Plant Gene 4 (2015) 69–77

Contents lists available at ScienceDirect

Plant Gene

journal homepage: www.elsevier.com/locate/plant-gene

An RNA-Seq analysis of the pear (*Pyrus communis* L.) transcriptome, with a focus on genes associated with dwarf

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ARTICLE INFO

Article history:
Received 6 April 2015
Received in revised form 30 June 2015
Accepted 10 August 2015
Available online 22 October 2015

Keywords:
Transcriptome
Pear
Dwarf
Gene transcription
Phytohormones

ABSTRACT

Dwarfing rootstocks, such as that of the Chinese pear variety 'Zhongai 1', are an important resource in modern fruit production. An RNA-Seq analysis of 'Zhongai 1' and its progenitor non-dwarfing variety 'Jinxiang' revealed a set of 234 genes which were differentially transcribed in the two varieties. Among the differentially transcribed gene set were some potential candidates for the dwarf trait: one encoded a gibberellin 3-beta-dioxygenase, four encoded auxin-associated proteins, one encoded LRR receptor-like serine/threonine-protein kinase, four encoded cytochrome P450s, two encoded enzymes involved in abscisic acid synthesis, three were ethylene-responsive transcription factors, six were moisture status related proteins, two were NAC and four WRKY transcription factors. The assessment of transcript abundance derived by the RNA-Seq analysis was validated using quantitative real time PCR for ten of the differentially transcribed genes. The transcript levels of these genes in other dwarf and non-dwarf varieties were also analyzed by the qPCR, but no completely consistent regularity was found. The concentration of some phytohormones (GA_3, IAA and ABA) was also determined, the results accorded with the transcript levels of some related genes in some varieties, but did not accord in all the survey varieties.

1. Introduction

The development and adoption of dwarfing rootstocks have improved the productivity of temperate fruit trees (Prassinos et al., 2009), by allowing for an increase in planting density and a reduction in production costs. Dwarfing rootstocks are also beneficial because their deployment reduces the length of the juvenility period, improves levels of disease/insect/virus resistance and enhances fruit quality (Prassinos et al., 2009; Zhu et al., 2003; Gonçalves et al., 2005; Lauri et al., 2006; Maas, 2006; Seleznyova et al., 2008). Attempts have been made to exploit dwarfing rootstocks in pear (*Pyrus communis* L.), but their use to date has been associated with scion incompatibility, a reduced abiotic stress tolerance and a heightened susceptibility to disease (Maas, 2006; Du Plooy and van Huyssteen, 2000; Jacob, 2002; Simard and Michelesi, 2002; Webster, 2002; Campbell, 2003; Johnson et al., 2005; Itai, 2007; Rachel et al., 2012). Until the physiological and the molecular bases of compatibility between the scion and its rootstock are properly understood, it will remain difficult to develop a predictive evaluation method for dwarfing rootstocks. If the dwarfing mechanisms are understood completely, it will speed breeding programs by identifying molecular markers (Atkinson and Else, 2001).

The mechanistic basis of scion dwarfing has been well explored in apple (Kamboj et al., 1999a, 1999b; Bulley et al., 2005; Pilcher et al., 2008; Van Hooijdonk et al., 2011; Li et al., 2012; Vattiprolu, 2012), sweet cherry (Lang et al., 2002; Olmstead et al., 2006; Hajagos and Végvári, 2013), beech (Weibel et al., 2003; Solari and DeJong, 2006; Weibel, 2008; Tombesi et al., 2010) and kiwifruit (Clearwater et al., 2004, 2006, 2007). Several genes implicated with the dwarfing effect have been tagged and isolated (Prassinos et al., 2009; Zhu et al., 2008), although not so far in pear. Meanwhile, the causal signals of the mechanisms for scion control by rootstocks are still uncertain and may differ between crop species (Gregory et al., 2013). The Chinese pear rootstock 'Zhongai 1' is effective in inducing the dwarf habit, reducing juvenility and improving fruit yield when combined with a wide range of scions. 'Zhongai 1' also is a dwarf variety and its one years old shoot length is less than 50 cm in average, otherwise, most of no-dwarf varieties are more than 100 cm. A deal of research has been directed at characterizing its anatomical and physiological features, but so far none has focused on gaining an understanding of the molecular

Abbreviations: RNA-Seq, RNA sequencing; Gb, gigabase(s); cDNA, DNA complementary to RNA; LRR, leucine-rich repeat; qPCR, quantitative real-time PCR; GA, gibberellin; IAA, indole-3-acetic acid; ABA, abscisic acid; ZR, zeatin riboside; ELISA, enzyme-linked immunosorbent assay; Mb, millionbase(s); bp, base pair(s); GA3ox, gibberellin 3-beta-dioxygenase; RPKM, reads per kilobase per million mapped reads; FDR, false discovery rate; BLAST, basic local alignment search tool; GO, gene ontology; COG, cluster of orthologous groups of proteins; KEGG, Kyoto encyclopedia of genes and genomes; WEGO, web gene ontology annotation plot; Nr, NCBI non-redundant sequence database; Nt, NCBI nucleotide sequence database; COL, constans-like; DTGs, differentially transcribed genes.

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http://dx.doi.org/10.1016/j.plgene.2015.08.003

events underlying its dwarf trait and the interaction between scion and rootstock. In our past research, we found that all of our tested pear rootstocks with dwarfing effect on scions are dwarf themselves. Understanding the dwarf mechanism of 'Zhongai 1' may provide theoretical base to the research on its dwarfing mechanism.

With the development of high throughput transcriptomic platforms such as RNA-Seq (Wang et al., 2009), it has now become feasible to quantify the transcription of large numbers of genes simultaneously in a way which is more reliable than was achieved by microarray-based methods (Dai et al., 2013). It has been successful in revealing the genic basis of a number of physiological characteristics in apple (Zhang et al., 2012), pear (Liu et al., 2012; Wang et al., 2014), Chinese bayberry (Feng et al., 2012) and hawthorn (Dai et al., 2013). Here, an RNA-Seq analysis of the 'Zhongai 1' transcriptome is presented in the form of a comparison between a dwarf and a non-dwarf variety. The intention was to identify candidate genes underlying the dwarf mechanism and the dwarfing effect induced by the 'Zhongai 1' rootstock further.

