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Transcriptome profiling of peanut (*Arachis hypogaea*) gynophores in gravitropic response

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Abstract. Peanut (*Arachis hypogaea* L.) produces flowers aerially, but the fruit develops underground. This process is mediated by the gynophore, which always grows vertically downwards. The genetic basis underlying gravitropic bending of gynophores is not well understood. To identify genes related to gynophore gravitropism, gene expression profiles of gynophores cultured *in vitro* with tip pointing upward (gravitropic stimulation sample) and downward (control) at both 6 and 12 h were compared through a high-density peanut microarray. After gravitropic stimulation, there were 174 differentially expressed genes, including 91 upregulated and 83 downregulated genes at 6 h, and 491 differentially expressed genes including 129 upregulated and 362 downregulated genes at 12 h. The differentially expressed genes identified were assigned to 24 functional categories. Twenty pathways including carbon fixation, aminoacyl-tRNA biosynthesis, pentose phosphate pathway, starch and sucrose metabolism were identified. The quantitative real-time PCR analysis was performed for validation of microarray results. Our study paves the way to better understand the molecular mechanisms underlying the peanut gynophore gravitropism.

Additional keywords: gravitropism, peanut gynophore, transcriptome profiling.

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Introduction

Many plants have remarkable abilities to perceive and respond to a variety of environmental stimuli like light, moisture and gravity, and adapt their physiological development to obtain successful growth. Tropism – the directional growth response – is the most obvious adaptable response, and includes phototropism and gravitropism. This adaptive response allows shoots to grow upwards and makes roots grow downwards. Therefore, shoots can have better light perception for photosynthesis and roots can easily acquire water and minerals in the soil. Among the various stimuli, gravity plays the most important and persistent role in plant growth. Gravitropism is a morphological and developmental process by which plants perceive and respond to gravity. It is a well coordinated regulation process including several sequential steps: gravity perception, molecular signal production, signal transduction, and asymmetric organ growth (Fukaki and Tasaka 1999; Haswell 2003). Several genetic studies have shown that a lack of an endodermal cell layer resulted in gravitropism defects in *Arabidopsis* (Benfey *et al.* 1993; van den Berg *et al.* 1995; Di Laurenzio *et al.* 1996). The results implied the endodermis is the gravity response site. Some models have been proposed to explain the sequential processes in plant gravitropism. First, the starch-statolith theory (Sack 1997) is used to explain gravity perception: according to this theory, starch-filled amyloplasts act as statoliths to sediment in the direction of gravity for plant gravity perception (Weise and Kiss 1999; Kiss 2000). In support of this theory, amyloplast endodermal sedimentation responding to gravity stimulation was determined in flowering shoots of snapdragon (Friedman *et al.* 1998; Philosoph-Hadas *et al.* 2001). Following the gravity perception, the Cholodny-Went hypothesis, was proposed to explain the signal transduction processes. Auxin, typically indole-3-acetic acid (IAA), has been defined as the signal molecule in gravitropism. An asymmetry in auxin distribution leads to unequal cell elongation rate on opposite sides of organ, thus, results in organ bending (Goldsmith 1977; Muday 2008). Auxin gradients identified by radiolabelled auxin and reporter gene systems provided a strong support for this hypothesis (Muday and DeLong 2001; Blancaflor and Masson 2003; Morita and Tasaka 2004).

The genetic basis of plant response to gravity induction has been reviewed (Blancaflor and Masson 2003; Hashiguchi *et al.* 2013). However, only a few studies have attempted to analyse gravity-induced changes at the transcriptional level in a large scale level (Moseyko *et al.* 2002). To date, a few of genes, especially auxin-related genes, have been found to be

associated with gravitropism (Philippar *et al.* 1999; Kato *et al.* 2002; Boccalandro *et al.* 2008; Koprivova *et al.* 2010; Nakamura *et al.* 2011; Band *et al.* 2012). Actin disruption with *MDR* (multidrug resistance)-like genes knockout or latrunculin B drug treatment enhanced gravitropic response, possibly through auxin transporter targeting. Due to auxin's role as a signal molecule in gravitropism, auxin influx carriers *AUX1* family and auxin efflux carriers *AGR*/*PIN* family, specific *MDR*-like proteins all contribute to plant gravity responses. For instance, disruption of *AUX1* and *AGR1*/*EIR1*/*PIN2*/*WAV6* leads to severe defects in root gravitropism, suggesting the essential function of auxin transport during gravitropism. Furthermore, the function of *ARG1*, *ARL2*, *SGR2*, *SGR3*, *and ZIG*/*SGR4* in early phases of gravity signal transduction in shoots has also been characterised. Because of defect to develop a curvature under gravity stimulations, the *gps* (gravity persistence signal) gene was identified for plant gravitropism. The effort to find auxin receptors led to the discovery of auxin response factors (*ARFs*) and *Aux*/*IAA* genes, mutations of which result in gravitropism defects.

Peanut (*Arachis hypogaea* L.) displays a unique growth process called geocarpy during its life cycle. The unique process is that the peanut produces flowers aerially, and subsequently buries the fertilised ovules into the soil for the fruit and seeds to develop and mature underground (Smith 1950; Shushu 1990). The specialised downward growth organ that carries and sows the young seeds into the soil is known as the gynophore. The morphology and anatomy of peanut gynophore have been well established (Ziv 1975; Periasamy 1984; Pattee 1987). Following fertilisation, the gynophore begins to form from an intercalary meristem into a pointed stalk-like structure. Embryo development tentatively stops at this time and the gynophore elongates and bends downward to the ground. When its tip goes into the soil, the gynophore stops elongation and its tip containing the developing embryo begins to swell and elongate on the dorsal side. Finally, the tip forms mature peanut seed in the horizontal orientation.

