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IN VITRO INDUCTION OF YACON TUBEROUS ROOT AND IDENTIFICATION OF GENES ASSOCIATED WITH TUBEROUS ROOT EXPANSION

Y. Duan, T. Xue, J. Li, J. Teng, A. Zhang, W. Sheng, Y. Zhu, L. Chang and J. Xue*

Key Laboratory of Resource Plant Biology of Anhui Province, College of Life Sciences, Huaibei Normal University, Huaibei 235000, China
*Corresponding authorE-mail:xuejp@163.com

ABSTRACT

Yacon (*Smallanthussonchifolius*) has become increasingly popular worldwide owing to the nutritional and prebiotic function of its tuberous root. Elucidating the molecular mechanism controlling the expansion of the tuberous root in yacon is important for its breeding and *in vitro*manipulation. However, both the *in vitro* induction and the development mechanism of yacon tuberous root are still unclear. In this study, we optimized the medium formula for efficient induction of tuberous root *in vitro* from leaf-derived adventitious roots using an orthogonal design. The optimal medium for *in vivo* tuberous root initiation was determined as Murashige and Skoog medium supplemented with 3 mgL^{-1} 6-benzylaminopurine, 0.1 mgL^{-1} naphthalene acetic acid, and 70 gL^{-1} sucrose. Suppression subtractive hybridization (SSH) was performed to identify the transcripts up-regulated during the expansion of yacon tuberous root. The cDNAs of expanded and unexpanded tuberous roots were used as the 'tester', and 'driver', respectively. SSH library sequencing yielded 302 expressed sequence tags (ESTs). Finally, 97 ESTs were retained after screening, 15 of which had no significant homology to any of the previously identified genes. Real-time quantitative RT-PCR analysis of the expression patterns showed that all 11 transcripts were up-regulated during the tuberous root expansion process. These ESTs were highly homologous with expansion, β-xyloglucan endotransglycosylase, acyl-CoA-binding protein, asparagine synthetase, seed storage protein, lignin-related peroxidase, cytochrome P450, polyubiquitin, translation initiation factor, and nutrient storage protein. These results would facilitate the functional characterization of the genes associated with yacon tuber expansion and subsequent *in vitro*manipulation.

Keywords:*in vitro*manipulation,*Smallanthussonchifolius*, suppression subtractive hybridization, tuberous root expansion.

INTRODUCTION

Yacon (*Smallanthussonchifolius*; family Asteraceae), a perennial plant native to the Andean region of South America, usually forms a clump of more than 20 big sweet-tasting underground tubers weighing 100–500 g, rarely over one kilogram (Valentová andUlrichová, 2003). Due to its strong adaptability to a wide range of climates and soils, yacon has gradually been introduced into North America, Oceania, Asia, and Europe. It was originally used by local inhabitants as a source of food energy, and rediscovered since the isolation and functional characterization of its bioactive components in the late 1990s. Yacon tubers are composed of 86% water and 14% dry matter (DM), and about 90% of the DM consists of low-molecular-weight substances that are extractable in 80% ethanol (Ohyama *et al.*, 1990). Further evidence suggested that yacon tubers are rich in soluble carbohydrates including fructose, glucose, sucrose, fructo-oligosaccharides (FOS) or inulin (Grau and Rea, 1997), as well as flavones, terpenoids, and phenolic acids. This, together with the absence of starch in yacon tubers, endows yacon with prebiotic potential (Lachman *et al*., 2003). Of the soluble carbohydrates identified from yacon tubers, FOS, an inulin-type fructan, has attracted the greatest attention for its beneficial effects in health and nutrition.

The bioactive function of yacon tubers has most intensively been studied; the tubers are found to be effective in alleviating diabetes and digestive diseases (Yan *et al*., 1999; Geyer*et al*., 2008), improving insulin resistance and reducing body weight in obese individuals (Genta *et al.*, 2009; Satoh *et al.*, 2013), showing immunomodulatory and hypolipidemic actions (Geyer*et al*., 2008; Habib *et al.*, 2011), as well as improving the element bioavailability (Lobo *et al.*, 2007; Lobo*et al.*, 2011). Their potential use as a prebiotic was also investigated using *in vitro*and *in vivo* rat and guinea pigmodel systems, respectively (Pedreschi *et al.*, 2003; Campos *et al*., 2012). Besides the bioactive functions, processing technologies such as spray drying encapsulation were widely optimized for maintaining the nutritional benefits of products without compromising their technological properties (Torres-Valenzuela *et al*., 2014; Bernstein and Noreña, 2014). Yacon was mainly investigated with regard to its intraspecific genetic variability and germplasm discrimination via internal transcribed spacer regions (Milella *et al*., 2011; Žiarovská *et al*., 2014).

The regeneration system of yacon has been established using adventitious buds or stem as explants,

and virus-free seedlings have been successfully obtained (Zeng, 2004). Yacon tubers store many important nutrients and are also used for harvesting. However, the *in vitro* initiation technique of tuberous root, the major storage organ of yacon, has not yet been established. The molecular mechanism of yacon tuber development is also unclear. Uncovering the genes controlling the yacon tuber expansion would facilitate its prebiotic application and molecular breeding.Expressed sequence tags (ESTs) provide a tool to mine the unknown functional genes, especially for the unsequenced species. In order to investigate the gene expression profile during tuberous root development, we initially optimized the medium for *in vitro* induction of tuberous root in yacon. We then identified the differentially expressed genes during tuberous root expansionusing SSH, and confirmed the expression pattern of some candidate genes by quantitative RT-PCR. This study is important for tissue culture-based large-scale production of virus-free seedlings, for unraveling the gene expression profile in response to tuberous root expansion, and for the subsequent molecular manipulation to improve its prebiotic action.