2. Materials and methods

2.1. Plant materials

'Zhongai 1' (dwarf) is a naturally pollinated seedling of 'Jinxiang' (non-dwarf). 'Jinxiang' is a selection from the cross 'Nanguoli' (*P. ussuriensis* Maxim.) × 'Bartlete' (*P. communis* L.). Tender leaves of newly emerged shoots from 'Zhongai 1' and 'Jinxiang' were collected and the length of 30 shoots was measured during 2012 (on May 7, 14, 21 and 28, on June 4, 11, 25 and on July 9). The same leaves from other dwarf pear varieties including 'Zhongai 1', 'Zhongai 3', 'Zhongai 4' and 'Zhongai 5' (all are selected from naturally pollinated seedlings of 'Jinxiang') and non-dwarf pear varieties including 'Jinxiang', 'Zaosu' (*P. bretschneideri* Rehd.) and 'Huasu' ['Zaosu' × 'Yakumo' (*P. pyrifolia* (Burm.) Nakai)] were also collected and the length of 30 shoots was measured during 2013 (on May 22, on June 7, 20 and on July 3). All above varieties were grown at the Research Institute of Pomology, Chinese Academy of Agricultural Sciences (120° 44′ 38″ E, 40° 37′ 9″ N). The trees were all at least 20 years old and had been grafted onto a *P. betulaefolia* rootstock. Each sampling featured at least ten trees per variety. The material was snap-frozen and stored at −80 °C until processed for RNA extraction or phytohormone determination.

2.2. RNA extraction, library preparation and RNA-seq

Total RNA was isolated using the TRIzol reagent (Invitrogen, San Diego, USA) following the manufacturer's protocol, then purified using oligo (dT) magnetic beads. The integrity of the RNA was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) and lodged with Biomarker Technology Company (Beijing, China) to prepare and sequence the derived cDNA. The paired-end library preparation and sequencing were achieved using a TruSeq RNA sample preparation kit (Illumina Inc., San Diego, USA) and the libraries were sequenced using a HiSeqTM2000 device (Illumina).

Table 1
The output of the RNA-seq analysis and sequence assembly of the two cDNA libraries.

Sample	'Zhongai 1'[a]	'Jinxiang'[b]
Total nucleotides (Mb)	2460	2510
GC (%)[c]	48.80	48.48
Cycle Q20 %[d]	100	100
Number of >200 bp transcripts	61,894	73,111
Mean length of transcripts (bp)	737	746
N50 length of transcripts (bp)	1048	1070
Number of unigenes	32,995	35,274
Mean length of unigenes (bp)	738	745
N50 length of unigenes (bp)	1144	1192

[a] Dwarf.
[b] Non-dwarf.
[c] GC base content.
[d] The proportion of cycle, of which the average quality value is ≥20.

Table 2
Length of *Pyrus communis* unigenes.

Unigene length (bp)	Total number	Percentage (%)
200–300	14,723	31.58%
300–500	11,655	25.00%
500–1000	9402	20.17%
1000–2000	7914	16.98%
2000+	2925	6.27%
Total length	33,966,145	
Count	46,619	
Mean length	729	
N50 length	1177	

2.3. Sequence assembly

Prior to the sequence assembly, adapter sequences and low quality (proportion of non-called bases >5%) and short (<13 bp) reads were deleted. The 'Zhongai 1' library comprised 2460 Mb of filtered sequence, and the 'Jinxiang' library 2510 Mb. Both of the cycleQ20 were 100%. The global GC percentages were 48.80% and 48.48%, respectively (Table 1). The two libraries were assembled separately using Trinity software (Grabherr et al., 2011), first into contigs and then into transcripts using paired-end information. Only contigs of length > 200 bp were used. The settings for the relevant parameters were: K-mer = 25, seqType: fq, group_pairs_distance = 150. The application of Trinity software identified 61,894 transcripts in 'Zhongai 1' and 73,111 in 'Jinxiang'. The mean transcript and N50 lengths in 'Zhongai 1' were, respectively 737 bp and 1048 bp, and the equivalents in 'Jinxiang' were 746 bp and 1070 bp (Table 1). Following clustering, 32,995 unigenes were identified in 'Zhongai 1' and 35,274 in 'Jinxiang'. The mean unigene and N50 lengths in 'Zhongai 1' were, respectively 738 bp and 1144 bp, and the equivalents in 'Jinxiang' were 745 bp and 1192 bp (Table 1). Combining the two sets of unigenes realized a global number of 46,619 unigenes, of mean length 729 bp and N50 length 1177 bp (Table 2).The longest transcript within each cluster was regarded as a unigene for the purpose of annotation. The full set of sequence data has been deposited in the NCBI Sequence Read Archive (SRA) under accession number SRP048768.

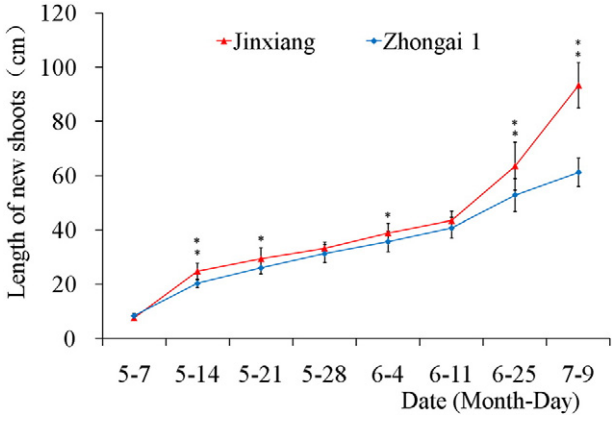

Fig. 1. The temporal growth of new shoots in 'Zhongai 1' and 'Jinxiang'. Individual points represent data values from the mean of 30 new shoots ± standard error bar ($n = 30$). Asterisks indicate significant difference, one is at $P \le 0.05$ and two is at $P \le 0.01$.