Gravitropism plays an essential role in the successful completion of peanut gynophore function, and the gravitropic behaviour of peanut gynophore is distinctive (Schwuchow *et al.* 1990). Although the peanut gynophore has structural and anatomical features similar to a typical shoot, it responds to gravity like a root, growing vertically downwards but not like the shoot growing upwards (Jacobs 1947). The positively gravitropic response plays a crucial role in peanut development: the embryo will not develop further unless it is buried underground. The unique gravitropic behaviour of peanut gynophores and its importance in peanut maturation have led many groups to study the underlying mechanisms that regulate the gynophore gravitropism. Auxin, especially IAA, has been well studied in relation to gynophore gravity responses. In 1951, Jacobs has found that IAA located at the distal 10 mm in vertically growing gynophores (Jacobs 1951). Shushu (1990) showed that IAA together with gibberellic acid (GA) near the tip promoted the vertical growth of gynophores. However, the molecular mechanism, especially the genes and pathway related to gravitropic response in peanut gynophores is still unknown.

In this study, a customised NimblerGen oligonucleotide microarray was used to survey the transcriptome profiling of peanut gynophores in gravitropic response for understanding of the molecular mechanism underlying the gynophore gravitropism. The objectives of the present study were to: (1) compare gene expression profiles between peanut gynophores cultured *in vitro* with tips pointing upward and downward; and (2) identify potential genes and corresponding regulation pathways that are involved in gynophore response to gravity stimuli.

Materials and methods

Plant material

A peanut (*Arachis hypogaea* L.) cultivar Yueyou7, provided by Crops Research Institute, Guangdong Academy of Agricultural Sciences (GAAS, Guangzhou, China), was grown normally in experiment field in the spring season of 2010. Healthy peanut plants were selected 12–15 days after flowering. Similar size (4–5 cm length) gynophores from the 2nd or 3rd joints of the plants were cut, rinsed with water, sterilised with 70% ethanol for 5 s and 0.1% hydrargyrum solution for 10 min then rinsed three times, each for a few seconds in sterilised ddH_2O. Gynophore tips of 1 cm in length were cut and vertically inserted in MS medium with downward or upward tipping, and then cultured in the dark at 28°C. After 6 and 12 h, the 6–8 mm fragments from gynophore tips were cut, immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction.

Total RNA extraction

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. To minimise biological and technological variance, for each experiment, RNA samples were extracted and pooled from a total of 100–150 gynophores of each treatment, and each treatment had two biological replicates. RNA samples were extracted from the two biological replicates individually in each treatment and were treated with RNase-free DNase I (Takara) then cleaned up with RNeasy Cleanup Kit (Qiagen, Shanghai, China). The concentration and quality were assessed using Nano Drop ND-100 spectrophotometer (Nano Drop Technologies Inc., Wilmington, DE, USA) and electrophoresis on a 1% agarose gel. The resulting total RNA was stored at −80°C until further analysis.

Microarray hybridisation and data acquisition

To measure gene expression in peanut, a customised NimblerGen (Nimblegen Systems, Inc., Madison, WI, USA) oligonucleotide microarray (4 × 72 K) has been developed. This microarray with 60-mer oligonucleotide probes contains 36 155 peanut unique transcripts (Chen *et al.* 2012). The microarray was used in this study and the hybridisation procedure was performed as described previously by Wang *et al.* (2013). Total RNA (5 μg) was used to synthesise double-stranded cDNA (ds-cDNA) using an Invitrogen SuperScript ds-cDNA synthesis kit. Resultant ds-cDNA was cleaned and fluorescently labelled using Klenow enzyme in accordance with the NimbleGen gene expression analysis protocol. Microarrays were hybridised, stained and conducted at CapitalBio Corporation (Beijing, China) using Roche (Shanghai, China) NimbleGen systems. All microarrays were scanned with a LuxScan 10 KA scanner using LUXSCAN

4.0 software (CapitalBio, Beijing, China). Fluorescence data were processed with SpotData software at CapitalBio Corporation as described previously (Graubert *et al.* 2007). Faint spots were removed with signal intensity less than 400 units after subtraction of the background and the data normalisation was performed at CapitalBio Corporation based on a robust multichip analysis (RMA, CapitalBio). The microarray analysis was employed to evaluate global gene expression gynophores cultured *in vitro* with tip pointing upward and downward at 6 and 12 h, respectively, then comparatively analysed differentially expressed genes (DEGs) between gynophores were cultured *in vitro* with tip pointing upward and downward at the same time. DEGs were defined as the probe sets having a *P*-value < 0.01 and >2-fold changes in at least one of the comparisons.

Gene identification and function analysis

The gene annotation was performed according to the method described by Shi *et al*. (2006). The DEGs were mapped to gene ontology (GO) terms using the molecule function annotation system (MAS, http://bioinfo.capitalbio.com/mas, accessed 20 December 2012) to organise genes into hierarchical categories on the basis of biological process and molecular function.

Quantitative real-time RT–PCR

All qRT–PCRs were performed as described previously (Wang *et al*. 2010). Four microgram of total RNA was reverse transcribed to cDNA using PrimeScript II 1st strand cDNA synthesis kit (Takara, Dalian, China) according to the manufacturer's protocols. Quantitative real-time RT–PCR was performed with SYBR Premix Ex Taq II kit (Takara) in LightCycler 480 instrument (Roche) equipped with Light-Cycler Software ver. 1.5 (Roche) according to the manufacturer's instructions (Alos *et al.* 2008). All the primers specific for selected peanut genes were designed using the Primer ver. 6.0 (Premier Biosoft International, Palo Alto, CA, USA). Standard curves for each genes and *actin* were generated based on serial dilutions of cDNAs reverse-transcribed from one sample in which target genes were expressed at an appropriate level. The actin gene was used as an internal control for calculating relative transcript abundance. The same RNA samples for the microarry analysis were used for real-time PCR analsysis with three technical replicates for each sample. The relative quantification of RNA expression was calibrated using formula $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

In vitro *culture system of peanut gynophore explants responding to gravity stimulation*

Gynophores of similar size from the 2nd or 3rd joints of peanut plants 12–15 days after flowering were cut, sterilised and inserted into MS agar medium for culture in darkness. As shown in Fig. 1, the gynophores with tip pointing downward kept their original growth direction for 12 h. Conversely, the tips of gynophores with tip pointing upward began to bend down at 6 h, and the angle of the bend reaching 90° at 12 h. The observation implies that the peanut gynophores *in vitro* culture system can respond to gravity stimulation in the same way as in peanut plant seedlings, and is valuable for use in studying the gravitropic responses in peanut gynophores.

