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THE DIFFERENTIAL GENE EXPRESSION OF KEY ENZYME IN THE GIBBERELLIN PATHWAY IN THE POTATO (*SOLANUM TUBEROSUM*) MUTANT *M4P-9*

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Abstract

In the present study, the expression patterns of the key genes in the gibberellin synthesis pathway in the potato dwarf mutant *M4P-9* were detected using quantitative real-time PCR. Using *Actin* as an internal control, *CPS1*, *KS*, *KO*, *GA20ox1*, and *GA2ox1*, genes for key gibberellin synthesis enzymes, were evaluated, along with a gibberellin receptor gene. The standard curves were obtained from dilutions of PCR product; the correlation coefficient for Actin was 0.995, and those for the target genes varied from 0.994 to 1.000. The expression patterns of gibberellin pathway genes in different growth stages and tissues were calculated according to the method of Pfaffl. These genes showed expression patterns that varied based on growth stage and tissue type. The higher expression levels of *CPS1* and *GA2ox1* in roots, the lower expression levels of *GA20ox1* in roots during tuber formation stage; as well as the increased expression of *GA20ox1* and *GA2ox1* genes in stems during the tuber formation stage, likely play key roles in the plant height phenotype in *M4P-9* mutant materials. This article provides a basis for researching the mechanism of gibberellin synthesis in potato.

Key words: Gene expression, Gibberellin pathway, *Solanum tuberosum*.

Introduction

The gibberellins (GAs) are a large family of terpenoids that play important regulatory roles throughout plant growth and development. As an important plant hormone, gibberellin is involved in controlling various aspects of plant development, including germination, stem elongation, root growth, leaf expansion, epidermal hair development, pollen tube growth, and flower and fruit development (Sakamoto *et al*., 2004; Huang *et al*., 2010). The gibberellin biosynthetic pathway can be divided into three stages, and the key enzymes involved in this process are copalyl pyrophosphate synthase (*CPS*), ent-kaurene synthase (*KS*), ent-kaurene oxidase (*KO*), GA-20 oxidase (*GA20ox*) and GA-2 oxidase (*GA2ox*) (Bou-Torrent *et al*., 2011; Colebrook *et al*., 2014).

CPS is an important enzyme in the regulation of the gibberellin biosynthetic pathway: it mediates the conversion of *GGPP* to *GA*, it is located in the proplastid as well as the ent-kaurene synthase, and it contains the guide sequence. During gibberellin biosynthesis, *CPS* catalyses the conversion of geranyl pyrophosphate (*GGPP*) to copalyl pyrophosphate (*CPP*), which is the first step in the formation of the cyclic diterpene (Hedden & Phillips, 2000). Ent-kaurene synthase catalyses the conversion of copalyl pyrophosphate to ent-kaurene, which is the precursor of gibberellin. Ent-kaurene oxidase is a membrane-bound mono-oxygenase; it is located in the endoplasmic reticulum and requires cytochrome P450 and NADPH, and ent-kaurene can be oxidized to ent-kaurenoic acid by ent-kaurene oxidase after a three-step reaction (Davidson *et al*., 2004). GA-20 oxidase and GA-2 oxidase are two important biosynthetic and regulatory enzymes of gibberellin; they are soluble dioxygenases encoded by a small family of genes. To date, GA-20 oxidase and GA-2 oxidase genes have been cloned from many species. The GA-20 oxidase enzyme is strictly controlled and is regulated by both a feedback cycle and light. GA-20 oxidase has low oxidation specificity to its substrates, and its affinity for the substrate is associated with the hydroxylation of C-13. This variable affinity allows GA-20 oxidase to form two or more parallel metabolic pathways for gibberellin, which is consistent with the bioactive GAs in plants (Vidal *et al*., 2003). GA-2 oxidase acts mainly on GA1 and GA4 in biological contexts, ensuring that GA1 and GA4 are hydroxylated into inactive GA8 and GA34, and GA-2 oxidase maintains the balance between GAs and C19-GAs in the plant (Huang *et al*., 2010). Gibberellin insensitive dwarf1 (*GID1*) is a soluble receptor protein that can bind with gibberellin. Through these interactions, the gibberellin signal is passed to downstream elements that mediate the effect of gibberellin on the plant (McGinnis *et al*., 2003). Since the 1960s, because of the use of the rice sd1 gene and the wheat Rht1 gene in crop breeding, the global production of major food crops has improved greatly through a phenomenon called the "Green Revolution" (Monna *et al*., 2002). Recent studies have shown that the "Green Revolution" of major crops is closely related to gibberellin activity. Currently, the expression and regulation of gibberellin at the molecular level have become hot topics in plant hormone research (Cao *et al*., 2005; Davière & Achard 2013; Araújo *et al*., 2014; Fukazawa *et al*., 2014). However, little research has been conducted on gibberellin in potato than in grain crops.

Quantitative real-time PCR is a method being commonly used to detect the gene expression in plant, it has the characteristics of low cost, saving both time and labour, high accuracy and high sensitivity, and can detect the mRNA of low expression. It has become the first choice for gene expression analysis (Callaway *et al*., 2012; Litao *et al*., 2005).

In this article, to further understand the molecular mechanism of gibberellin activity in potato, the gene expression levels of key enzymes in the gibberellin synthesis pathway were analysed using quantitative real-time PCR from multiple stages and organs of the dwarf mutant *M4P-9*.

Materials and Methods

Potato material: The potato (*Solanum tuberosum*) material *M4P-9* and wild type (*WT*) *Plateau 4* were obtained from the Biotechnology Center of the Qinghai Academy of Agriculture and Forestry. *M4P-9* is a dwarf mutant line identified through a T-DNA insertion, and has single copy number detected by southern blot (Fig. 1). After many years of phenotype observation, the *M4P-9* line showed the phenotypic trait of dwarf mutant, and the mutant stable genetic in the offspring. In this research, the both types of plant were grown on MS culture medium. After 30 days of cultivation (25°C during the day, 21°C at night, with fill-in illumination for 14 hours/day), the tissue cultured seedlings were planted in a greenhouse. Three months later, the micro-potatoes were produced. Micro-potatoes of the same size were selected, and their tubers were planted in flowerpots, with three replicates. During the seedling, tuber formation and tuber expansion stages, the root, stem, leaf and tuber tissues of *M4P-9* and *WT* plants were obtained. These tissues were used for RNA extraction as described below.

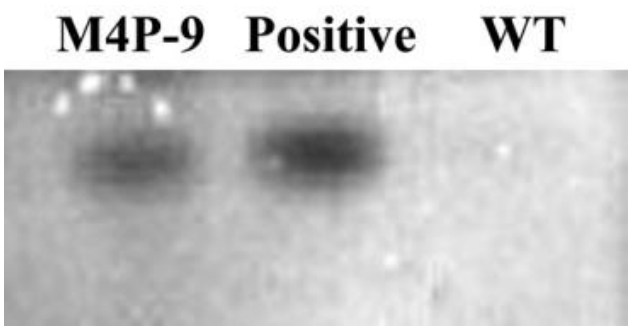

Fig. 1. The T-DNA detection by using southern blot.