MATERIALS AND METHODS

Plant materials and growth conditions: The crown buds of yacon (purchased from yacon production base, Pei County, Xuzhou City, Jiangsu Province, China) were sand-cultured at room temperature. Shoots were sheared from the seedlings, washed thoroughly under tap water for 2 h, 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. The explants were sectioned into segments each with two or three nodes, plated in 100 mL-Erlenmeyer flasks at 2–3 explants per container with 40 mL MS medium (Murashige and Skoog, 1962) solidified with 8 gL^{-1} agar and supplemented with 2 mgL^{-1} 6-benzylaminopurine (BA), 0.2 mgL^{-1} naphthalene acetic acid (NAA), and 3% sucrose. The explants were incubated at (25 ± 1)C under illumination of 30–40 $\mu molm^{-2}s^{-1}$) with a photoperiod regime of 12 h light/12 h dark cycle.

Optimization of tuberous root initiation medium: Leaves with petioles were collected from the above regenerated plantlets by cutting at the petiole base. They were transferred to MS medium supplemented with 0.2 mgL^{-1}indolebutyric acid and 3% sucrose, with their petioles vertically inserted into the medium. When the adventitious roots formed in the petioles and the leaf vein reached to about 0.5 cm, the leaves were transferred to media with different treatments of orthogonal design by inserting the adventitious roots into the medium (Table 1). The initiation effect was presented by average weight, average diameter, and average length. The experimental design was completely randomized with three replications per treatment, each containing 30 explants. Data were subjected to variance analysis and range analysis using the software Minitab 15.

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 the ‘tester’ cDNA, resulting in a subtraction library consisting of genes that were up-regulated in the ‘tester’ cDNA. Total RNA was extracted from the tuberous root before and after expansion according to a previously described protocol (Wu *et al*., 2008). Double-stranded ‘tester’ cDNA was reverse transcribed from 0.4 mg total RNA extracted from the expanded tuberous roots, and ‘driver’ cDNA was prepared similarly from unexpanded tuberous roots (5 days after induction culture; Fig. 1), using a SMART™ polymerase chain reaction (PCR) cDNA synthesis kit (Clontech Laboratories, 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 differentially expressed cDNA fragments were cloned into a PMD18-T simple vector (Takara Bio, Otsu, Shiga, Japan). A total of 476 white clones were selected and cultured for plasmid extraction.

Sequencing and sequence analysis: Recombinant plasmids containing cDNA fragments were extracted from *Escherichia coli* with the E.Z.N.A.® Plasmid Miniprep Kit I (Omega Bio-Tek, Norcross, GA, USA) and sequenced with the universal M 13 sequencing primer. DNA sequencing was performed using an ABI-PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All the inserted sequences were checked for homologies in the GenBank database using BLASTx (http://www.ncbi.nlm.nih.gov/BLAST).

Relative quantification of11 ESTs: Total RNA was isolated from unexpanded tuberous rootsand from the bulked samples with different expansion degree (days 5, 15 and 15 during expansion process), as described above. Total RNA from each sample was digested by DNase I, as previously described (Huang *et al*., 1996), and cDNA was synthesized using an RT-PCR Kit (Takara Bio) in accordance with the manufacturer’s instructions. Real-time fluorescence measurements were made from reactions containing SYBR® Green I dye (Takara Bio) using an ABI 7300 real-time PCR system (Applied Biosystems). The actin gene was selected as an endogenous control. Primers for 11 target genes were designed according to their expressed sequence tags (ESTs) in our library. The primers used, which were designed using PrimerSelect in Lasergene software (DNAStar, Madison, WI, USA), are listed in Table 2. All real-time PCR reactions were performed in quadruplicate.

Non-template control reactions were also carried out in quadruplicate, for each pair of primers, and four biological repeats were performed. Gene expression levels over the unexpanded state were measured as relative quantification values, which were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2011). The real-time quantitative RT-PCR data were analyzed by the Statistical Package for the Social Sciences (SPSS) version 17.0 (SPSS Inc, Chicago, IL, USA).