Identification of potential gravitropic response genes in peanut gynophore transcriptome

To identify differentially expressed genes related to gynophore gravitropic response, a customised NimblerGen onligonucleotide microarray assay was performed to analyse the mRNA expression profiles of gynophores cultured *in vitro* with tip pointing upward (gravitropic stimulation sample) and downward (natural growth control). The results showed that 28 029 genes, accounting for 77.52% of all genes in the microarray, were expressed. The remaining 8129 genes (22.48%) were not expressed in all samples – these were excluded in subsequent analyses.

Scatter plots could be used to show the gene expression difference between the two samples in microarray data analysis. In Fig. 2, *x*-axis and *y*-axis represent the hybridisation signal intensity of gynophores cultured *in vitro* with tip pointing downward (control) and upward (gravitropic stimulation sample) respectively. Each point in the figure indicates the hybridisation signal value of one gene. The red and green points represent upregulated expressed genes (ratio >2) and downregulated expressed genes (ratio <0.5) respectively. The black points represent genes that were not differentially expressed (ratio >0.5 and ratio <2). Both red and green points represent significantly differentially expressed genes that were further analysed. The results showed that there was a substantial number of differentially expressed genes between the control gynophores and the gravitropic stimulated gynophores (Fig. 2).

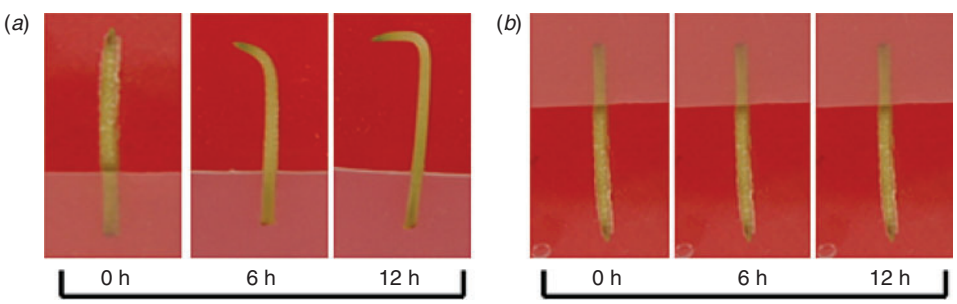

Fig. 1. Peanut gynophores planted vertically *in vitro* with the tip pointing upward (*a*) and downward (*b*) in MS medium at 0, 6 and 12 h respectively.

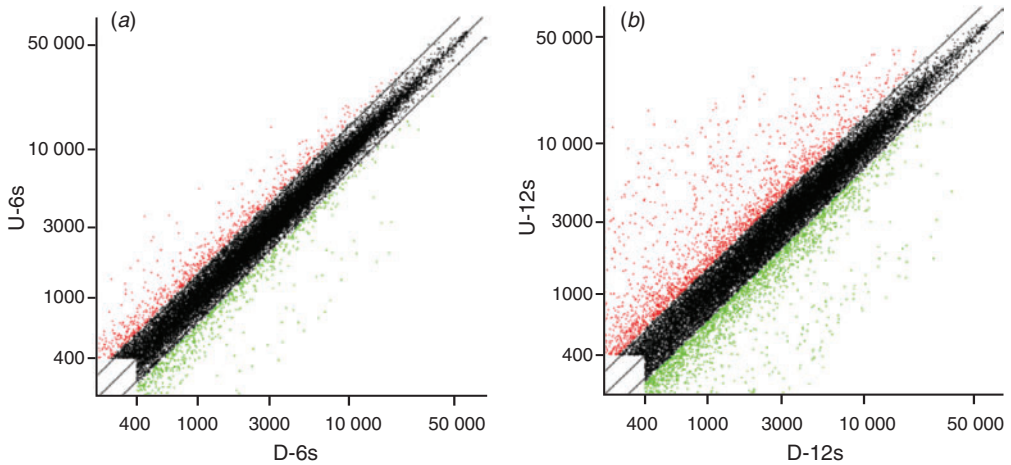

Fig. 2. Scatter plots of peanut gynophores grown *in vitro* with tip pointing upward and downward at 6 h (*a*) and 12 h (*b*). Intensity D-6s on the *x*-axis represents the fluorescence intensity of peanut gynophores grown *in vitro* with tip downward for 6 h. Intensity U-6s on the *y*-axis represents the fluorescence intensity of peanut gynophores grown *in vitro* with tip upward for 6 h. Intensity D-12s on the *x*-axis represents the fluorescence intensity of peanut gynophores grown *in vitro* with tip downward for 12 h. Intensity U-12s on the *y*-axis represents the fluorescence intensity of peanut gynophores grown *in vitro* with tip upward for 12 h. The red points represent upregulated expressed genes (ratio >2). The green points represent downregulated expressed genes (ratio <0.5). The black points represent gene expression almost indifferently (ratio >0.5 and ratio <2).