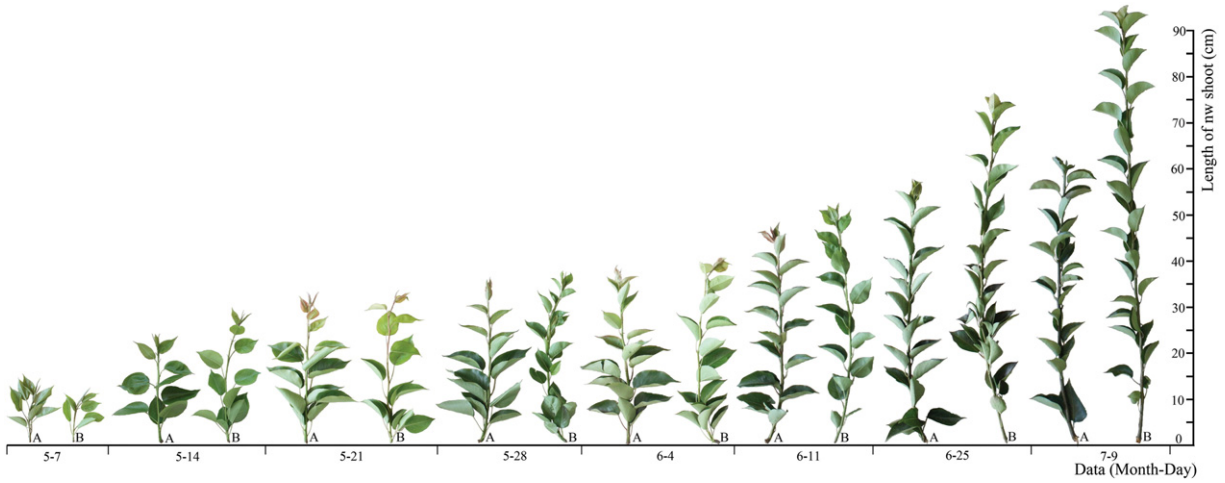

Fig. 2. The morphology of young shoots formed by (A) 'Zhongai 1' and (B) 'Jinxiang'.

2.4. Functional annotation

The sequences were functionally annotated by BLAST analysis, and classified according to the COG (http://www.ncbi.nlm.nih.gov/COG), KEGG (http://www.genome.jp/kegg), Swiss-Prot (http://www.expasy.ch/sprot), TrEMBL (http://www.uniprot.org/), InterPro (http://www.ebi.ac.uk/interpro/), NCBI non-redundant Nr and Nt databases (http://www.ncbi.nlm.nih.gov). The Blast2GO and WEGO packages (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) were used for GO annotation and functional classification.

2.5. Differential gene transcription

Transcript abundances were estimated from the number of reads per kilobase per million mapped reads (RPKM) (Mortazavi et al., 2008). IDEG6 software and the general Chi-squared hypothesis testing method were used to validate differential transcription (by at least two folds) between the two libraries, applying a false discovery rate (FDR) of <0.01 and a P threshold of <0.001.

2.6. Quantitative RT-PCR (qPCR) analysis

Total RNA was quantified by UV spectroscopy (GeneSpecV, Hitachi, Japan) and an 800 ng aliquot was reverse transcribed using a PrimeScript™ RT reagent kit (TaKaRa, Dalian, China) in a 20 μL reaction following the manufacturer's protocol. Each qPCR was processed using an MJ-Chromo4 Real Time PCR Detection System (Bio-Rad, Hercules, USA) based on SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China). The sequences of the relevant primers are given in Table S1. Each sample was technically replicated three times, and the pear *ACTIN* gene (GenBank accession number: GU830958.1) was used as the reference. Relative fold changes in transcript abundance were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7. Phytohormone extraction and concentration determination

Phytohormone extraction was performed according to the reference by Zhang et al.(2011). Four phytohormones of gibberellin (GA_3), indole-3-acetic acid (IAA), abscisic acid (ABA) and zeatin riboside (ZR) were extracted from frozen leaves and the concentration was determined by means of enzyme-linked immunosorbent assay (ELISA) using a Bio-Rad 680 Model Microplate Reader (Bio-Rad, Osaka, Japan). The ELISA kits were provided by China Agricultural University in Beijing, and all the test steps followed the manufacturer's protocol. The hormone level was calculated by the external standard curve method. Three biological replicates for each sample and three technical replicates for each biological replicate were done.

2.8. Statistical analysis

Experimental data were subjected to analysis of variance (ANOVA) by standard procedures using SPSS software (Version 13.0).

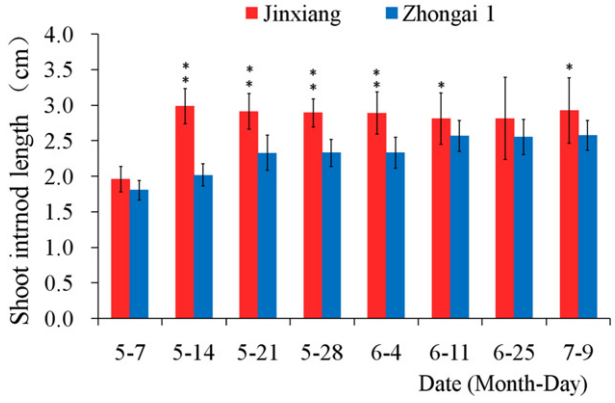

Fig. 3. Shoot internode length in 'Zhongai 1' and 'Jinxiang'. Individual poles represent data values from the mean of 30 new shoots ± standard error bar ($n = 30$). Asterisks indicate significant difference, one is at $P \leq 0.05$ and two is at $P \leq 0.01$.

Table 3
Annotation of the pear unigenes.

Category of annotation	Number of unigenes ≥300 bp and <1000 bp	Number of unigenes ≥1000 bp	Number of total annotated unigenes	Percentage (%)[c]
COG_annotation	3268	4829	8932	19.16
GO_annotation	5210	7393	13,772	29.54
Kegg_annotation	2547	2880	6356	13.63
Swiss-Prot_annotation	9629	9383	22,139	47.49
TrEMBL_annotation	14,272	10,689	30,312	65.02
InterProScan_annotation	7630	9098	18,495	39.67
Nr[a]_annotation	14,187	10,690	30,147	64.67
Nt[b]_annotation	11,951	10,379	26,547	56.94
All_annotation	15,554	10,741	33,151	71.11

[a] Nr = NCBI non-redundant sequence database.
[b] Nt = NCBI nucleotide sequence database.
[c] Proportion of the 46,619 assembled unigenes.