RNA extraction and cDNA synthesis: The root, stem, leaf and tuber tissues were ground into powder in liquid nitrogen, and total RNA from each potato sample was extracted and purified using an RNA extraction kit (TIANGEN, China). cDNA was synthesised using a reverse transcription kit (TIANGEN, China).

Primer design and synthesis: Based on the gene sequences of *CPS1* (NCBI: XM006352710), *KS* (NCBI: XM006345957), *KO* (NCBI: XM006354880), *GA20ox1* (GenBank: AJ291453.1), *GA2ox1* (NCBI: XM006359960), *GID1B* (NCBI: XM006362914) and Actin (GenBank: GQ339765.1), the oligonucleotide primers used in this study were designed using Primer Premier 5.0 software (Table 1). All primers were synthesized by Sangon Biotech (Shanghai, China). The Actin gene sequence used is from *S. tuberosum*. In research using transgenic potato, Actin is often used as the housekeeping gene for normalizing data from expression analysis (Dominguez & Holmes 2011; Gao *et al*. 2012).

Quantitative real-time PCR reaction: To generate the standard curves for the endogenous Actin and the target genes, the PCR product of each target gene was used as a template and serially diluted to final concentrations of 125, 25, 5, 1 and 0.2 ng. The standard curves for the Actin gene and the target genes were obtained by plotting cycle threshold (Ct) values against log-transformed concentrations. The amplifications of the target genes and Actin gene from *M4P-9* and *WT* material were performed simultaneously. Each reaction was quantified in six times repeated. Quantitative real-time PCR was conducted in a fluorescence quantitative PCR instrument (iQ™ 5: BIO-RAD, US), and the data were analysed using iQ™ 5 Optical System Software Version 2.1.

Quantitative real-time PCR reaction was carried out in fluorescence quantitative PCR instrument (iQ™ 5: BIO-RAD, US). 20μl reaction mixtures contain 2×SYBR Green I Mix 10μl, forward primer (10μmol/L) 1μl, reverse primer (10μmol/L) 1μl, and cDNA template 25ng, up ddH_2O to 20μl. The reactions were performed at 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 32 seconds, and then collect the fluorescent. The temperature of melting curve was increased from 65°C to 95°C by 0.2°C per second. The data were analyzed with the iQ™ 5 Optical System Software Version 2.1.

Relative quantitative PCR analysis: After the reaction, the logarithm of the initial concentration was plotted on the X-axis, and the Ct value was plotted on the Y-axis. The expression levels of target genes of *M4P-9* were calculated according to the *Pfaffl* method as described by Michael (Pfaffl 2001) and using the following formulas: $E = 10^{-1/r}$, $C = E^{\Delta Ct} / E^{\Delta Ct}$, $\Delta Ct = Ct_{Target} - Ct_{Actin}$, (*E* is the amplification efficiency of the standard curve; r is the slope of the standard curve; *C* is the expression level of the target gene.).

Statistical and analysis: The Excel software 2010 was used to statistics the data of plant phenotype and generate the bar diagram. The SAS 9.2 statistical software was used for analysis of variance.

Results

Material preparation: The copy number of T-DNA in potato mutant *M4P-9* is one (Fig. 1). Studies indicate that multiple transgene copies are more likely to affect the level and stability of gene expression one or two transgene copies are generally preferred for stable and high-level of gene expression (Iyer *et al*., 2000; James *et al*., 2002). This means that, usually, events with low copy numbers are preferred.

Potted samples of *M4P-9* showed the same characteristics as those demonstrated in the field (Fig. 2). The plant height of *M4P-9* is 35.2 cm and *WT* is 57.5 cm, *M4P-9* plants are significantly shorter ($p<0.01$) than *WT* plants throughout the growth process (Fig. 3). In addition, the roots of *M4P-9* are less developed than those of the *WT* ($p<0.01$), and the root length of *M4P-9* is 8.5 cm, *WT* is 15.4 cm (Fig. 3). There is no significant difference in leaf size.

Table 1. Primers used in SYBR Green I real-time PCR assays.

Gene			Sequence	Size
CPS1	qRT-PCR	Sense	AACAAGAGGACAAGGCTGCC	100
		Anti-sense	TCCAAGAATAACCCCGACGA	
KS	qRT-PCR	Sense	GCAAAGAGAGGGCAGCGTAG	100
		Anti-sense	CATTGAACTGAAACCATCGTCC	
KO	qRT-PCR	Sense	TCCACGAAGACACGCAGGT	159
		Anti-sense	AAATCGTTCAGGCTTCCACTCT	
GA2ox1	qRT-PCR	Sense	ATCACAACAAATCCATCA	100
		Anti-sense	AGCACCATACATCCCATA	
GA20ox1	qRT-PCR	Sense	TTTTGTGGACGATGAATGGC	135
		Anti-sense	GTCTTGTTGTTTACTACTGCTCTGT	
GID1B	qRT-PCR	Sense	TACATTTTGCCGCCGCCT	110
		Anti-sense	CCCATTTTAGAGCAGCCCATC	
Actin	qRT-PCR	Sense	AACAAGAGGACAAGGCTGCC	97
		Anti-sense	TCCAAGAATAACCCCGACGA	

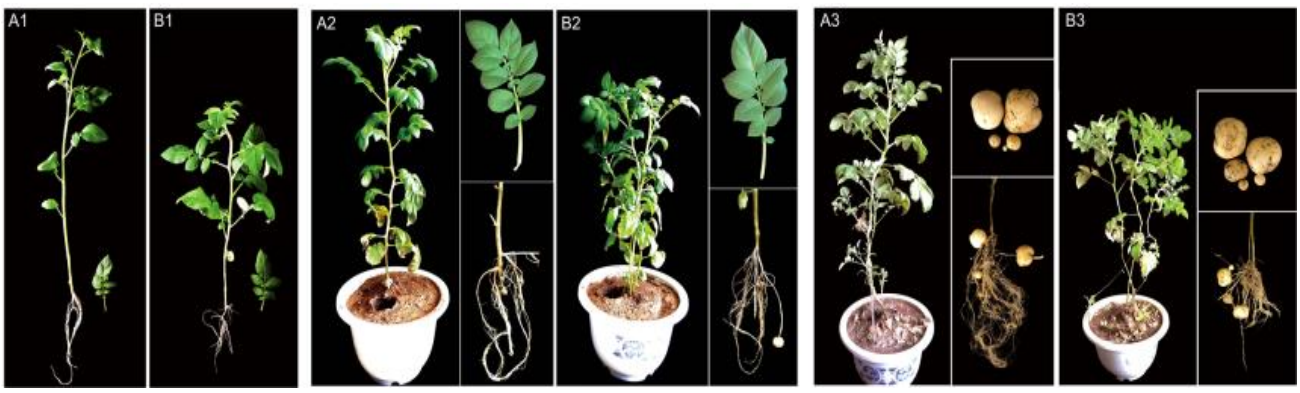

Fig. 2. The growth of *M4P-9* compared to *WT* potatoes
A1: *WT* seedling. B1: *M4P-9* seedling. A2: *WT* at the tuber-formation stage. B2: *M4P-9* at the tuber-formation stage. A3: *WT* at the tuber-expansion stage. B3: *M4P-9* at the tuber-expansion stage.