In gravitropic stimulated gynophores (with tip pointing downward), the number of upregulated genes was close the number of downregulated genes at 6 h, whereas the number of upregulated genes was more than that of the downregulated genes at 12 h (Fig. 2). According to the 2.0 : 0.5 (ratio >2.0 or ratio <0.5) screening standard, there were 174 differentially expressed genes including 91 upregulated genes and 83 downregulated ones in gravitropic stimulated gynophores after *in vitro* culture for 6 h (Figs 2, 3). After *in vitro* culturing for 12 h, there were 491 differentially expressed genes including 129 upregulated genes and 362 downregulated ones in gravitropic stimulated gynophores (Figs 2, 3). Among them, one gene (AHTC1034254, Peroxidase 4-like protein from *Glycine max*) was upregulated and 4(AHTC1006412, AHTC1010360, AHTC1036262 and AHTC1036618) downregulated at both 6 and 12 h in gravitropic stimulated gynophores after *in vitro* culture (Fig. 3; Table 1). In gravitropic stimulated gynophores, four genes (AHTC1003533, AHTC1004729, AHTC1012222 and AHTC1034957) were upregulated after *in vitro* culture for 6 h then downregulated after *in vitro* culture for 12 h (Fig. 3; Table 1). And one gene (AHTC1014746) was downregulated after *in vitro* culture for 6 h then upregulated after 12 h of *in vitro* culture (Fig. 3; Table 1). As shown in Fig. 3, the overall analysis of microarray data revealed that there were 655 differentially expressed genes including 219 upregulated genes and 441 downregulated ones in gravitropic stimulated gynophores after *in vitro* culture for 6 or 12 h. All upregulated and downregulated genes of gynophores grown *in vitro* with tip pointing upward at 6 and 12 h were listed in Tables S1 and S2 (available as Supplementary Material to this paper) respectively. The top 20 differently expressed genes of gynophores grown

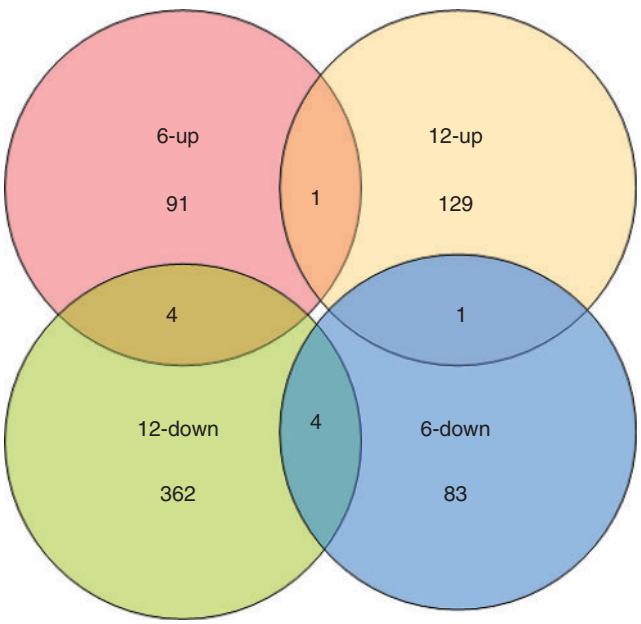

Fig. 3. Venn diagram depicting the number of differentially expressed genes in peanut gynophores grown *in vitro* with tip upward and downward. The red circle represents the number of upregulated expressed genes in peanut gynophores grown *in vitro* with tip upward at 6 h. The yellow circle represents the number of upregulated expressed genes in peanut gynophores grown *in vitro* with tip point upward at 12 h. The blue circle represents the number of downregulated expressed genes in peanut gynophores grown *in vitro* with tip pointing upward at 6 h. The green circle represents the number of downregulated expressed genes in peanut gynophores grown *in vitro* with tip pointing upward at 12 h.

Table 1. Identity and known functions of 10 differentially expressed genes of gynophore cultured *in vitro* with tip pointing upward at 6 and 12 h in response to gravitropic stimulation

Probe set ID	Hit name	E-value	Gene description	Species	Expression status
AHTC1034254	I1JHK1	1E-69	Peroxidase 4-like protein	*Glycine max*	Upregulated at 6 and 12 h
AHTC1003533	Q9MB42	8E106	Beta-amyrin synthase	*Glycyrrhiza glabra*	Upregulated at 6 and downregulated at 12 h
AHTC1004729	I1MRA7	8E-179	Amine oxidase	*Glycine max*	Upregulated at 6 and downregulated at 12 h
AHTC1012222	Q8VX11	1E-82	Diphosphonucleotide phosphatase 1	*Lupinus luteus*	Upregulated at 6 and downregulated at 12 h
AHTC1034957	G7KBW6	2e-76	Laccase	*Medicago truncatula*	Upregulated at 6 and downregulated at 12 h
AHTC1014746	[A]	–	–	–	Downregulated at 6 and upregulated at 12 h
AHTC1006412	I3S2G7	1E-83	Uncharacterised protein	*Lotus japonic*	Downregulated at 6 and 12 h
AHTC1010360	G7IFJ0	2E-48	Ethylene-responsive transcription factor	*Medicago truncatula*	Downregulated at 6 and 12 h
AHTC1036262	Q4VT47	2E-47	RD22-like protein	*Vitis vinifera*	Downregulated at 6 and 12 h
AHTC1036618	I1 L776	5E-104	Uncharacterised protein	*Glycine max*	Downregulated at 6 and 12 h

[A]No hits found.

Table 2. Top 20 differently expressed genes of gynophores grown *in vitro* with tip pointing upward at 6 h