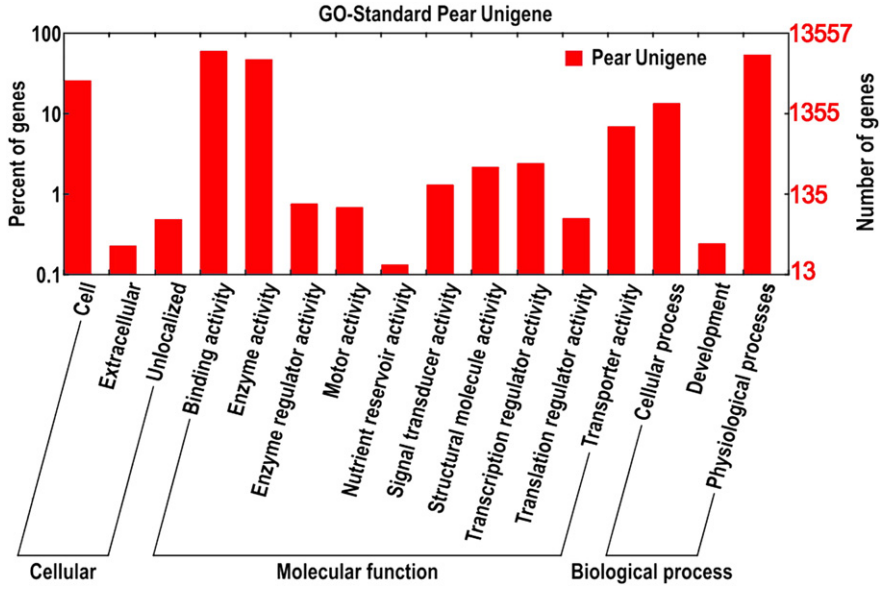

Fig. 4. GO classification of pear unigenes. Three major GO categories were identified: cellular component, molecular function and biological process.

3. Results

3.1. Shoot development in 'Zhongai 1' and 'Jinxiang'

There were no obvious differences in the length of newly emerging shoots between the two pear varieties prior to June 11 (Figs. 1 and 2), but after this date, their lengths began to diverge. The internode length of 'Zhongai 1' shoots was less than that of 'Jinxiang' shoots throughout the survey period, and peaked on June 11 (Fig. 3). As a result, June 11 was identified as the critical date for recognizing differential transcription between the two varieties, and the material sampled at the next time point (June 25) was therefore used as the source of RNA for the RNA-Seq analysis.

3.2. Illumina sequencing and de novo assembly

Through sequencing and assembling, we obtained a global number of 46,619 unigenes from the two pear varieties. To confirm the quality of sequence assembly, the integrality of coding sequence of unigene was checked. Altogether, 63 of the unigenes which had a RPKM of >50

Table 4
DTGs putatively associated with plant growth.

Gene ID	RPKM value		$\log_2$ (Jinxiang/ Zhongai 1)	Putative name and function
	Jinxiang	Zhongai 1		
Pear_158-T2-I_unigene_BMK.30804	27	1	4.75	Gibberellin 3-beta-dioxygenase(GA3ox)
Pear_158-T1-I_unigene_BMK.27443	76	180	−1.24	Auxin-induced in root cultures protein 12
Pear_158-T2-I_unigene_BMK.646	68	185	−1.44	Auxin-responsive family protein
Pear_158-T2-I_unigene_BMK.30090	32	95	−1.57	Auxin-induced protein
Pear_158-T2-I_unigene_BMK.18246	46	101	−1.13	IAA-amino acid hydrolase
Pear_158-T1-I_unigene_BMK.70	21	97	−2.21	LRR receptor-like serine/threonine-protein kinase (FLS/GSO/BRI)
Pear_158-T2-I_unigene_BMK.35176	16	0	13.97	Cytochrome P450 (CYP82G1)
Pear_158-T1-I_unigene_BMK.28132	114	230	−1.01	Cytochrome P450 (CYP716B2)
Pear_158-T1-I_unigene_BMK.8471	8	75	−3.23	Cytochrome P450 (CYP77A3)
Pear_158-T1-I_unigene_BMK.2532	23	59	−1.36	Cytochrome P450 (CYP94A1)
Pear_158-T1-I_unigene_BMK.28029	198	95	1.06	9-cis-epoxycarotenoid dioxygenase (NCED)
Pear_158-T2-I_unigene_BMK.5745	108	49	1.14	Zeaxanthin epoxidase (ZEP)
Pear_158-T2-I_unigene_BMK.31115	122	11	3.47	EID1-like F-box protein 3
Pear_158-T1-I_unigene_BMK.7652	45	95	−1.08	Ethylene-responsive transcription factor (ERF073)
Pear_158-T2-I_unigene_BMK.14387	39	87	−1.16	Ethylene-responsive transcription factor 1A
Pear_158-T2-I_unigene_BMK.5402	87	27	1.69	Ethylene-responsive transcription factor (ERF060)
Pear_158-T1-I_unigene_BMK.29037	0	71	−16.12	Dehydration responsive protein (RD22-like)
Pear_158-T1-I_unigene_BMK.28953	16	61	−1.93	Dehydration responsive protein
Pear_158-T2-I_unigene_BMK.7925	9	297	−5.04	Dehydration-responsive protein (RD22-like)
Pear_158-T1-I_unigene_BMK.3667	35	79	−1.17	Aquaporin (TIP1-3)
Pear_158-T2-I_unigene_BMK.8404	47	5	3.23	Dehydrin (DHN)
Pear_158-T2-I_unigene_BMK.29609	54	17	1.67	Desiccation-related protein PCC13-62-like
Pear_158-T2-I_unigene_BMK.32554	21	54	−1.36	NAC transcription factor 25
Pear_158-T1-I_unigene_BMK.29995	248	107	1.21	NAC domain-containing protein 72-like
Pear_158-T2-I_unigene_BMK.33538	104	265	−1.35	WRKY transcription factor 33
Pear_158-T2-I_unigene_BMK.11034	92	198	−1.11	WRKY transcription factor 31
Pear_158-T1-I_unigene_BMK.30540	37	122	−1.72	WRKY transcription factor 40
Pear_158-T1-I_unigene_BMK.24581	8	37	−2.21	WRKY transcription factor 18