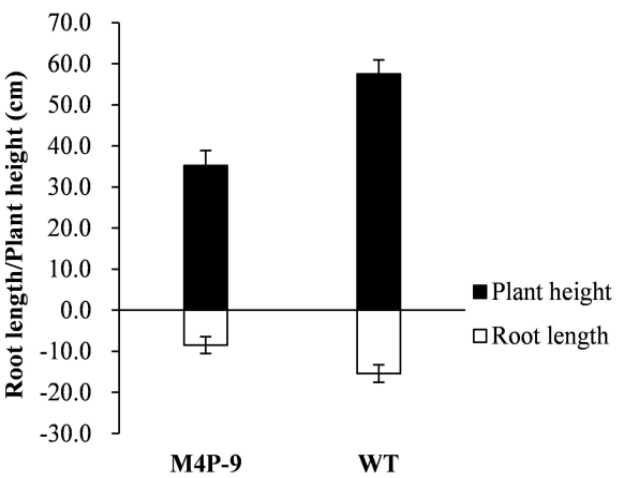

Fig. 3. The plant height and root length of *M4P-9* compared to *WT* potatoes
Compared with the *WT*, the differential of plant height and root length of *M4P-9* reached significance ($p<0.01$).

Establishment of standard curves: Total RNA was measured using a nucleic acid analyser (Eppendorf, Germany) and 1.2% gel electrophoresis (Fig. 4). cDNA was generated by reverse transcription, followed by conventional PCR. Using dilution gradients of the PCR products as the templates for quantitative real-time PCR, standard curves were established for each of the genes (Fig. 5), showing a linear relationship between template initial concentration and Ct value. The amplification efficiency of the Actin gene is 110.90%, and its correlation coefficient is 0.995. The amplification efficiency of the gibberellin genes are 85.0%-112.1%, and their correlation coefficients are 0.994-1.000. All the correlation coefficients of the standard curves are near 1, the melting curves are unimodal (Fig. 6), and the specificity of amplification products is good. These values indicate that the PCR products repeat in each cycle (Wilkening & Bader 2004), showing that the PCR efficiency is acceptable.

Expression of genes in the gibberellin pathway in potato seedlings: The expression levels of genes in the gibberellin pathway in the seedling stage of *M4P-9* and *WT* were calculated using the *Pfaffl* method (Table 2). The relative expression of gibberellin pathway genes in *M4P-9* roots varied from 0.27 to 2.29, with the lowest expression observed for *CPS1* and the highest for *GID1B* in the stem, the gene expression in *M4P-9* varied from 0.11 to 1.42, with the lowest for *GA20ox1* and the highest for *KS*. The expression of gibberellin pathway genes in *M4P-9* leaves varied from 0.34 to 1.96, with the lowest expression for *GID1B* and the highest for *GA20ox1*. In addition, the relative expression levels of *CPS1* and *GA2ox1* were lower than for the housekeeping gene (*Actin*) in roots and stems. *KS* and *KO* were expressed at lower levels than *Actin* in

roots and leaves. *GA20ox1* was expressed at lower levels in stems but at higher levels in roots and leaves than *Actin*. *GID1B* was expressed at lower levels than *Actin* in leaves but at higher levels in roots.

The differential expression patterns of key enzymes in the gibberellin pathway in seedlings of the mutant *M4P-9* are shown in Fig. 7. The gene expression changes of key enzyme genes differed among tissue types. Compared with the *WT*, the expression levels of the genes *CPS1*, *KS*, *KO* and *GA2ox1* were down-regulated in roots, at 1.73, 1.60, 1.58 and 1.49 times the *WT* levels, respectively. By contrast, *GA20ox1* and *GID1B* expression were up-regulated in roots, at 2.22 and 2.29 fold the level in *WT*, respectively. The expression levels of the *CPS1*, *GA20ox1* and *GA2ox1* genes were lower in the stems, at 1.81, 1.89, and 1.63 times the *WT* levels, respectively. Meanwhile, in leaves, the expression levels of *KS*, *KO* and *GID1B* were down-regulated in *M4P-9*, at 0.67, 0.49 and 0.34 times the *WT* levels, and *GA20ox1* and *GA2ox1* were up-regulated, at 1.96 and 1.46 fold the *WT* levels, respectively.

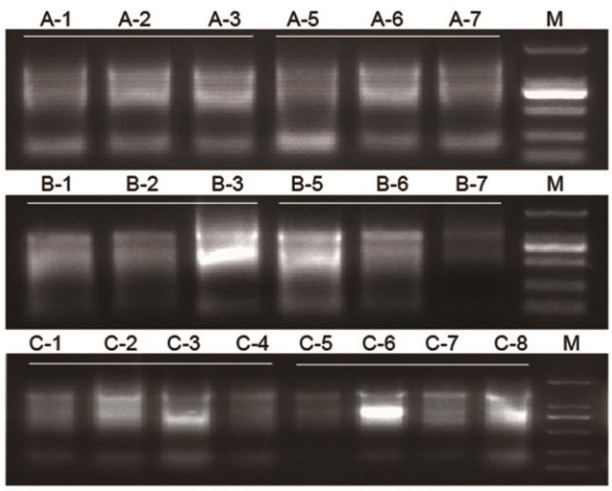

Fig. 4. RNA detection
A: Seedling. B: Tuber formation. C: Tuber expansion.
Lanes 1-4: Root, stem, leaf and tuber of *WT*. Lanes 5-8: Root, stem, leaf and tuber of *M4P-9*.