Probe set ID	Hit name	E-value	Gene description	Species
AHTC1002058	Q39448	5.00E-48	Specific tissue protein 2 (fragment)	*Cicer arietinum*
AHTC1002229	C5I848	7.00E-126	12-oxophytodienoic acid 10,10-reductase	*Astragalus sinicus*
AHTC1002760	B7FHT3	7.00E-75	Putative uncharacterised protein	*Medicago truncatula*
AHTC1003375	Q9ZWQ5	0	UDP-glycose:flavonoid glycosyltransferase	*Vigna mungo*
AHTC1004729	Q70EW1	1.00E-142	Diamine oxidase (copper amino oxidase)	*Glycine max*
AHTC1005973	B9S545	4.00E-115	Putative uncharacterised protein	*Ricinus communis*
AHTC1009432	C6T013	1.00E-46	Non-specific lipid-transfer protein	*Glycine max*
AHTC1011526	P39866	1.00E-130	Nitrate reductase (NADH) 2	*Phaseolus vulgaris*
AHTC1012231	A5BDB9	4.00E-39	Putative uncharacterised protein	*Vitis vinifera*
AHTC1015381	C6TEH1	1.00E-34	Putative uncharacterised protein	*Glycine max*
AHTC1015595	C6T3H3	9.00E-18	Putative uncharacterised protein	*Glycine max*
AHTC1028353	A2SY66	1E-42	Vicianin hydrolase (fragment)	*Vicia angustifolia*
AHTC1030550	B9HQH6	1.00E-36	Predicted protein	*Populus trichocarpa*
AHTC1031331	B7FKQ0	5.00E-40	Putative uncharacterised protein	*Medicago truncatula*
AHTC1032035	B7FJN1	1.00E-46	Putative uncharacterised protein	*Medicago truncatula*
AHTC1032263	B9MTJ6	2.00E-43	Beta-alanine n-methyltransferase related	*Populus trichocarpa*
AHTC1032481	Q45NI5	6.00E-53	Monooxygenase (fragment)	*Medicago sativa*
AHTC1033068	C6T3Y8	2.00E-09	Putative uncharacterised protein	*Glycine max*
AHTC1034957	B9HP74	4.00E-64	Predicted protein	*Populus trichocarpa*
AHTC1035241	C6SY68	8.00E-15	Non-specific lipid-transfer protein	*Glycine max*

in vitro with tip pointing upward at 6 and 12 h were listed in Tables 2 and 3 respectively.

Validation of the microarray results using quantitative RT–PCR

To confirm the microarray results, five differentially expressed genes were randomly selected for real-time RT–PCR analysis. The primers were designed on the basis of expressed sequence tags (ESTs) corresponding to the microarray hybridisation probes (Table 4). The expression level of *actin* gene was used as an internal control. All real-time PCR reactions were repeated biologically two times and repeated technologically three times. The changes of gene expression level among gynophores cultured *in vitro* with tip pointing upward and downward both at 6 and 12 h were examined. The results showed that, with the exception of AHTC1004450 (auxin efflux carrier), the remaining four genes showed similar patterns with real-time RT–PCR and microarray analyses. Although the extent of expression was slightly different between microarray and real-time RT–PCR, the expression trends were the same between two analysing systems (Table 4).

Functional classification of potential gravitropic response genes in peanut gynophores

To identify biological process of differentially expressed genes, gene ontology (GO) analysis was conducted using molecule annotation system (MAS, http://bioinfo.capitalbio.com/mas3, accessed 20 December 2012). The GO analysis obtained with the annotation procedure through homology analysis generated a concise functional annotation. As shown in Fig. 4, the known differentially expressed genes were classified into 24 functional categories (Fig. 4). These differentially expressed genes were mainly distributed in cellular process (13.5%), physiological process (13.5%), catalytic activity (10.6%), metabolism (8.7%), cell (6.4%), cell part (6.4%), response to stimulus (5.7%); other items (4.7%), binding (4.6%) and organelle

Table 3. Top 20 differently expressed genes of gynophores grown *in vitro* with tip pointing upward at 12 h

Probe set ID	Hit name	E-value	Gene description	Species
AHTC1000080	Q45W72	3.00E-19	Metallothionein-like protein	*Arachis hypogaea*
AHTC1000102	A4PF88	0	Repressor of silencing 1	*Nicotiana tabacum*
AHTC1000102	A4PF88	0	Repressor of silencing 1	*Nicotiana tabacum*
AHTC1000379	C6TMG1	0	Fructose-bisphosphate aldolase	*Glycine max*
AHTC1000379	C6TMG1	0	Fructose-bisphosphate aldolase	*Glycine max*
AHTC1000464	Q2PK12	1.00E-71	Actin depolymerising factor-like protein	*Arachis hypogaea*
AHTC1000464	Q2PK12	1.00E-71	Actin depolymerising factor-like protein	*Arachis hypogaea*
AHTC1000548	C6T529	1.00E-49	Ribulose bisphosphate carboxylase small chain	*Glycine max*
AHTC1000548	C6T529	1.00E-49	Ribulose bisphosphate carboxylase small chain	*Glycine max*
AHTC1001087	B6ZK00	4.00E-114	Peroxisomal biogenesis factor 11 family protein	*Glycine max*
AHTC1001087	B6ZK00	4.00E-114	Peroxisomal biogenesis factor 11 family protein	*Glycine max*
AHTC1001326	C6TH42	5.00E-34	Putative uncharacterised protein (Fragment)	*Glycine max*
AHTC1001685	A5HIJ1	3.00E-156	Cysteine protease Cp1	*Actinidia deliciosa*
AHTC1001685	A5HIJ1	3.00E-156	Cysteine protease Cp1	*Actinidia deliciosa*
AHTC1001804	B9T3S5	0	Cyclic nucleotide-gated ion channel, putative	*Ricinus communis*
AHTC1002079	B0M197	2.00E-35	Peroxisomal voltage-dependent anion-selective channel protein	*Glycine max*
AHTC1002171	C6TGV1	2.00E-179	3-isopropylmalate dehydrogenase	*Glycine max*
AHTC1002176	A9P7U9	0	Predicted protein	*Populus trichocarpa*
AHTC1002307	Q9FVD6	0	Ser/Thr specific protein phosphatase 2A A regulatory subunit β isoform	*Medicago varia*

(4.3%) (Fig. 4). To identify the metabolic pathways of these potential gravitropic genes, the MAS was employed to assign the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the differential expression genes. Twenty top pathways (all q-values <0.05) are listed in Table 5, which includes carbon fixation, aminoacyl-tRNA biosynthesis, pentose phosphate pathway, and starch and sucrose metabolism.