and a log2 of the ratio of their abundance in 'Zhongai 1' and 'Jinxiang' either >1 or <−1 were selected for checking this. Three of these unigenes were annotated as non-coding RNA, while for 55 of the remaining 60 unigenes the entire coding sequence was represented. A BLAST analysis of these 55 full-length unigenes indicated that 52 had a likely homolog in apple and the other three matched a plum sequence. The sequence identity of the coding sequences between pear and apple (or plum) ranged from 50.54% to 98.78%, with the mean of 93.54% (Supplementary Table 2).

3.3. Functional annotation and functional classification

A BLASTx analysis of the 46,619 unigenes showed that 33,151 of them had at least one significant match to an existing gene model

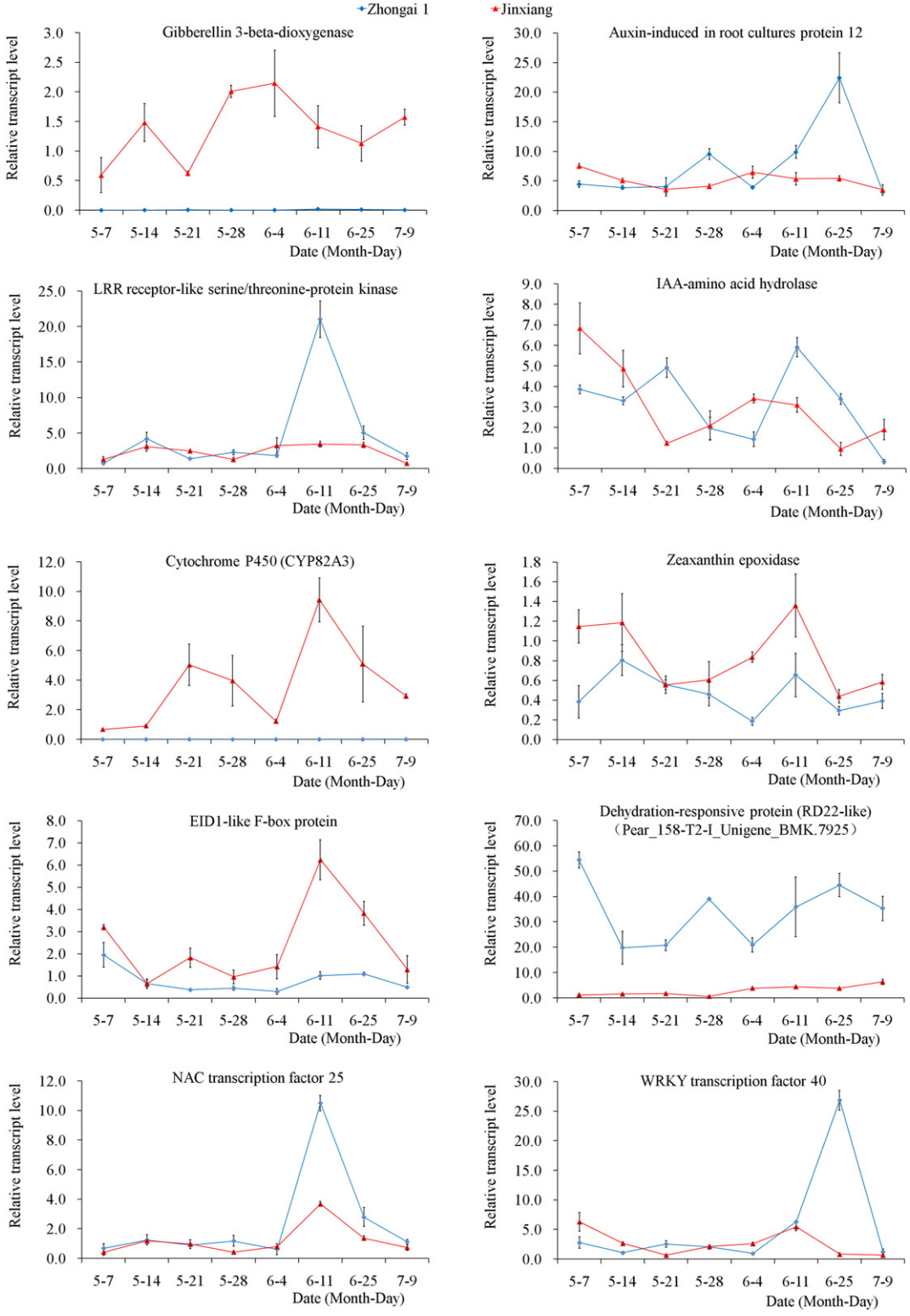

Fig. 5. Transcript abundance of ten putative plant growth related genes as assessed by qPCR. The blue lines refer to 'Zhongai 1' and the red ones to 'Jinxiang'. The bars represent standard error bars ($n = 3$).

from eight public databases (Table 3). Based on GO analysis, 13,772 of the unigenes were categorized into 16 functional groups, belonging to three main GO ontologies: cellular component, molecular function and biological process (Fig. 4). The majority of the unigenes were classified as associated with 'binding activity', 'physiological process', 'enzyme activity', 'cell' or 'cellular process', with only a few classified as associated with 'extracellular', 'development' or 'nutrient reservoir activity'.