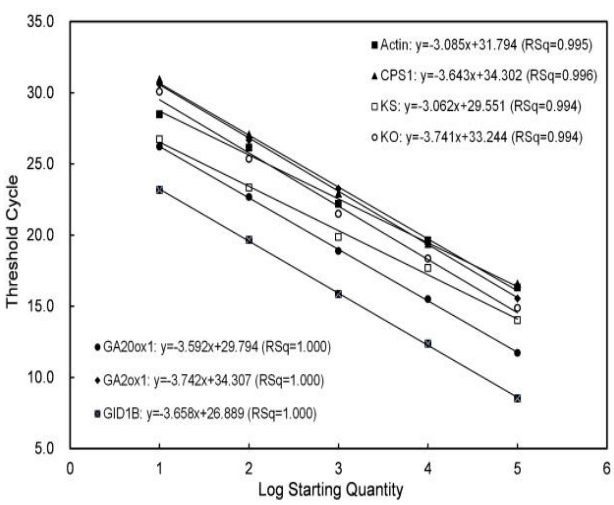

Fig. 5. Standard curves of *Actin* and the gibberellin genes.

Expression of genes in the gibberellin pathway during tuber formation in potato: The gene expression levels of key enzymes in the gibberellin pathway during the tuber formation stage were calculated for *M4P-9* and *WT* (Table 3). The relative expression levels of *CPS1*, *KS*, *KO*, *GA20ox1*, *GA2ox1* and *GID1B* gene in the roots of *M4P-9* were 3.57, 0.71, 2.32, 0.14, 2.10 and 1.61, respectively; in the stems of *M4P-9* were 1.02, 1.22, 1.92, 1.66, 3.58 and 2.34, respectively; and in the leaves of *M4P-9* were 0.44, 1.33, 0.28, 0.82, 2.47 and 0.55, respectively. In *M4P-9*, *CPS1*, *KS* and *GID1B* were expressed at lower levels in leaves but at higher levels in roots and stems than the housekeeping gene *Actin*. *GA2ox1* was expressed at higher levels than *Actin* in all tissues.

The differential expression of key enzymes of the gibberellin pathway during the tuber formation stage of mutant material *M4P-9* is shown in Fig. 8. Compared with the *WT*, the gene expression changes of *CPS1*, *KO*, *GA2ox1*, and *GID1B* were up-regulated in roots, at 3.57, 2.32, 2.10 and 1.61 fold those in *WT*, respectively; the *GA20ox1* gene was down-regulated in roots, at 1.86 times that in *WT*. The gene expression changes of *KO*, *GA20ox1*, *GA2ox1* and *GID1B* were up-regulated in stems, at 1.92, 1.66, 3.58 and 2.34 fold those in *WT*, respectively. In leaves, *GA2ox1* was up-regulated, with expression at 2.47 fold that of *WT*; *CPS1*, *KO*, *GA20ox1* and *GID1B* were down-regulated, at1.56, 1.72, 1.18 and 1.45 times the levels in *WT*, respectively.

Expression of genes in the gibberellin pathway during tuber expansion in potato: The gene expression levels of key enzymes in the gibberellin pathway during the tuber expansion stage were calculated for *M4P-9* and *WT* plants (Table 4). In *M4P-9*, the relative expression of *CPS1*, *KS*, *KO*, *GA20ox1*, *GA2ox1* and *GID1B* varies from 0.19 to 1.94 in roots, with the lowest expression for *KO* and the highest for *KS*; from 0.46 to 2.66 in stems, with the lowest expression for *KO* and the highest for *GA20ox1*; from 0.42 to 3.56 in leaves, with the lowest expression for *GA20ox1* and the highest for *GA2ox1*; from 0.22 to 2.05 in tubers, with the lowest expression for *GA2ox1* and the highest for *CPS1*. The relative gene expression of *KO* was lower than for the housekeeping gene *Actin* in each tissue. *GA20ox1* was expressed at higher levels in roots and stems than housekeeping gene (*Actin*). *GA2ox1* was expressed at lower in roots but at higher levels in stems, leaves and tubers.

The differential expression levels of key enzymes in the gibberellin pathway during the tuber expansion stage of the mutant *M4P-9* are shown in Fig. 9. Compared with the *WT*, the gene expression changes of *KO*, *GA2ox1* and *GID1B* were down-regulated in roots, at 1.81, 1.73 and 1.64 times the levels in *WT*, respectively; *KS* and *GA20ox1* were up-regulated, at 1.94 and 2.54 fold that in *WT* respectively. *KO* was down-regulated in stems, at 1.54 times that in *WT*; the genes *GA20ox1*, *GA2ox1* and *GID1B* were up-regulated, at 2.66, 2.26 and 1.30 fold those in *WT*, respectively. In the leaves of *M4P-9*, the genes *CPS1*, *KO* and *GA20ox1* were down-regulated, at 1.34, 1.36 and 1.58 times the levels in *WT*, respectively; *GA2ox1* and *GID1B* were up-regulated, at 3.56 and 2.49 fold those in *WT*. Meanwhile, in the tubers of *M4P-9*, the expression level of *CPS1* was up-regulated to 2.05 fold that in *WT*, and the expression levels of *KO*, *GA20ox1*, *GA2ox1* and *GID1B* were down-regulated to 1.77, 1.52, 1.78 and 1.65 times those in *WT*, respectively.