RESULTS

Screening of optimal medium for *in vitro* tuberous root initiation: Based on the statistical data of tuberous root induction in the orthogonal experiment, the *K* (average of certain factor and level) and *R* (range of *K*) values were calculated, as shown in Table 3. Five days after initiation, the adventitious roots transferred to media of orthogonal design began to expand, except those in medium No. 1. At day 15, some calli formed on the roots induced in the media supplemented with 3% and 5% sucrose (Fig. 2 A); however, no callus initiation was observed on the roots induced with 7% sucrose. Experimental results showed that the *K* value of average weight was in order $K_2 > K_3 > K_1$ (Table 3), while that of average diameter was $K_3 > K_2 > K_1$. This indicates that tuberous root induced in the medium with 5% sucrose was more compact while that induced in the medium with 7% sucrose was thicker. Based on the *K* value of average length, the average length of tuberous root was found to reduce with increasing sucrose concentration from 30 to 70 gL^{-1}. The slightly higher weight of the tuberous root induced with 7% sucrose may have accounted for the elongation of tuberous root. However, the elongating root could not expand (Fig. 2B). With comprehensive comparison, it was concluded that 7% sucrose would be beneficial for *in vitro* tuberous root initiation. A similar phenomenon was observed for the *K* value for BA. With the increase in the concentration of BA from 1 to 3 mgL^{-1}, the average diameter was enhanced, but the average length reduced. The tuberous root formed in the medium with 2 mgL^{-1} BA was more compact than that in the other media.

An obvious elongation but slight expansion of adventitious roots was observed after they were transferred to the medium without NAA (Fig. 2C). However, when adventitious roots were cultured in the medium supplemented with 0.1 mgL^{-1} NAA for several days, a uniform growth was observed in the length and diameter of the roots. During this experiment, three indices of the induced root were measured and analyzed. Results of extreme deviation indicated that NAA was the factor that influenced the average weight and diameter of tuberous root the most, which played a significant role in the two important indices of tuberous root ($P < 0.05$). Based on the *K* values, it is easy to obtain a sharply ascending trend of the two indices when the concentration of NAA changes from 0 to 0.1 mgL^{-1}, but a slight decreasing trend when the concentration increases from 0.1 to 0.2 mgL^{-1}.

From comprehensive analysis on the data in Table 3, the optimal medium for tuberous root initiation from yacon leaves was determined as $A_3B_3C_2$, i.e., MS supplemented with 70 gL^{-1} sucrose, 3 mgL^{-1} BA, and 0.1 mgL^{-1} NAA. This medium was combination No.9, which was included in 9 combinations of the orthogonal experiment. This verifies that all adventitious roots could develop into tuberous roots with a good shape (Figs. 2D–F).

Single-pass sequencing and classification of SSH Library: The cDNA inserts varied in size from 200 to 750 bp (Fig. 3). The vector leader sequences and poor quality sequences among the 302 sequencing reactions attempted were trimmed. 3'-Vector and linker sequences were removed if poly(A+) were included in the results. Sequences of bacterial origin were also excluded on the basis of BLASTn search results. The resultant 97 ESTs were retained after screening, 15 of which had no significant homology to any previously identified genes. These functional categories of ESTs 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 Table 4.

Relative expression patterns of 11 tuberous root expansion-related candidate genes: Relative quantification of mRNA expression was carriedout on the bulked tuberous roots at different expanding periods. The concentrations of the 11 mRNAs of interest were calculated from the threshold cycle (Ct) values of the samples. The expression patterns of 11 candidate genes analyzed are shown in Fig. 5. The mRNA levels of all 11 candidate genes were found to be higher during the expansion process of tuberous roots than in the other two stages. Of these, expansin, β-xyloglucan endotransglycosylase (XET), acyl-CoA-binding protein, asparagine synthetase, and seed storage protein were up-regulated during the early development period and reduced sharply at the late development stage. In particular, XET changed most remarkably at mRNA level, with its expression level up-regulated 12-fold at the remarkably expanding stage and 10-fold at growth stagnation stage over the initial developmental stage. Meanwhile, the expression of lignin-related peroxidase and cytochrome P450 was also up-regulated and then down-regulated with the development of tuberous root; polyubiquitin, translation initiation factor, and nutrient storage protein assumed a relatively stable express pattern during the development. This indicates that the temporal expression of related genes further determines the developmental process of tuberous root.

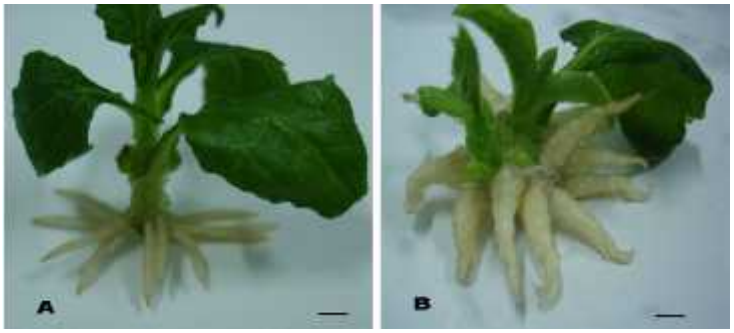

Figure 1.Yaconplantletscultured on tuberous root initiation medium for 5 d (A) and 15 d (B). The size of roots in Fig. 1A was similar with that cultured in hormone free medium and considered to be unexpanded and that in B was as expanded. Scale bar, 1.0 cm.