3.4. Differentially transcribed genes (DTGs)

Only 234 of the 46,619 unigenes appeared to be differentially transcribed between 'Zhongai 1' and 'Jinxiang'. Of these, 143 were up- and 91 were down-regulated in 'Zhongai 1'. A few genes (17 in 'Zhongai 1' and 13 in 'Jinxiang') were recovered from only one of the two transcriptomes. It was possible to assign a function to all but 44 of the DTGs (14 unigenes could not be annotated, and 30 were annotated as encoding either "uncharacterized protein", "predicted protein" or an "alternative splicing product"). Among the 190 functionally assigned genes, 84 encoded various enzymes (such as transferases, synthases, kinases, dioxygenases, oxidoreductases, oxidases, phosphatases, reductases and anhydrases), 14 encoded a transcription factor (C2H2L, AP2/ERF, MYB, NAC, WRKY or COL), and the other 92 encoded various functional proteins and RNAs (such as ribosomal protein and RNA, cytochrome P450, auxin-induced proteins and dehydration-responsive proteins) (Supplementary Table 3).

In an attempt to identify key genes involved in the dwarf characteristic of 'Zhongai 1', the focus was placed on those DTGs likely to be associated with plant growth (Table 4). Several were implicated in interactions with the phytohormones gibberellin, auxin or brassinosteroid. One gibberellin 3-beta-dioxygenase, a key enzyme in gibberellin synthesis (Poupin et al., 2013), was down-regulated in 'Zhongai 1'. Four auxin associated genes were up-regulated in 'Zhongai 1', three of which likely encoded auxin-induced or auxin-responsive proteins of unknown function, but one IAA-amino acid hydrolase able to release free IAA (auxin) and implicated in the regulation of auxin levels (Le Clere et al., 2002). One of the genes up-regulated in 'Zhongai 1' encoded LRR receptor-like serine/threonine-protein kinase which is highly homologous with brassinosteroid insensitive (BRI) gene. BRI genes are known to be important for plant growth as they regulate brassinosteroid signaling (Li et al., 2002). Four genes (three up- and one down-regulated in 'Zhongai 1') encoded cytochrome P-450s. Cytochrome P-450 mono-oxygenase genes participate in the synthesis and catabolysis of the brassinosteroids and mutations to some of these have been associated with dwarf phenotype (Nomura et al., 2005; Kim et al., 2008; Turk et al., 2003).

Abscisic acid (ABA) and ethylene are two further phytohormones involved with plant growth and development. Three genes associated with ABA were identified as being down-regulated in 'Zhongai 1': two encoded enzymes (respectively, 9-cis-epoxycarotenoid dioxygenase and zeaxanthin epoxidase) participating in ABA synthesis (Iuchi et al., 2001; Thompson et al., 2000), while the third encoded EID1-like F-box protein which may be involved in ABA signaling (Koops et al., 2011). Three genes annotated as ethylene-responsive transcription factors were identified: two were up- and the other down-regulated in 'Zhongai 1'.

Peach scions grafted onto dwarfing rootstocks have been shown to express a lower stem water potential than those grafted onto a non-dwarfing rootstock (Basile et al., 2003). A number of genes associated with water relations were identified as DTGs up-regulated in 'Zhongai 1'. Three encoded dehydration responsive proteins and one gene an aquaporin. The down-regulated DTGs included dehydrin and desiccation-related protein.

Plant growth is dependent on the activity of a range of transcription factors. Two NAC transcription factors were among the DTGs: one was up- and the other down-regulated in 'Zhongai 1'. A further four of the DTGs encoded a WRKY transcription factor; these were all up-regulated in 'Zhongai 1'.

3.5. Real-time quantitative RT-PCR analysis

The transcript abundance of ten of the putatively plant growth related genes was assayed by qPCR as a check on the RKPM-based assessment. The outcome of these assays (Fig. 5) was fully consistent with the conclusions drawn from the RNA-Seq analysis and the correlation coefficient of the two test methods was 0.898 (Fig. 6).

To estimate the connection between the candidate genes and plant growth further, the transcript levels of these ten genes in other dwarf and non-dwarf varieties in the next year were also measured by qPCR. The order of the final new shoot length of the test varieties from short to long is 'Zhongai 4', 'Zhongai 5', 'Zhongai 1', 'Zhongai 3', 'Jinxiang', 'Huasu', 'Zaosu' (Fig. S1). The qPCR results showed that the temporal transcript trend of the ten genes was similar between the two years in 'Zhongai 1' and 'Jinxiang' but without clearly consistent regularity in all the test dwarf or non-dwarf varieties. But there were some sectional consistency for some genes such as lower transcript levels of gibberellin 3-beta-dioxygenase gene and higher transcript levels of dehydration-responsive protein gene in 'Zhongai 1' and 'Zhongai 4', and higher transcript levels of auxin-induced protein, IAA-amino acid hydrolase, LRR receptor-like serine/threonine-protein kinase and WRKY transcription factor gene in 'Zhongai 1' and 'Zhongai 5' (Fig. 7).

3.6. Phytohormone content analysis

Several identified differentially transcribed genes are associated with phytohormones, the levels of some phytohormones were also measured in tender leaves of some dwarf and non-dwarf varieties collected on June 20, 2013. The results indicated that dwarf varieties had a lower level of gibberellin (GA_3) than non-dwarf varieties. In addition, compared with other varieties, 'Zhongai 1' and 'Zhongai 5' had a higher level of IAA and a lower level of ZR, respectively. The levels of ABA in dwarf varieties were lower than that in non-dwarf varieties (Fig. 8).

4. Discussion

New generation sequencing technologies have had a major impact on the characterization of plant transcriptomes, including those of fruit species (Dai et al., 2013; Zhang et al., 2012; Liu et al., 2012; Wang et al., 2014; Feng et al., 2012). The application of RNA-Seq resulted in the two pear varieties in the recognition of over 46,000 unigenes. The greater number (69,393) was identified in the close relative *P. bretschneideri* (Liu et al., 2012), perhaps because gene

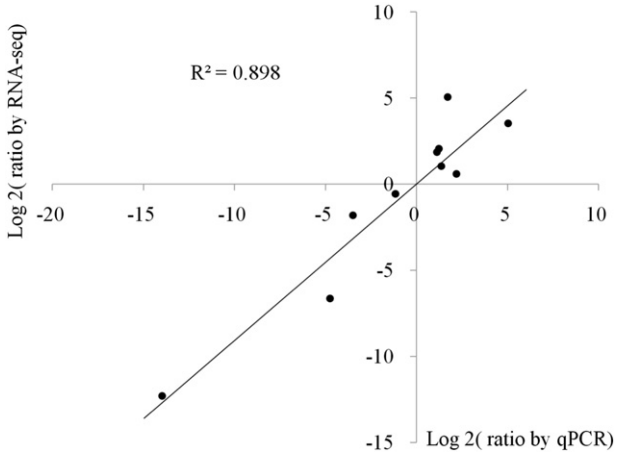

Fig. 6. Transcribed profile comparison of ten putative plant growth related genes by RNA-Seq and qRT-PCR.