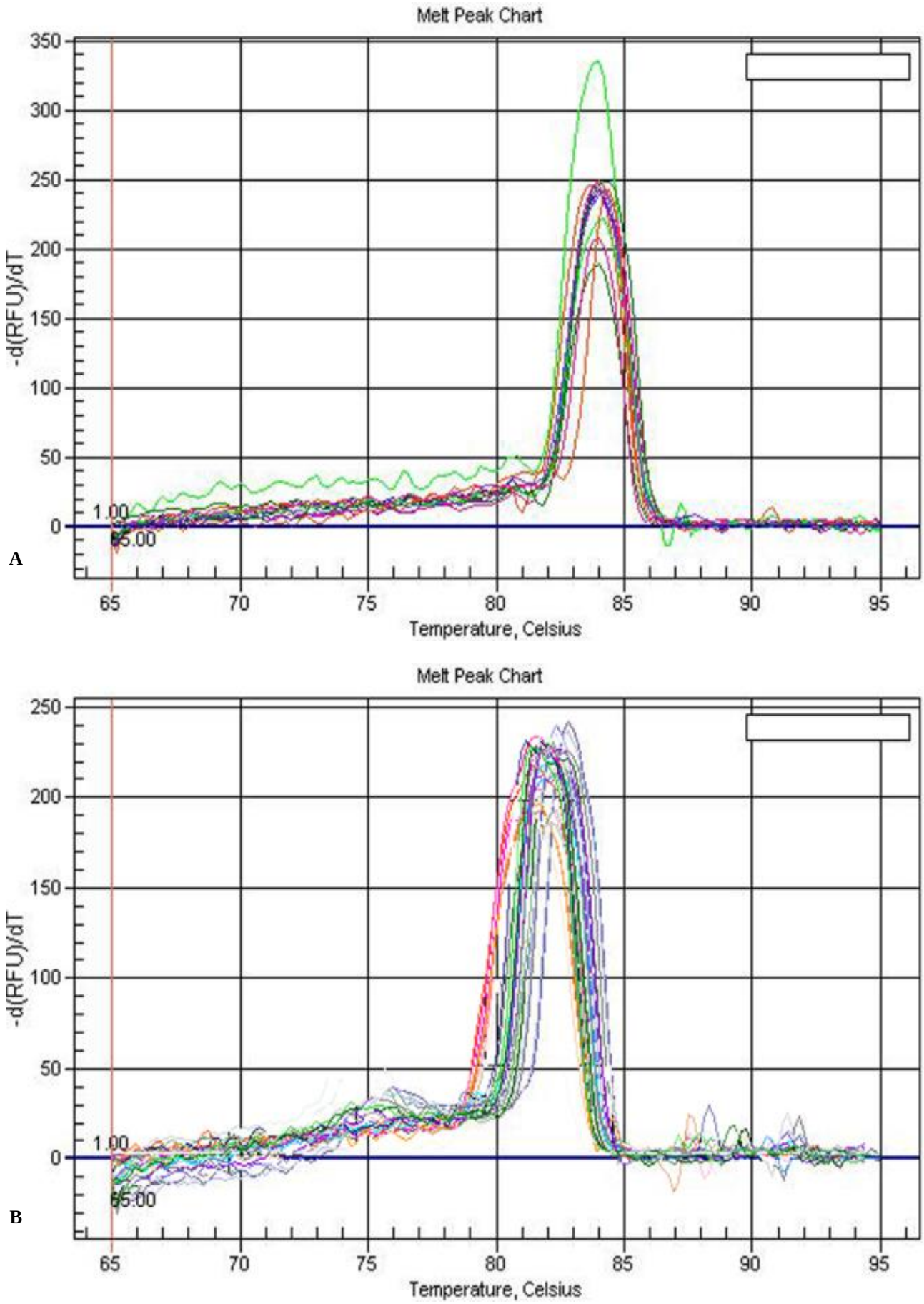

Fig. 6. Melting curves of *Actin* and the gibberellin genes.
A: Melting curve of the *Actin* gene. B: Melting curves of the gibberellin genes

Table 2. Gene expression levels of key enzymes in the GA pathway in potato seedlings.

Gene	Tissue	*E*	ΔCt_{Target}	ΔCt_{Actin}	$E^{\Delta Ct_{Target}}$	$E^{\Delta Ct_{Actin}}$	Expression (C)
Actin	Root	2.11	-1.44	-1.44	0.34	0.34	1.00
	Stem	2.11	1.10	1.10	2.27	2.27	1.00
	Leaf	2.11	1.22	1.22	2.49	2.49	1.00
CPS1	Root	1.88	-3.77	-1.44	0.09	0.34	0.27
	Stem	1.88	-1.30	1.10	0.44	2.27	0.19
	Leaf	1.88	1.63	1.22	2.80	2.49	1.13
KS	Root	2.12	-2.65	-1.44	0.14	0.34	0.40
	Stem	2.12	1.56	1.10	3.23	2.27	1.42
	Leaf	2.12	0.68	1.22	1.67	2.49	0.67
KO	Root	1.85	-3.15	-1.44	0.14	0.34	0.42
	Stem	1.85	1.37	1.10	2.32	2.27	1.02
	Leaf	1.85	0.32	1.22	1.22	2.49	0.49
GA20ox1	Root	1.90	-1.19	-2.09	0.47	0.21	2.22
	Stem	1.90	-2.17	1.10	0.25	2.27	0.11
	Leaf	1.90	2.47	1.22	4.87	2.49	1.96
GA2ox1	Root	1.85	-2.85	-1.44	0.17	0.34	0.51
	Stem	1.85	-0.30	1.10	0.83	2.27	0.37
	Leaf	1.85	2.10	1.22	3.64	2.49	1.46
GID1B	Root	1.88	-0.64	-1.65	0.67	0.29	2.29
	Stem	1.88	1.23	1.10	2.17	2.27	0.95
	Leaf	1.88	-0.26	1.22	0.85	2.49	0.34

Table 3. Gene expression levels of key enzymes in the GA pathway during tuber formation in potato.

Gene	Tissue	*E*	ΔCt_{Target}	ΔCt_{Actin}	$E^{\Delta Ct_{Target}}$	$E^{\Delta Ct_{Actin}}$	Expression (C)
Actin	Root	2.11	1.14	1.14	2.34	2.34	1.00
	Stem	2.11	-0.05	-0.05	0.96	0.96	1.00
	Leaf	2.11	-0.42	-0.42	0.73	0.73	1.00
CPS1	Root	1.88	3.36	1.14	8.36	2.34	3.57
	Stem	1.88	-0.03	-0.05	0.98	0.96	1.02
	Leaf	1.88	0.45	1.47	1.33	3.00	0.44
KS	Root	2.12	0.68	1.14	1.67	2.34	0.71
	Stem	2.12	0.22	-0.05	1.18	0.96	1.22
	Leaf	2.12	-0.04	-0.42	0.97	0.73	1.33
KO	Root	1.85	2.75	1.14	5.43	2.34	2.32
	Stem	1.85	1.00	-0.05	1.85	0.96	1.92
	Leaf	1.85	-1.33	0.63	0.44	1.60	0.28
GA20ox1	Root	1.90	-0.75	1.99	0.62	4.42	0.14
	Stem	1.90	0.73	-0.05	1.60	0.96	1.66
	Leaf	1.90	-0.79	-0.42	0.60	0.73	0.82
GA2ox1	Root	1.85	2.59	1.14	4.92	2.34	2.10
	Stem	1.85	2.01	-0.05	3.44	0.96	3.58
	Leaf	1.85	2.15	0.56	3.75	1.52	2.47
GID1B	Root	1.88	2.11	1.14	3.77	2.34	1.61
	Stem	1.88	1.29	-0.05	2.25	0.96	2.34
	Leaf	1.88	-1.45	-0.42	0.40	0.73	0.55

Table 4. Gene expression levels of key enzymes in the GA pathway during tuber expansion in potato.