Discussion

In this study, a high-density peanut gene microarray was employed to compare the expression profiles of peanut gynophores cultured *in vitro* with tip pointing upward and downward at both 6 and 12 h. The collection of gravity responsive genes provides a valuable resource for understanding the molecular mechanism underlying the peanut gynophore gravitropism. The differentially expressed genes under gravity stimuli were determined and further confirmed for some of them by quantitative RT–PCR. Potential gravitropic genes and their putative functions presented in this study can help our understanding of the biological processes involved in gravitropism. The microarray data also provided clues for identification of potential molecular markers in peanut gynophores, especially in their gravity perception and response. The MAS assignments demonstrate that except aminoacyl-tRNA biosynthesis, and ribosome 2 translation pathways, all other pathways are metabolic pathways, which potentially functioned in the gynophore gravitropism. Among the metabolism pathways identified, carbohydrate metabolism and amino acid metabolism are the two major pathways, following with energy metabolism, biosynthesis of other secondary metabolites, metabolism of terpenoids and polyketides, glycan biosynthesis and metabolism, and nucleotide metabolism. Similar to our results, transcriptome analysis in *Arabidopsis* exposed to gravity stimulation also revealed that metabolism is one of major gene groups responding to gravitropism (Kimbrough *et al.* 2004; Kittang *et al.* 2004) Among the metabolism pathways identified in this study, energy metabolism was also found to function in plants under gravitropic stimulation according to proteomic analysis (Azri *et al.* 2009; Herrera *et al.* 2010). In *Arabidopsis*, secondary metabolism genes were altered by magnetically induced hyper- and microgravity at both transcription and translation levels (Manzano *et al.* 2012; Herranz *et al.* 2013). Proteome analysis of poplar stems exposed to gravitropic stimulation has led to determination of several pathways also identified in our study, including nucleotide metabolism, carbohydrate metabolism, photosynthesis and primary metabolism (energy) (Azri *et al.* 2009). Millar and Kiss (2013) also showed the metabolic profile of *Arabidopsis thalianna* during gravitropism and phototropism, and identified carbohydrate metabolism, secondary metabolism and amino acid biosynthesis as the three main responding pathways. They are all included in identified pathways responding to gravitropism in the present study. Consistent with previous results, carbohydrate metabolism, energy metabolism, biosynthesis of other secondary metabolites, and nucleotide metabolism were also identified in our results. Additionally, several novel pathways including aminoacyl-tRNA biosynthesis, ribosome, amino acid metabolism, metabolism of terpenoids and polyketides, glycan biosynthesis and metabolism were identified for the first time to function in gravitropic responses.

Carbohydrate metabolism in peanut gynophore gravitropism

Five carbohydrate metabolism pathways, including pentose phosphate pathway (PPP), pyruvate metabolism, starch and sucrose metabolism, glyoxylate and dicarboxylate metabolism, fructose and mannose metabolism, were discovered in peanut gynophores in response to gravity in our microarray assay. Metabolism of glucose through the pentose phosphate pathway is one of the main antioxidant cellular defence systems (Riganti *et al.* 2012). It supplies cells nucleic acid and lipid biosynthesis

Table 4. Comparison of expression levels (log2 transformation) of selected target genes between qRT–PCR and microarray data

Probe no.	Gene description	Forward primer (5′-3′)	Reverse primer (5′-3′)	Length (bp)	Primer efficiency	Fold change (microarray)		Fold change (qRT–PCR)	
						6 h	12 h	6 h	12 h
AHTC1024090	Calcium-activated outward-rectifying potassium channel	CACCAGTCTTGATGAGGAGGC	CGTGTCTTTGCCCTGAATCTT	121	1.868	0.624	0.954	1.205	0.746
AHTC1035324	Ubiquitin-protein ligase	AGTGACCTTGATTCATTAGCCG	CACCAGTCTTGATGAGGAGGC	73	1.955	1.2964	0.260	1.734	1.341
AHTC1000339	Tubulin α chain	CGTGTCTTTGCCCTGAATCTT	CGTGTCTTTGCCCTGAATCTT	215	1.901	1.169	1.158	1.788	1.349
AHTC1004450	Auxin efflux carrier (Fragment)	GCTTATCCGTAATCCCAACACT	CATTCCAAGACCAGCATCAGA	136	1.984	0.4189	1.037	1.734	1.299
AHTC1035268	Phosphoinositide-specific phospholipase C	GAAGCGAAACTTAGAGCGATG	TTCCTCCTCCGTCGCTGT	204	2.052	1.343	0.895	1.450	0.980

with NADPH, ribose 5-phosphate, and glycolytic intermediates for defence against oxidative stress. Recently, the PPP was determined to also play roles in cell cycle, apoptosis, invasion, angiogenesis, and respond to anti-tumour therapy (Riganti *et al.* 2012). Pentose level is responsive to gravitropism in cell walls of wheat coleoptiles (Wakabayashi *et al.* 2003). Pyruvate is the major product of glycolysis. Metabolism of pyruvate is complicated and involves various cycles to generate NADPH, acetyl-CoA (Sugden and Holness 2011). It has a broad application in food industry. Disorder of the pathway is associated with human diseases. Starch and sucrose metabolism contains two major pathways. First, sucrose is converted to starch for carbohydrate storage in the amyloplasts (Czyzewska and Marczewski 2009). Second, starch may be degraded into glucose and maltose either hydrolytically or phosphorolytically for energy within the plant (Zeeman *et al.* 2007). In cereal grass shoots, starch statoliths function as the gravisensors (Song *et al.* 1988, and in excised wheat leaves, alteration of gravity dramatically influence carbohydrate metabolism by reducing synthesis of fructose, sucrose, starch and fructan (Obenland and Brown 1994). In plant gravitropism, the widely accepted model is the starch-statolith hypothesis: that sediment of starch filled amyloplasts converts gravity into subsequent cellular signalling for plant growth (Sack 1997). The mutant experiments in roots, hypocotyls and stems showing reduced gravitropism provide supporting evidence for the starch-statolith theory (Kiss *et al.* 1996, 1997; Weise and Kiss 1999). Glyoxylate and dicarboxylate metabolism refers to the set of glyoxylate or dicarboxylates involving reactions. Among these, the glyoxylate cycle functions in the biosynthesis of carbohydrates from lipids. Fructose-2, 6-bisphosphate regulates the balance between glycolysis and gluconeogenesis (Hampp *et al.* 1997). In etiolated corn and pea, mannose can increase gravitropic bending by 2-deoxy-D-glucose, an inhibitor of protein glycosylation, and callose deposition may be a biochemical component of gravitropism in plant shoots (Jaffe and Leopold 1984).