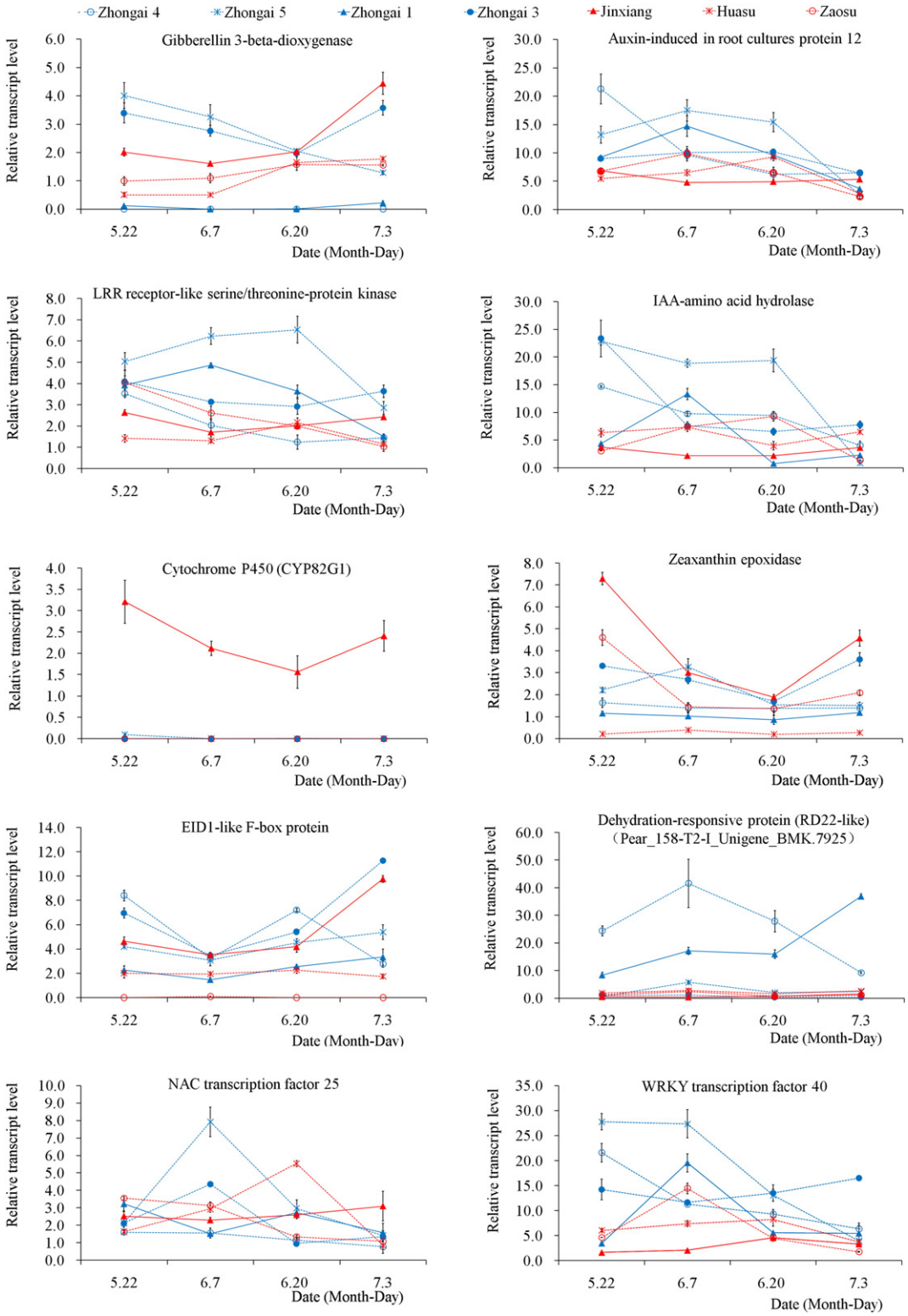

Fig. 7. Transcript abundance of ten putative plant growth related genes in different growth potential varieties by qPCR. The blue lines refer to dwarf and the red ones to non-dwarf varieties. The bars represent standard error bars ($n = 3$).

activity in buds is greater than in the tissue sampled here; more consistent with the present estimate of the pear genome's gene content was the suggestion by Wu et al. (2013) that the number of genes present in *P. bretschneideri* is around 43,000.

Plant growth, although strongly modulated by the environment, is under the genetic control of a large number of genes and transcription factors. Several phytohormones are known to exert a major influenced over plant growth, in particular gibberellin, auxin and the brassinosteroids. Dwarf phenotype in numerous plant species results from the disruption or differential expression of genes encoding the synthesis (Nomura et al., 2005; Kim et al., 2008; Sasaki et al., 2002), metabolism (Dijkstra et al., 2008), signal transduction (Li et al., 2002; Ueguchi-Tanaka et al., 2005) or transport (Ye et al., 2013) of these phytohormones. Here, a number of the DTGs between 'Zhongai

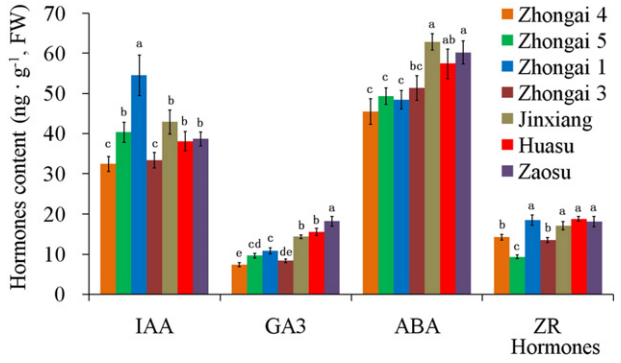

Fig. 8. Phytohormone content of tender leaves from different growth potential varieties. Individual poles represent data values from the mean of 3 samples ± standard error bar ($n = 3$). Different lower-case letters up the bars are significantly different at $P \leq 0.05$ (Duncan multiple range test).