Gene	Tissue	*E*	ΔCt_{Target}	ΔCt_{Actin}	$E^{\Delta Ct_{Target}}$	$E^{\Delta Ct_{Actin}}$	Expression (C)
Actin	Root	2.11	-1.19	-1.19	0.41	0.41	1.00
	Stem	2.11	-0.22	-0.22	0.85	0.85	1.00
	Leaf	2.11	-0.55	-0.55	0.66	0.66	1.00
	Tuber	2.11	-0.54	-0.54	0.67	0.67	1.00
CPS1	Root	1.88	-1.16	-1.19	0.48	0.41	1.17
	Stem	1.88	-0.11	-0.22	0.93	0.85	1.10
	Leaf	1.88	-1.31	-0.55	0.44	0.66	0.66
	Tuber	1.88	0.50	-0.54	1.37	0.67	2.05
KS	Root	2.12	-0.30	-1.19	0.80	0.41	1.94
	Stem	2.12	-0.18	-0.22	0.87	0.85	1.03
	Leaf	2.12	-0.45	-0.55	0.71	0.66	1.07
	Tuber	2.12	-0.13	-0.54	0.91	0.67	1.36
KO	Root	1.85	-2.14	0.44	0.27	1.39	0.19
	Stem	1.85	-1.54	-0.22	0.39	0.85	0.46
	Leaf	1.85	-1.39	-0.55	0.43	0.66	0.64
	Tuber	1.85	-3.06	-0.54	0.15	0.67	0.23
GA20ox1	Root	1.90	-0.42	-1.61	0.76	0.30	2.54
	Stem	1.90	1.27	-0.22	2.26	0.85	2.66
	Leaf	1.90	-0.94	0.35	0.55	1.30	0.42
	Tuber	1.90	-1.77	-0.54	0.32	0.67	0.48
GA2ox1	Root	1.85	0.97	2.53	1.82	6.61	0.27
	Stem	1.85	2.61	1.06	4.98	2.21	2.26
	Leaf	1.85	1.76	-0.25	2.95	0.83	3.56
	Tuber	1.85	-3.15	-0.54	0.14	0.67	0.22
GID1B	Root	1.88	-3.02	-1.19	0.15	0.41	0.36
	Stem	1.88	0.15	-0.22	1.10	0.85	1.30
	Leaf	1.88	1.78	0.28	3.07	1.23	2.49
	Tuber	1.88	-2.31	-0.54	0.23	0.67	0.35

Changes of gene expression in different growth stages: In roots, the expression of gibberellin-related genes varied with developmental stages. As shown in Fig. 10, the expression levels of *CPS1*, *KO* and *GA2ox1* gene were lower than *WT* during the seedling stage, then higher than *WT* during the tuber-formation stage, and returned to a lower level than *WT* during the tuber-expansion stage. The expression of the *GA20ox1* gene was higher than *WT* in seedlings, but lower than in *WT* during the tuber-formation stage and expressed more highly than in *WT* during the tuber-expansion stage. The expression of the *KS* gene was lower than *WT* in seedlings, and then gradually increased to a higher level than in *WT* during the tuber-expansion stage. By contrast, the expression of the *GID1B* gene was higher than in *WT* during the seedling stage, after which it gradually decreased to a lower level than in *WT*. A previous study showed that *GA2ox1* is a member of a small family of genes that are mainly expressed in flowers, roots, and seeds (Lester *et al*., 1999; Achard *et al*., 2006). The different results in *M4P-9* indicate the presence of changes in the expression of *GA2ox1*, which may partly explain the mutant phenotype.

In stems, the expression of *CPS1* was lower in *M4P-9* than in *WT* during the seedling stage but returned to the normal level during the tuber-formation and tuber-expansion stages. The expression levels of *KO* and *GID1B* were higher than in *WT* during the tuber-formation stage, but at the tuber-expansion stage, *GID1B* returned to the normal level, and *KO* expression decreased to a level below that in *WT*. The expression levels of *GA20ox1* and *GA2ox1* increased gradually and surpassed the *WT* levels beginning at the tuber-formation stage (Fig. 11).

The expression of gibberellin-related genes in leaves is shown in Fig. 12. Expression of *GA20ox1* was higher in *M4P-9* than in *WT* during the seedling stage, after which it gradually decreased to a lower level than in *WT* during the next stages. The expression of *GA2ox1* increased to 2.47 times that in *WT* at the tuber-formation stage from the overexpression observed during the seedling stage, and it then increased to 3.56 times that in *WT* during the tuber-expansion stage. The expression of *GID1B* was lower than that in *WT* during the seedling stage and increased gradually until it reached 2.49 times that in *WT* during the tuber expansion stage. There were no obvious changes in the expression of *KS*, which was below the level observed in *WT* and stable.

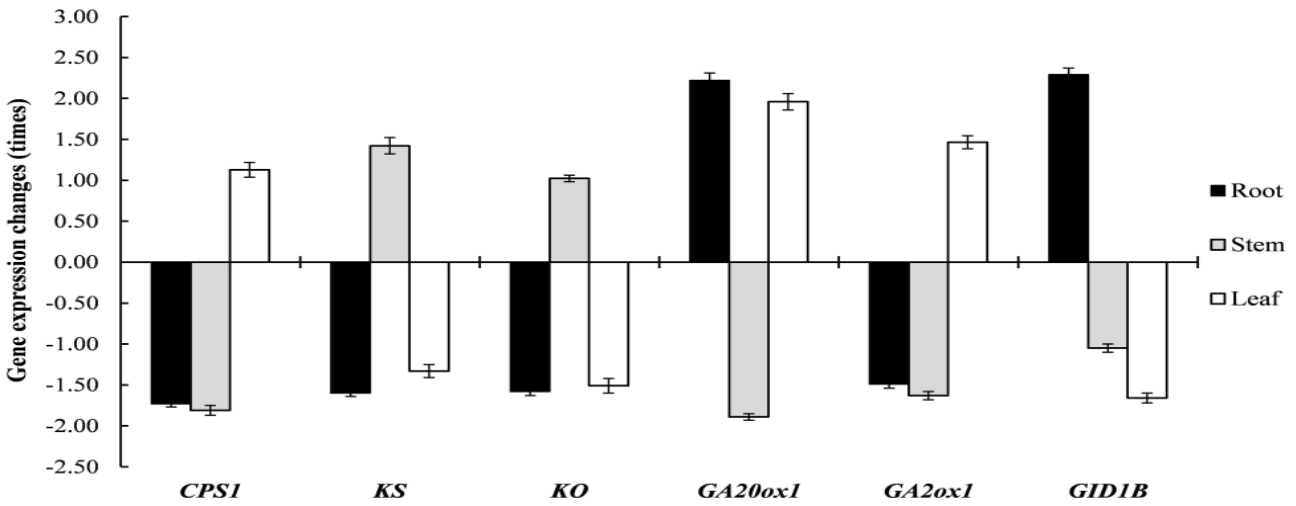

Fig. 7. Gene expression patterns of key enzymes in the gibberellin pathway in *M4P-9* seedlings
Compared with the *WT*, the differential expression of genes in each tissue reached significance ($p<0.01$).