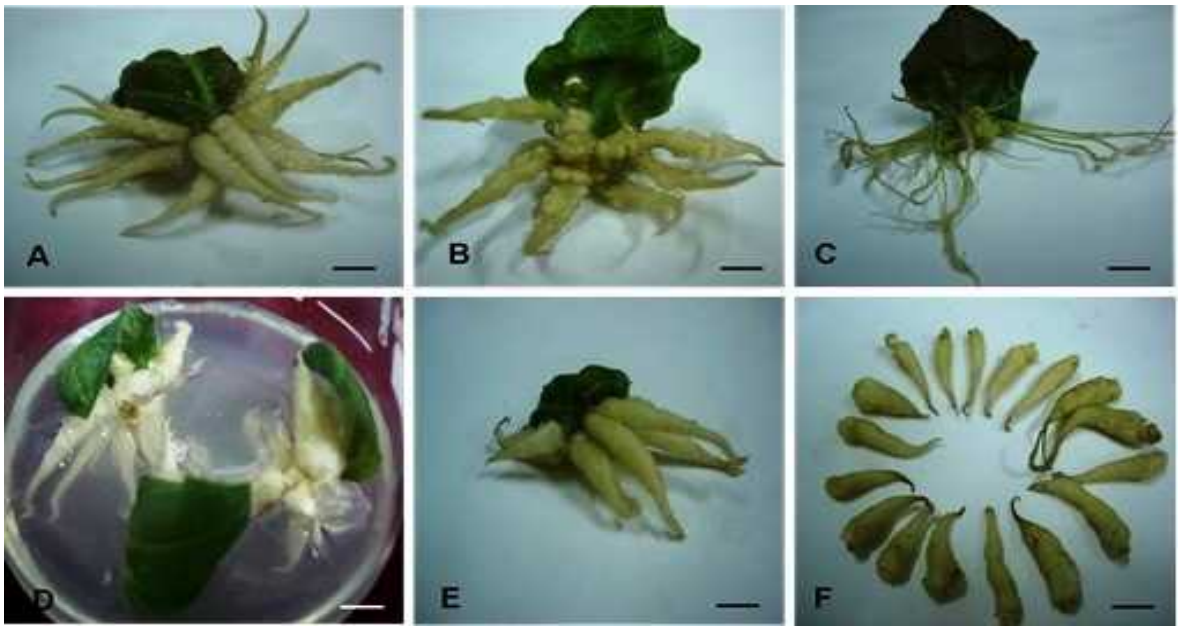

Figure 2.Development of yacon tuberous roots in different media.A, Tuberous roots of yacon formed in the medium supplemented with 5% sucrose, 2 mgL^{-1} BA, and 0.2 mgL^{-1} NAA;B, callus formed on the tuberous roots induced in the medium supplemented with 3% sucrose, 3 mgL^{-1} BA, and 0.2 mgL^{-1} NAA;C, adventitious roots could not develop into tuberous roots in the medium supplemented with 3% sucrose and 1 mgL^{-1}BA;DandE, tuberous roots of yacon formed in the screening medium: MS supplemented with 70 gL^{-1} sucrose, 3 mgL^{-1} BA, and 0.1 mgL^{-1} NAA;F, tuberous roots of yacon formed in the screening medium. Scale bar, 0.5 cm.

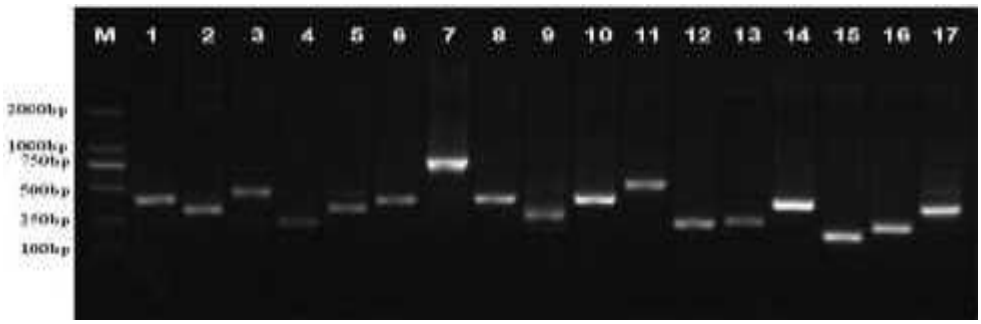

Figure 3. The length of clones in SSH library. M, D2000 marker (Tiangen, China); 1-17, PCR products of 17 clones.