1' and 'Jinxiang' were associated with these growth-related phytohormones. Prominent among these was a gene encoding gibberellin 3-beta-dioxygenase (GA3ox), which was down-regulated in 'Zhongai 1'. GA3ox catalyzes the final step in gibberellin synthesis (Poupin et al., 2013), so its suppression may have restricted the gibberellin content of the 'Zhongai 1' leaves and thereby shortened internodes. The lower levels of GA_3 in dwarf varieties verified this prediction (Fig. 8), but the transcript levels of GA3ox gene were not down-regulated in all test dwarf varieties except 'Zhongai 1' and 'Zhongai 4' (Fig. 7), this indicates that GA3ox is not the only factor impacting the level of GA, and the dwarf mechanism may be different in various dwarf genotypes. Genes encoding three putative auxin-induced or auxin-responsive proteins and one IAA-amino acid hydrolase were all up-regulated in 'Zhongai 1'. In rice, auxin-responsive genes have been shown to be involved in metabolism, transcription, signal transduction and transport (Jain and Khurana, 2009), while IAA-amino acid hydrolase is responsible for the release of auxin and so contributes to the regulation of the plant's auxin content (Le Clere et al., 2002). The up-regulation of these genes in 'Zhongai 1' may therefore have had the effect of increasing the endogenous level of auxin, and through this the synthesis of gibberellins. This corresponded with the high level of IAA in 'Zhongai 1' (Fig. 8). The brassinosteroids regulate the plant growth and development via the formation of a complex which includes the leucine-rich repeat receptor-like protein kinase brassinosteroid-insensitive 1 (BRI1) (Li et al., 2002). The brassinosteroids and gibberellins interact with one another by the former activating BZR1 and the latter inactivating DELLA proteins (Bai et al., 2012). Here, a putative LRR receptor-like serine/threonine-protein kinase was up-regulated in 'Zhongai 1' and 'Zhongai 5'; this gene may encode BRI type protein and play important roles in the plant growth and development of the two varieties. The synthesis and catabolysis of brassinosteroids involve cytochrome P-450 monooxygenases (Nomura et al., 2005; Kim et al., 2008; Turk et al., 2003), and here, four cytochrome P-450 encoding genes were differentially transcribed between 'Zhongai 1' and 'Jinxiang', but the results of qPCR showed that one of the four genes nearly had no transcription in other varieties except in 'Jinxiang' (Fig. 7); so the possibility that they have an effect on plant growth by mediating the level of brassinosteroid present requires experimental validation.

The growth of 'Zhongai 1' shoots ceased earlier than those of 'Jinxiang'. Among the major endogenous factors which are antagonistic to shoot growth are the phytohormones ABA and ethylene levels. Several genes associated with these two phytohormones were among the DTGs identified between 'Zhongai 1' and 'Jinxiang'. The genes encoding two enzymes involved in ABA synthesis (9-cis-epoxycarotenoid dioxygenase and zeaxanthin epoxidase (Iuchi et al., 2001; Thompson et al., 2000)) and one EID1-like F-box protein gene involved in ABA signal transduction (Koops et al., 2011) were all down-regulated in 'Zhongai 1'. A reduced level of their expression could limit the synthesis and ABA signal transduction. The lower levels of ABA in 'Zhongai 1' were consistent with this, but other dwarf varieties also exhibited a low ABA content without down-regulated transcription of these genes (Figs. 7 and 8), this indicates that ABA may be not the growth inhibitor of these dwarf varieties. Ethylene controls many aspects of plant growth and development and participates in a variety of stress responses (Ohta et al., 2000), but is also an inducer of senescence (Li et al., 2013). Here, three putative ethylene-responsive transcription factors were identified, two up-regulated and one down-regulated in 'Zhongai 1'; their potential role in growth may reflect an effect on the timing of senescence of the shoot.

Stem extension is highly dependent on the stem's moisture status (Basile et al., 2003). Several of the DTGs were associated with the plant's water status: these included three putative dehydration responsive protein genes, one putative aquaporin gene, one putative dehydrin gene and one putative desiccation-related protein gene. Of these, the former four were up-regulated and the latter two were down-regulated in 'Zhongai 1'. The qPCR results showed that 'Zhongai 4' had a similar transcript trend of one dehydration responsive protein gene with 'Zhongai 1' (Fig. 7), but whether it could cause plant dwarf also requires experimental validation. The final class of DTGs potentially implicated in stem growth was transcription factors. Two differentially transcribed NAC and four WRKY transcription factors were identified. NAC and WRKY transcription factors (both comprising large families) are prominent in the response to moisture stress and in ABA signaling (Zheng et al., 2009; Rushton et al., 2010, 2012). A test WRKY transcription factor also exhibited high transcript levels in other dwarf varieties, but its function has not been clear.

Although so many putative plant growth related genes were detected, it is uncertain which gene is the most critical and actually plays roles in generating the dwarf phenotype for 'Zhongai 1' and other dwarf genotypes. There were 44 of the DTGs between the two pear varieties without any functional information, which may also actively participate in the growing process of 'Zhongai 1'. According to existing research results, the detected gibberellin 3-beta-dioxygenase, dehydration responsive proteins and WRKY transcription factors must be researched further as chief genes, because their transcribed differentiate between the two pear varieties were throughout the survey period. Therefore, there was no any gene that exhibited a completely consistent transcriptional regularity in all the test dwarf or non-dwarf varieties, this also revealed that the process of plant growth is very complex, the dwarf mechanism of different genotypes may be diverse, and even in the same genotype the dwarf character may be controlled by many factors.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.plgene.2015.08.003.

Acknowledgments

The authors thank professor Zhihong Zhang (College of Horticulture, Shenyang Agricultural University, Shenyang, China) for suggestions and guidance to this paper. Funding by National Natural Science Foundation of China (Grant No. 31101516) was appreciated.

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