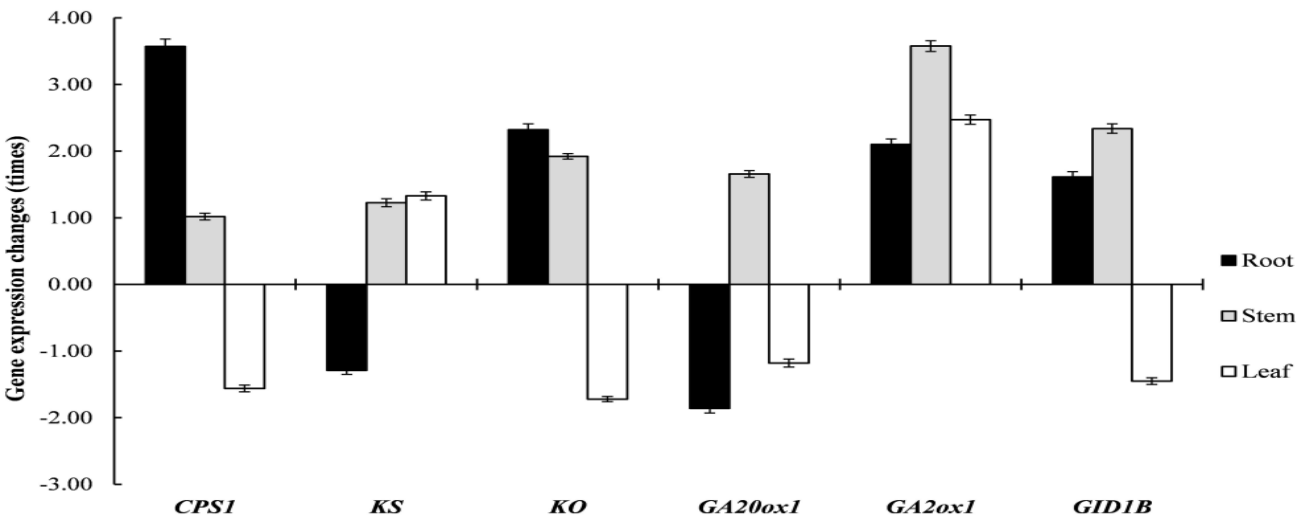

Fig. 8. Gene expression levels of key enzymes in the gibberellin pathway during tuber formation in *M4P-9*
Compared with the *WT*, the differential expression of *KS* in each tissue, *CPS1* in stems, and *GA2ox1* in leaves did not reach significance. The differential expression of other gene and tissue combinations reached significance ($p<0.01$).

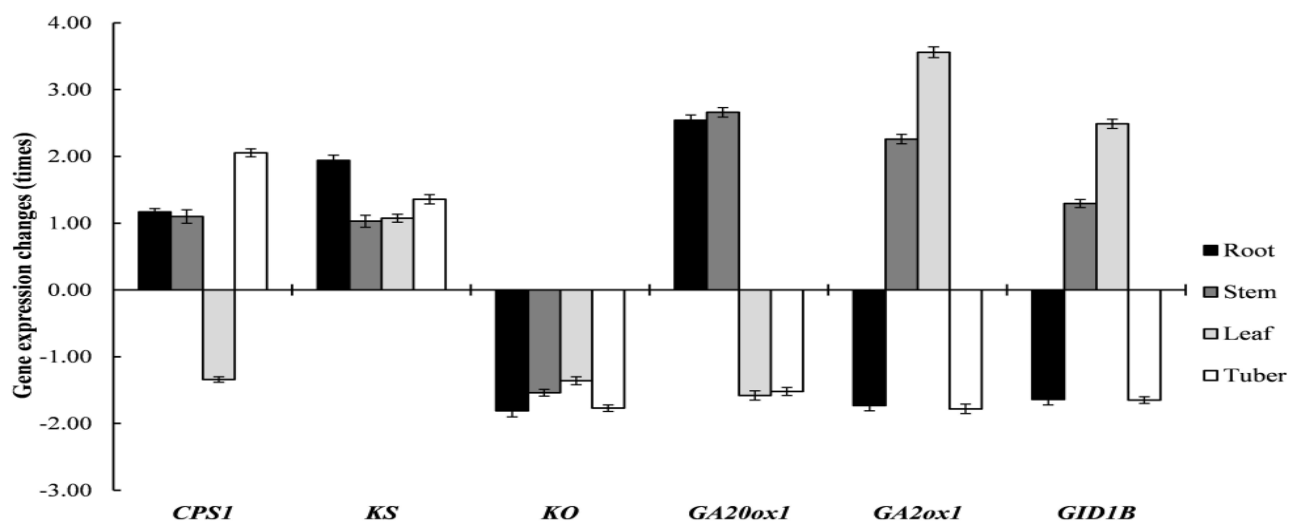

Fig. 9. Gene expression levels of key enzymes in the gibberellin pathway during tuber expansion in *M4P-9*
Compared with the *WT*, the differential expression of *KS* in stems, leaves, tubers and of *CPS1* in root and stems did not reach significance. The differential expression of other gene and tissue combinations reached significance ($p<0.01$).

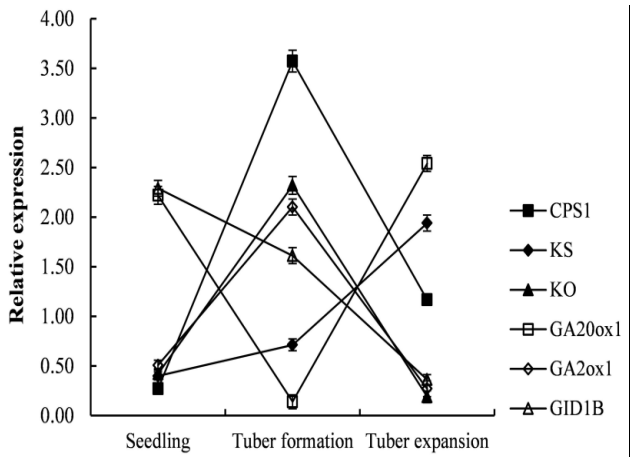

Fig. 10. Changes in gene expression in root at different stages
Compared with the *WT*, the differential expression of the *CPS1* gene in tuber expansion did not reach significance. The differential expression of other gene and stage combinations reached significance ($p<0.01$).

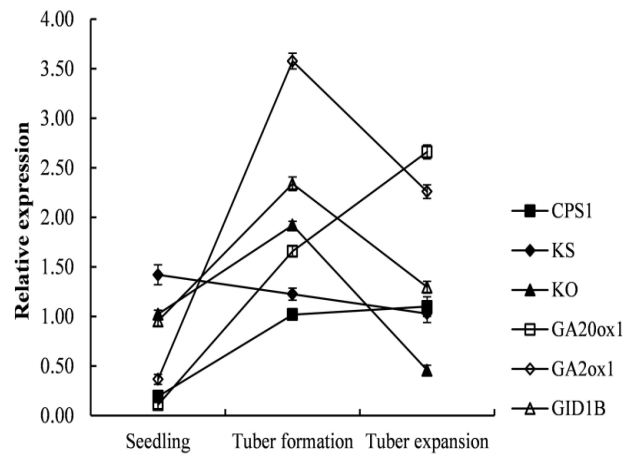

Fig. 11. Changes in gene expression in stems at different stages
Compared with the *WT*, the differential expression of *KS* in each stage, *CPS1* in tuber formation and tuber expansion, and *GID1B* in tuber expansion did not reach significance. The differential expression of other gene and stage combinations reached significance ($p<0.01$).