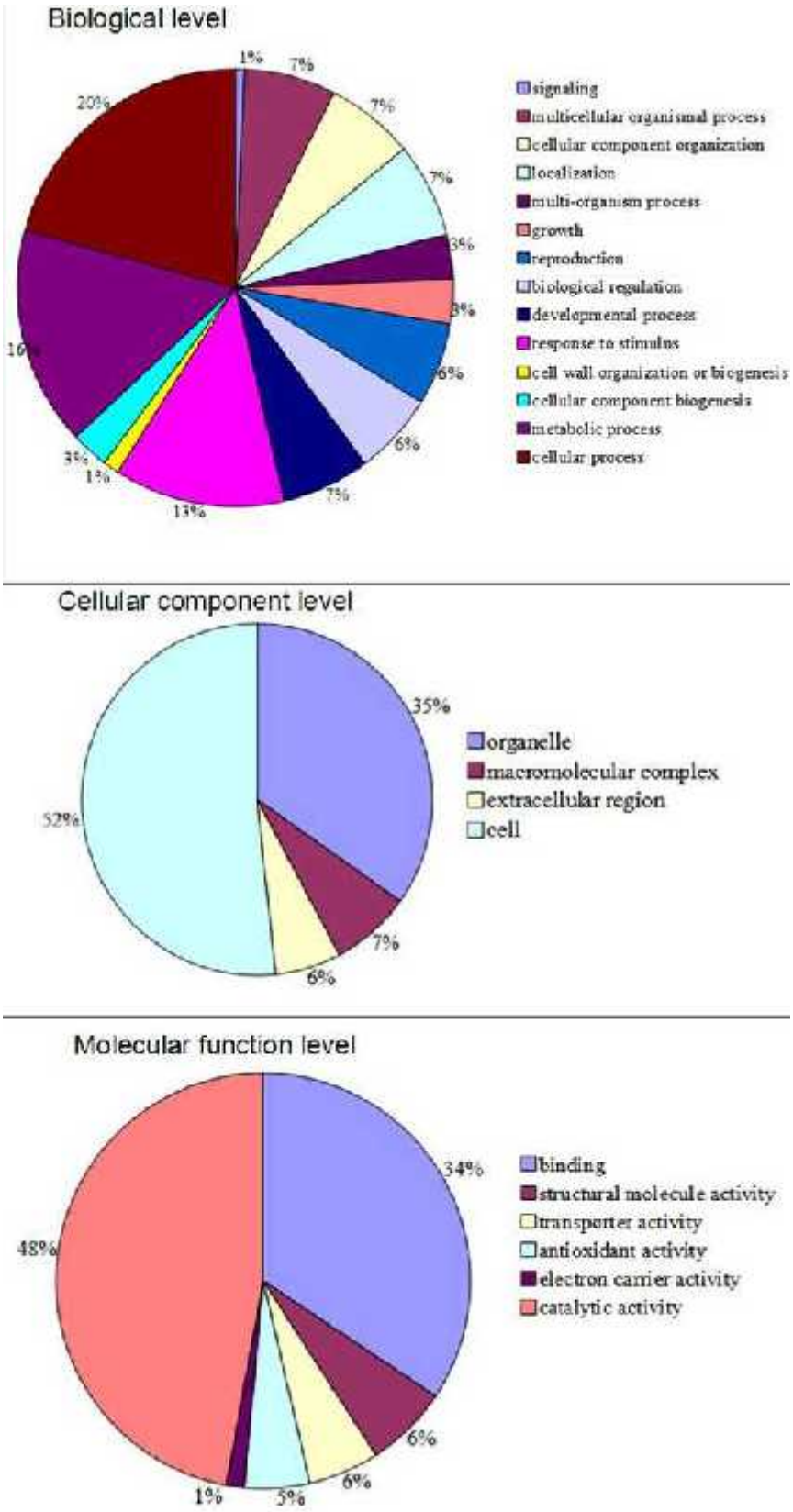

Figure 4. Biological process classification analysis of the ESTs screened out during tuberous root expansion