Other metabolism in peanut gynophore gravitropism

Other metabolism pathways identified in our study include energy metabolism, biosynthesis of other secondary metabolites, metabolism of terpenoids and polyketides, glycan biosynthesis and metabolism, and nucleotide metabolism. Carbon fixation is one of the major energy metabolism pathways. Plant photosynthesis is the typical carbon fixation process, which serves to transform energy from sunlight into chemical compound (Zelitch 1975; Ducat and Silver 2012). Ortiz *et al.* (2000) revealed that hypergravity inhibited CO_2 fixation during photosynthesis through decreasing chlorophyll *a*/*b* ratios. Carbon fixation increased under microgravity environment (Salisbury 1984; Adamchuk *et al.* 1999). In addition to energy production, the mitochondrial oxidative phosphorylation system also plays important roles in free radical generation and apoptosis (Hüttemann *et al.* 2007). Altered gravity changes the mitochondrial structure and increases both of the size and activity of mitochondria in cells (Hüttemann *et al.* 2007). Flavonoid biosynthesis and phenylpropanoid biosynthesis are the two pathways found in the biosynthesis of

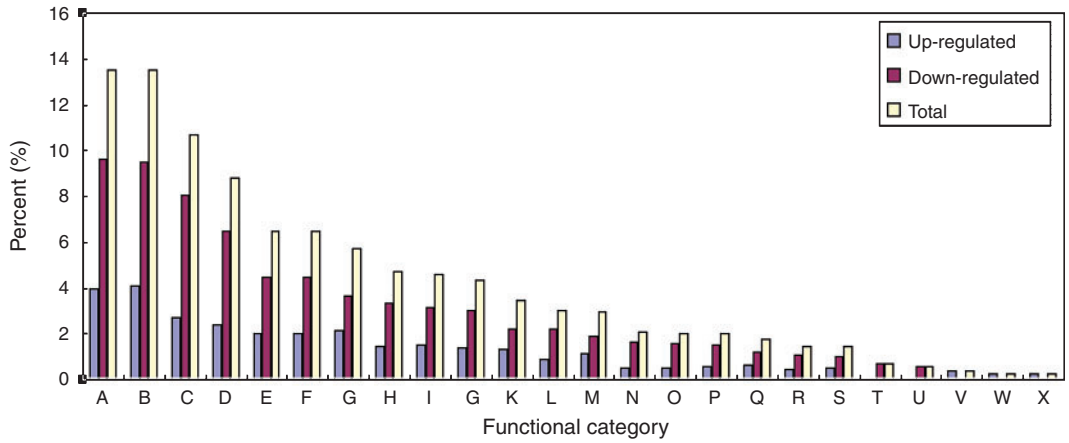

Fig. 4. Functional classification of differentially expressed genes in gynophores cultured *in vitro* with tip pointing upward at 6 and 12 h in response to gravitropic stimulation. A, cellular process; B, physiological process; C, catalytic activity; D, metabolism; E, cell; F, cell part; G, response to stimulus; H, other items; I, binding; G, organelle; K, biological regulation; L, organelle part; M, regulation of biological process; N, localisation; O, establishment of localisation; P, macromolecular complex; Q, developmental process; R, multicellular organismal process; S, transporter activity; T, envelope; U, reproduction; V, interaction between organisms; W, membrane-enclosed lumen; X, transcription regulator activity.

Table 5. Representative peanut gynophore gravitropic-preferred metabolic pathways identified by the molecule annotation system (MAS)
Pathways with a *P*-value higher than that of 'pyrimidine metabolism' are not listed consecutively

Kyoto Encyclopedia of Genes and Genomes pathways	No. of identified differentially expressed genes	*P*-value	*q*-value
Carbon fixation	5	1.38E-07	2.76E-07
Aminoacyl-tRNA biosynthesis	4	1.05E-06	9.04E-07
Ribosome	6	1.36E-06	9.04E-07
Oxidative phosphorylation	4	3.63E-05	1.81E-05
Pentose phosphate pathway	3	7.97E-05	3.19E-05
Pyruvate metabolism	3	1.64E-04	4.83E-05
Flavonoid biosynthesis	2	1.69E-04	4.83E-05
Starch and sucrose metabolism	3	2.93E-04	7.33E-05
Carotenoid biosynthesis - General	2	5.85E-04	1.16E-04
Carotenoid biosynthesis - Organism-specific	2	5.85E-04	1.16E-04
Cyanoamino acid metabolism	2	6.40E-04	1.16E-04
N-glycan biosynthesis	2	8.20E-04	1.37E-04
Glyoxylate and dicarboxylate metabolism	2	0.001167	1.78E-04
Glutamate metabolism	2	0.001244	1.78E-04
Phenylpropanoid biosynthesis	3	0.002303	3.06E-04
Phenylalanine, tyrosine and tryptophan biosynthesis	2	0.002445	3.06E-04
Fructose and mannose metabolism	2	0.003256	3.62E-04
Glycine, serine and threonine metabolism	2	0.003256	3.62E-04
Glutathione metabolism	2	0.003256	3.62E-04
Pyrimidine metabolism	2	0.005502	5.50E-04