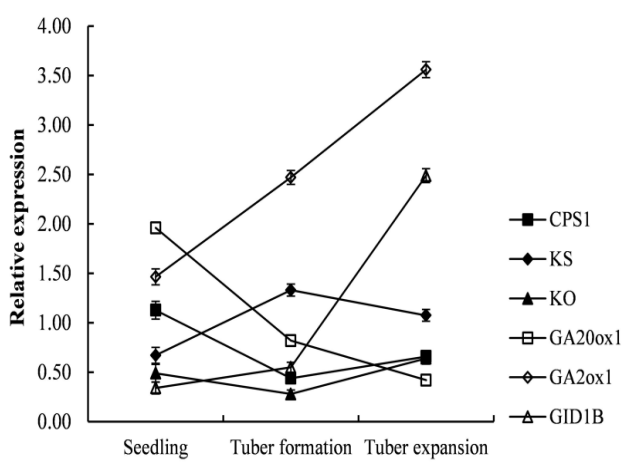

Fig. 12. Changes in gene expression in leaves at different stages
Compared with the *WT*, the differential expression of *CPS1* in seedling and *GA20ox1* in tuber formation did not reach significance. The differential expression of other gene and stage combinations reached significance ($p<0.01$).

Discussion

The molecular mechanism underlying the development of mutant materials: Exogenous genes are randomly integrated into the plant genome. Therefore, integration of exogenous genes usually causes changes to the genome of the host cells, leading to long-term effects whether or not the introduced genes are expressed (Iyer *et al*., 2000; James *et al*., 2002). Random integration of genes in potato results in the rearrangement of the genome, possibly including the deletion and repetition of the integration sites in the plant's genome, the deletion and repetition of the exogenous gene, and the translocation and inversion of the chromosome (Omar *et al*., 2008). Changes in the gene sequence at a specific location can lead to changes in gene expression. In this article, the changed expression of genes in the gibberellin synthesis pathway is considered to be one of the reasons for the plant height phenotype of *M4P-9* material.

The application of quantitative real-time PCR: The Semi-quantitative PCR and Northern blot are the most commonly methods to investigate the gene expression in transcriptional level. While the fluorescence quantitative real-time PCR is developed in recent years, which has the characteristics of accurate and high sensitivity. It can effectively eliminate the pollution of PCR products, such as the nucleic acids and ethidium bromide, and has been widely applied to investigate the differential expression of genes.

There are two ways to detect the changes of gene expression, absolute and relative quantitative. Absolutely quantitative need to clone the amplified fragments of target and reference gene, construct a transcriptional system in vitro, and build standard curve using standard substance of known copy number, this method is complicated, time-consuming, high cost, and has rarely used in the analysis of gene expression. In the practice of the study, people's attention is often not the gene copy number of transcriptional gene, but the change of gene expression under different external processing and physiological state, therefore tend to adopt the method of relative quantitative. The analysis of traditional relative quantitative usually adopts $2^{-\Delta\Delta Ct}$ method, but the amplification efficiencies are difficult to achieve consistent, making the results inaccurate. Using *Pfaffl* method can calculate the amplification efficiency directly by the slope of standard curve, and thus the influence of amplification efficiency on results can be eliminated.

The effect of genes for key gibberellin synthesis enzymes on *M4P-9*: Gibberellin is involved in many aspects of plant growth and development, and plants must therefore precisely regulate the expression levels of gibberellin-related genes. This regulation occurs mainly by controlling the expression of genes involved in gibberellin synthesis and metabolism. Research shows that gibberellin plays an important role in regulating plant height during growth and development (Peng *et al*., 1999). *CPS1* is the first key enzyme of the formal gibberellin biosynthetic pathway; if *CPS1* is completely mutated, the plant will not produce any gibberellin, and the seeds will fail to germinate. As an early key gene in the synthesis of GAs, *CPS1* controls the conversion of

GGDP to CDP (Prisic & Peters, 2007). When overexpressed in zucchini, the GA20 oxidase gene can produce phenotypes of gibberellin deficiency, such as dwarf plants, deepened leaf colour, and reduced gibberellin content (Curtis *et al*., 2000; Niki *et al*., 2001). GA2-oxidase has the capacity to smooth out the biological activity of C19 gibberellin (Middleton *et al*., 2012). The GA2 oxidase gene of spinach has been cloned by Lee & Zeevaart (2002), and it encodes a 337-amino acid protein that can be expressed in *E. coli*. Their results showed that that GA2 oxidase can catalyse the conversion of GA9 and GA20 into GA51 and GA29, maintaining the balance between bioactive GAs and C19-GAs. Overexpression of GA2 oxidase often leads to dwarf plants and to decreased gibberellin content (Huang *et al*., 2010; Lee & Zeevaart 2002). Ueguchi-Tanaka has identified the first *GID1B*, a type of GA receptor, in rice, and they believe that the excessive expression of GID1 will lead to supersensitivity to gibberellin. GID1 is a soluble receptor that mediates gibberellin signalling by binding to active GAs and then passing that signal to the DELLA proteins, thereby causing a series of downstream signalling events (Ueguchi-Tanaka *et al*., 2007).

The results presented here show changes in the gene expression patterns of the key enzymes in the gibberellin pathway in the *M4P-9* mutant material. The expression of *CPS1* showed a large variation in roots, with expression lower than normal in seedlings and excessive during tuber formation. The expression of *KO* was lower than in *WT* in seedling tissues, excessive in roots and stems during tuber formation, and then lowers than in *WT* during tuber expansion. The genes *GA20ox1* and *GA2ox1* were overexpressed in stems during tuber formation; this overexpression ultimately caused a decrease in the gibberellin content in the metabolic pathway, prompting the decreased expression of the gibberellin receptor gene *GID1B*, likely a factor in the plant height phenotype of *M4P-9* mutant material. This result is consistent with those of previous studies (Huang *et al*., 1998; Zhang *et al*., 2007).

Conclusion

The gene expression patterns of key enzymes in gibberellin metabolic pathways were evaluated in the potato mutant *M4P-9*, and the results show tissue- and stage-dependent changes in the expression of gibberellin-related genes. The higher expression levels of *CPS1* and *GA2ox1* in roots, the lower expression levels of *GA20ox1* in roots during tuber formation stage; as well as the increased expression of *GA20ox1* and *GA2ox1* genes in stems during the tuber formation stage, may play key roles in the plant height phenotype of *M4P-9* mutant materials.

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