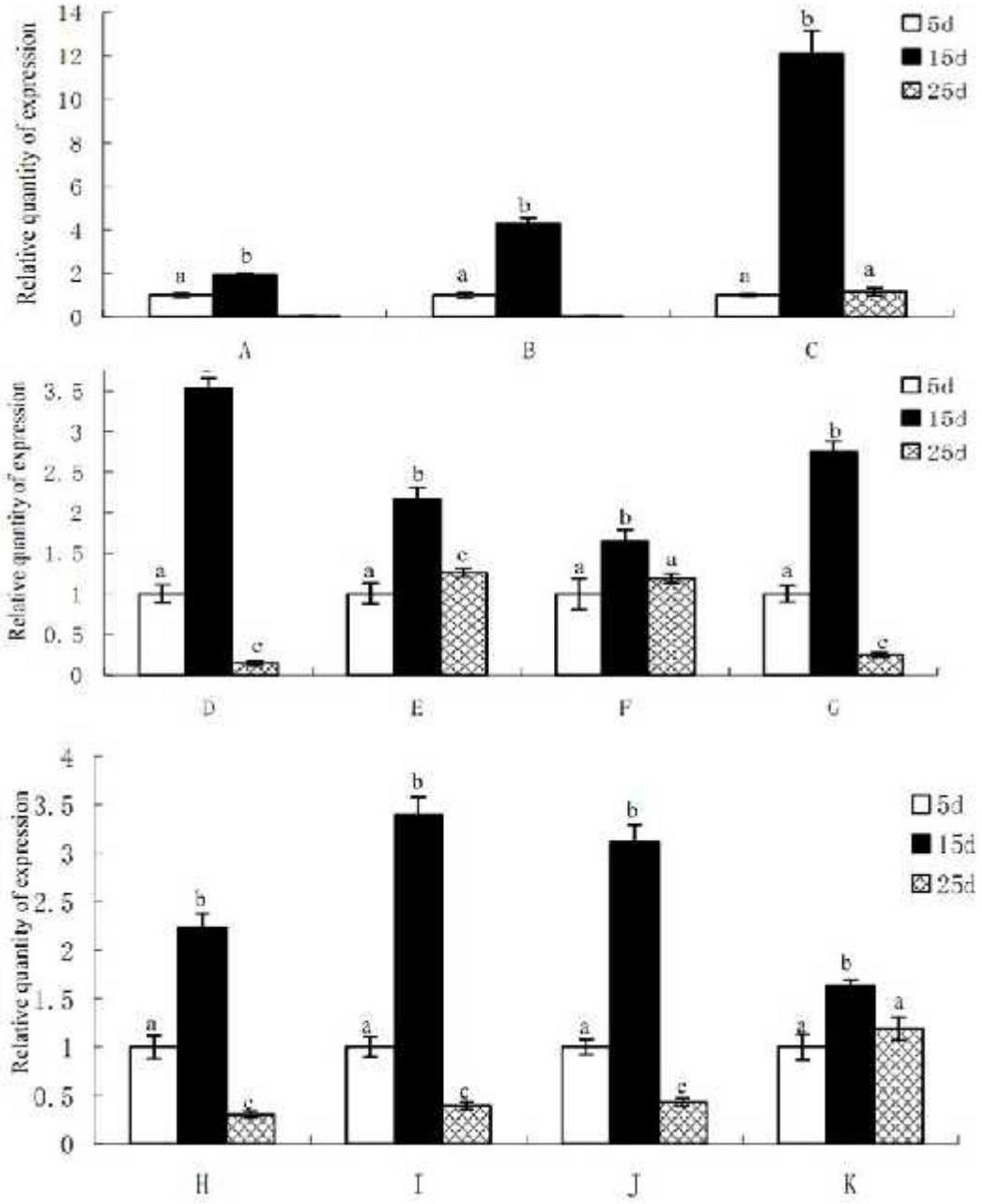

Figure 5. Expression of 11 candidate genes tested during tuberous root expansion process. A, Expansin; B, β-glucanase; C, XET; D, anionic peroxidase; E, polyubiquitin; F, translation initiation factor; G, cytochrome P450; H, acyl-binding protein; I, asparagine synthetase; J, seed storage protein; K, vegetative storage protein. For each gene, different lowercases indicate statistically significant difference ($P< 0.05$).

Table 1.Parameters of orthogonal design.

Level	Factor		
	A **Sucrose (gL^{-1})**	***B*** **BA (mgL^{-1})**	***C*** **NAA (mgL^{-1})**
1	30	1	0
2	50	2	0.1
3	70	3	0.2

Table 2. Sequences of primers used for real time-PCR analysis.

Clone No.		Sequence
Xlg103	-F	5'-GCCCGTTCAGGTAAGAGTTG-3'
	-R	5'-CGGTGTCTATCAAAGGGTCAA-3'
Xlg129	-F	5'-ACATCCTCATAACCAACCACAA-3'
	-R	5'-CCAGACCAGCAGAGGTTGAT-3'
Xlg140	-F	5'-TTCCTGCGGGTAAGGTTAAGT-3'
	-R	5'-CAGCACACAGATTGGCAGATA-3'
Xlg157	-F	5'-ACACAAAACACCCATCACAGA-3'
	-R	5'-CATCCGCTTTCTTGACTCGT-3'
Xlg238	-F	5'-TCACGAAGAAATCACACATCCT-3'
	-R	5'-AGACCTTGCCGACCTTGAG-3'
Xlg262	-F	5'-ATAAGTTTCGGGATGAGCAAC-3'
	-R	5'-TTCAGATGGAGGAAGTGTGTTC-3'
Xlg268	-F	5'-CCTGAAGGGTCCAGATTGTTAG-3'
	-R	5'-CCGAGGTCTAAGTTGACTGTCC-3'
Xlg271	-F	5'-GTGCTAACTGCCCACAGACTC-3'
	-R	5'-GTATTCGGTCACAATGCTATCG-3'
Xlg282	-F	5'-GCTGGCTAAAATCCAAATCAAC-3'
	-R	5'-TGTTCAGCATGAGAGAAAGAGC-3'
Xlg295	-F	5'-GCCGCTGATACAGAACAATC-3'
	-R	5'-TGGGTCCATACAATAATCATAC-3'
Actin	-F	5'-GAGAGATTCCGTTGCCCTGA-3'
	-R	5'-CTCAGGAGGAGCAACCACC-3'

Table 3.Analysis of orthogonal experiment results.

		A	***B***	***C***
Average weight	K_1	0.0832	0.0730	0.0453
	K_2	0.1064	0.1143	0.1313
	K_3	0.1052	0.1074	0.1181
	R	0.0232	0.0413	0.086
Average diameter	K_1	0.2647	0.2133	0.1327
	K_2	0.2993	0.3093	0.3970
	K_3	0.3027	0.3440	0.3370
	R	0.038	0.1307	0.2643
Average length	K_1	3.363	3.560	3.287
	K_2	2.890	2.867	2.747
	K_3	2.603	2.430	2.823
	R	0.760	1.130	0.540

Table 4. SSH cDNA library associated with development of *in vitro* tuberous root of yacon.