other secondary metabolites. Flavonoids are the major pigments of red, blue, and purple in plants and constitute a diverse family including the chalcones, flavones, flavonols, flavandiols, anthocyanins, and condensed tannins (Winkel-Shirley 2001). Both microgravity exposure in spaceflight and clinorotation conditions resulted in enhanced accumulation of flavonoids and isoflavone glycosides in soybean seedlings (Levine *et al.* 2001). Gravity stimuli induced flavonoid accumulation in the site of gravity perception, i.e. columella cells (Levine *et al.* 2001). In addition, deficiency of flavonoid resulted in delayed gravitropism in *Arabidopsis thaliana* (Buer and Muday 2004). Aminocyclopropane carboxylic acid reduced flavonoid synthesis thereby inhibited gravity response in *A. thaliana* (Buer *et al.* 2006). These results suggest that flavonoid is one of the key components in the plant gravitropic regulation signalling. The way of flavonoid functions in gravity response was determined through regulation of auxin fluxes in roots (Santelia *et al.* 2008).

Gene expression in peanut gynophore gravitropism

Among the 10 differentially expressed genes at both 6 and 12 h in this study, three encode uncharacterised proteins and one is not found in the database based on sequence similarity analysis using BLASTX algorithm. Here, we will focus on other six proteins identified. Beta-amyrin synthase is a key enzyme in the plant signal pathway for secondary metabolites triterpenoid saponins production (Yendo *et al.* 2010), which is widely used pharmacological medicine for antiplatelets, antitumorals and immunoadjuvants. In *Glycyrriza uralensis*, beta-amyrin synthase was found only to be expressed in underground parts, where its expression level in the root tip is much higher than in the rootstock. Since the root tip is the responding part to gravity stimulation, its expression in this part indicates its possible role in gravitropism (Liu and Liu 2012). The upregulated expression at 6 h and downregulated expression at 12 h of beta-amyrin synthase indicates its potential role in responding to gravitropic signals at the early phase. In plants, amine oxidase includes diamine oxidases (DAO) and FAD-binding polyamine oxidases (PAO), which oxidatively catabolised polyamines and function in plant development, stress defence (Wimalasekera *et al.* 2011). As early as 1965, plant amine oxidase has been demonstrated to catalyse the oxidation of tryptamine and produce 3-indolylacetaldehyde (IAc), which may function as the precursor of 3-indolylacetic acid (IAA) (Clarke and Mann 1957). This may explain how amine oxidase plays a role in gravitropism. Further, it has been reported that the expression of animal-derived semicarbazide-sensitive amine oxidase (SSAO; E.C.1.4.3.6.) in *Escherichia coli* BL21 is dramatically influenced by simulated microgravity (Zhang *et al.* 2012). The result indicates amine oxidase's possible roles in responding to gravity. The expression alteration of amine oxidase in our study suggests that amine oxidase may be involved in the early stage of gravitropism signalling. diphosphonucleotide phosphatase 1 (PPD1) was first identified from yellow lupin (Olczak *et al.* 2000). It belongs to a novel group of metallophosphatases comprising PPD1, PPD2, PPD3 and PPD4. PPD1 can specifically hydrolyse diphosphonucleotides and phosphodiesters (Olczak *et al.* 2009). The mRNA expression of PPD1 accumulates at stems and leaves in *yellow lupin* (Olczak and Olczak 2002). However, so far the function of PPD1 in plant response to gravity has not been reported. The findings in our study suggest that PPD1 may be involved in plant gravitropic regulation. Laccases are another type of oxidase found in our study. They are copper-containing phenolic compounds metalloxidases (Jeon *et al.* 2012). Laccases are involved in fungal spore pigmentation, tobacco protoplasts regeneration, and the formation and degradation of organic substances like lignin (Mayer and Staples 2002; Claus 2003). Both auxin and laccase may be involved in root meristem formation in *Medicago truncatula* (Eyles *et al.* 2013). In white-rot fungus *Funalia trogii* (*Trametes trogii*), the production of auxin IAA and the activity of peroxidase-laccase were revealed to have a negative correlation (Ünyayar *et al.* 2001). The results in this study suggest that peroxidase and laccase may degrade IAA, and IAA may, in turn, repress activities of peroxidase and laccase. The expression alteration of laccase in different time points during gynophore gravitropism indicates that it may respond to gravity stimulation through its regulation of auxin concentration. Ethylene is well known as the inducer of fruit ripening. Moreover, it regulates a variety of metabolic and developmental processes including seed germination, organ senescence in plants (Bleecker and Kende 2000). Ethylene-responsive transcription factor belongs to the ethylene-responsive element binding factors (ERFs), which are characterised by the ERF domain, a highly conserved DNA binding domain (Fujimoto *et al.* 2000). It has been proposed that ethylene signalling-induced activation of the ethylene response factor 1 (ERF1) gene is associated to the inhibition of auxin signal response (Díaz and Alvarez-Buylla 2006). The downregulation of ethylene-responsive transcription factor at both 6 and 12 h indicates the increasing auxin response, which will enhance the gravitropism of peanut gynophore cultured *in vitro* with tip pointing upward. RD22-like protein is one subfamily of the BURP-domain protein family, which featured as the conserved BURP domain at the *C*-terminal. RD22-like protein is a known stress response gene in plants. Drought treatment induced RD22 expression in *Arabidopsis*, and then RD22 has often been used as drought stress marker (Yamaguchi-Shinozaki and Shinozaki 1993). Also, abscisic acid (ABA) and salt stress can induce RD22 expression (Iwasaki *et al.* 1995; Abe *et al.* 1997). Recently, it has been reported that transgenic expression of a RD22-like protein (GmRD22) can reduce salinity and osmotic stress in plants (Wang *et al.* 2012). Both downregulated expression of RD22-like protein at 6 and 12 h in the gynophores with pointing upward indicate its opposite role in gravitropism, but how the RD22-like protein functions in gravitropism needs further investigation.

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