wide effort to understand the GA-related molecular profiles of tubers development in *P. ternata*, which will provide a platform for future studies in this field.

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