Clone	Length (bp)	Origin of matched Sequence	Identity	*E* value	Putative function of genes(Blastn or Blastx)
xlg36	527	CAB65692.1	83%	3e-31	Rad23 Protein
xlg225	410	ABD61653.1	92%	1e-64	sucrose synthase
xlg28	334	ABV25504.1	51%	2e-24	X4 protein
xlg116	518	AAD50629.1	93%	1e-43	alpha-tubulin
xlg149	249	XP_002304869.1	47%	4e-08	predicted protein
xlg33	390	CBI35120.3	63%	1e-36	unnamed protein product
xlg277	441	CBI37807.3	59%	2e-43	unnamed protein product
xlg316	363	AES71699.1	76%	4e-21	Endo-1,4-beta-glucanase
xlg303	610	XP_002267261.1	55%	1e-29	hypothetical protein

xlg12	471	XP_002284211.1	77%	6e-66	hypothetical protein
xlg19	436	CBI30075.3	83%	4e-10	unnamed protein product
xlg106	392	AAB61421.1	95%	8e-81	cytosolic glutamine synthetase
xlg341	558	ACF74323.1	38%	7e-17	unknown
xlg17	465	XP_002271792.1	68%	3e-32	hypothetical protein isoform 1
xlg147	324	XP_002332752.1	84%	2e-40	high affinity inorganic phosphate transporter
xlg262	491	CAB71301.2	54%	3e-40	vegetative storage protein,VSP
xlg236	266	AAK62818.1	90%	4e-29	fructose-1,6-bisphosphate aldolase
xlg31	494	ACN40189.1	52%	3e-06	unknown
xlg268	395	AAF02775.1	84%	3e-30	asparagine synthetase
xlg329	347	AEA86296.1	93%	2e-26	aquaporin PIP1
xlg5	314	XP_002303308.1	64%	1e-15	predicted protein
xlg7	656	CAQ43070.2	61%	7e-11	putative puroindoline b protein
xlg26	482	XP_002299126.1	62%	8e-12	predicted protein
xlg183	450	ABK95087.1	50%	6e-06	unknown
xlg161	559	ACI31554.1	94%	5e-67	S-adenosyl homocysteine hydrolase
xlg172	606	XP_002509837.1	88%	5e-21	conserved hypothetical protein
xlg11	669	Q4W1I8.1	73%	5e-70	putative peroxidase
xlg18	384	AAM27914.1	94%	3e-04	defensin
xlg97	417	ABP35529.1	68%	8e-11	proline-rich protein
xlg157	259	AAM77753.1	91%	3e-12	translation initiation factor B04
xlg265	279	CAA06853.1	100%	2e-50	26S protease regulatory subunit 6
xlg103	472	AAF35902.1	93%	4e-47	expansin 3
xlg303	614	ABB29957.1	91%	2e-70	unknown
xlg93	464	BAJ96951.1	76%	2e-24	predicted protein
xlg27	346	AAF64453.1	90%	6e-08	putative heat-shock protein 90
xlg163	429	XP_002272081.1	92%	2e-14	hypothetical protein isoform 2
xlg63	610	XP_002267261.1	55%	1e-29	hypothetical protein
xlg296	439	XP_002530876.1	61%	5e-12	conserved hypothetical protein
xlg31	288	XP_002325963.1	61%	9e-05	predicted protein
xlg282	440	Q39315.1	88%	1e-25	Full=Acyl-CoA-binding protein
xlg30	629	XP_002331334.1	63%	1e-76	predicted protein
xlg229	296	BAC75923.1	91%	6e-42	cysteine protease-1
xlg129	409	CAD56223.1	98%	2e-48	polyubiquitin
xlg140	626	XP_002334834.1	62%	6e-76	cytochrome P450
xlg266	488	AAR31106.1	88%	1e-78	peroxidase precursor
xlg321	441	CBI37807.3	59%	2e-43	unnamed protein product
xlg84	286	Q01412.1	73%	3e-21	Glucan endo-1,3-beta-glucosidase A
xlg48	684	XP_002513044.1	66%	1e-80	Aspartic proteinase nepenthesin-1 precursor
xlg109	312	BAI67717.1	72%	3e-23	protein disulfide isomerase 1
xlg201	363	ABD62082.1	74%	5e-17	endo-1,4-beta-glucancase precursor
xlg273	253	CAN83845.1	67%	5e-22	hypothetical protein VITISV_001862
xlg377	571	ACN40189.1	65%	1e-11	unknown
xlg85	303	ACF82321.1	84%	1e-07	unknown
xlg13	250	BAH20421.1	87%	3e-42	AT5G46630
xlg72	481	F74323.1	39%	9e-18	unknown
xlg30	1073	CBL87542.1	52%	4e-06	hypothetical protein S18_873_0036
xlg265	232	ABM74181.1	61%	4e-27	Fe-superoxide dismutase
xlg314	573	XP_002270769.1	87%	1e-29	hypothetical protein isoform 1
xlg116	300	XP_002281000.1	82%	5e-45	hypothetical protein
xlg217	767	AAD01265.1	51%	4e-38	glucose acyltransferase
xlg192	317	XP_002303308.1	64%	1e-15	predicted protein
xlg221	350	AAM92570.1	95%	7e-08	S-adenosylmethioninesynthetase
xlg209	348	XP_002450330.1	83%	6e-22	hypothetical protein SORBIDRAFT_ 05g003875
xlg386	668	AEB37127.1	68%	7e-66	EIN3
xlg359	465	96951.1	73%	3e-22	predicted protein

xlg58	239	ADN43429.1	63%	6e-20	pathogenesis-related protein 1
xlg44	621	XP_002276450.1	71%	6e-72	hypothetical protein
xlg203	440	ABK92955.1	69%	2e-33	unknown
xlg238	262	NP_199500.1	80%	2e-08	seed storage 2S albumin-like protein
xlg175	621	XP_002276450.1	70%	2e-66	hypothetical protein
xlg166	436	9315.1	88%	1e-25	Acyl-CoA-binding protein
xlg35	826	E20246.1	100%	0.0	beta-tubulin
xlg295	406	ABK30788.1	76%	1e-49	xyloglucan endotransglycosylase 1
xlg99	627	CAN60567.1	61%	1e-38	hypothetical protein
xlg92	432	AAL77049.1	75%	1e-09	type 2 metallothionein-like protein
xlg135	843	XP_002304305.1	90%	2e-41	predicted protein
xlg271	530	XP_002509737.1	69%	8e-32	Lignin-forming anionic peroxidase precursor, putative
xlg49	393	AAF61733.1	98%	3e-58	catalase 3
xlg285	291	ABK96092.1	62%	4e-27	Unknown
xlg240	479	ACF74323.1	39%	6e-13	Unknown
xlg244	568	XP_002521083.1	88%	3e-12	conserved hypothetical protein

DISCUSSION

In vitro induction of yacon tuberous roots of great significance for unraveling the molecular mechanism controlling tuberous root expansion as well as its practical application in rapid propagation, virus-free culture, and artificial seed preparation. It has been established in sweet potato and Radix Rehmanniae. Using stem-derived adventitious root as explants, Nakatani (1994) for the first time developed the *in vitro* tuberous root induction system in sweet potato, with 1/2 MS medium supplemented with 6% sucrose, 0.2% casamino acids, and 10^{-5} $molL^{-1}$ JA. In a previous study, our research group investigated the tuberous root induction technique in Radix Rehmanniae, with MS basal medium supplemented with 2 mgL^{-1} BA and 0.1 mgL^{-1} NAA and 5% sucrose as optimal, and found that the commonly used GA_3 and activated carbon were unfavorable for tuberous root development (Xue *et al*., 2002). Using the leaf-derived adventitious roots as explants, we successfully achieved the *in vitro* induction of yacon tuberous root with MS medium supplemented with 3 mgL^{-1} BA, 0.1 mg L^{-1} NAA, and 70 gL^{-1} sucrose.

The development of tuberous root in yacon is a complicated biological process that requires the temporal and spatial regulation of a number of genes. Expansin belongs to a family of closely related non-enzymatic proteins that widely exist in the cell wall of plants, and play important roles in promoting cell wall expansion (Cosgrove, 1997; Li and Cosgrove, 2001). In this study, *expansin* gene was expressed at the initial stage of tuberous root expansion, peaked at the remarkably expanding stage, and down-regulated during the subsequent growth stage, suggesting its association with the development of tuberous root. This is consistent with the findings of Sun *et al*.in the study investigating the tuberous root development of *Rehmanniaglutinosa* (Sun*et al.*, 2010). β-glucanase and xylanase also participate in the regulation of cell wall extension via interaction with expansion, which have also been confirmed in pea (Fry*et al*., 1992), cucumber (McQueen-Mason *et al*., 1997), tomato (Jongedijk *et al*., 1995), tobacco (Anfoka and Buchenauer, 1997; Chi, 2001), and moso bamboo (Zhang *et al*., 2010). These two enzymes were also found to be differentially expressed during the expansion of yacon tuberous root, suggesting their probable contribution to tuberous root formation in yacon.

The growth and development of plants rely on the degradation and reconstruction of their cell wall. Lignin, as a major component of cell wall, is essential for its reconstruction, and its formation has been found to be closely related with anionic peroxidase (Koutaniemi *et al*., 2005). In the present study, anionic peroxidase level in yacon was confirmed to be increased during the tuberous root expansion process and peaked at the remarkably expanded stage at mRNA level. This implies that cell metabolism is vigorous at this stage, and results in an increase in lignin synthesis, cell number and size, finally initiating the formation of tuberous root. Chemical compounds are also quickly synthesized along with the development of yacon tuberous root. Thus, cytochrome P450, an important compound, not only assumes a key role in the formation of lignin, but is also involved in the synthesis of flavones (Zhao *et al*., 1999). Asparagine synthetase and acyl binding protein, which are associated with amino acid synthesis and metabolism, were also found to be differentially expressed during the expansion of yacon tuberous root.

Nutrient storage proteins are a variety of storage proteins broadly found in potato and sweet potato (Zhang, 2008). In yacon, for the first time, we detected ESTs correlated with seed storage protein and nutrient storage protein, implying that a specific storage protein may accumulate along with the development of tuberous root. Polyubiquitin, which is involved in light signal response, plant defense, and organogenesis (Dreher and Callis, 2007), and translation initiation factor, which directly regulates the synthesis of various proteins, were also

detected in the expansion process of yacon tuberous root.

In conclusion, we established the *in vitro* tuberous root initiation technique in yacon and identified the differentially expressed genes during the tuber expansion process. Most of the ESTs detected in the tuberous root expansion-based SSH were found to have putative functions from other plant species, whereas some others have not yet been recorded. These genes may assume important roles in the development of tuberous roots and the characterization of their functions, which would be useful for unraveling the mechanisms of tuberous root expansion in yacon.

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