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ORIGINAL ARTICLE

Improvement of soybean transformation via *Agrobacterium tumefaciens* methods involving α-aminooxyacetic acid and sonication treatments enlightened by gene expression profile analysis

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Abstract

Key message **Antagonists and sonication treatment relieved the structural barriers of *Agrobacterium* entering into cells; hindered signal perception and transmission; alleviated defense responses and increased cell susceptibility to *Agrobacterium* infection.**

Abstract Soybean gene expression analysis was performed to elucidate the general response of soybean plant to *Agrobacterium* at an early stage of infection. *Agrobacterium* infection stimulated the PAMPs-triggered immunity (BRI1, BAK1, BZR1, FLS2 and EFR) and effector-triggered immunity (RPM1, RPS2, RPS5, RIN4, and PBS1); up-regulated the transcript factors (WRKY25, WRKY29, MEKK1P, MKK4/5P and MYC2) in MAPK pathway; strengthened the biosynthesis of flavonoid and isoflavonoid in the second metabolism; finally led to a fierce defense response of soybean to *Agrobacterium* infection and thereby lower transformation efficiency. To overcome it, antagonist α-aminooxyacetic acid (AOA) and sonication treatment along with *Agrobacterium* infection were applied. This novel method dramatically decreased the expression of genes coding for F3′H, HCT, β-glucosidase and IF7GT, etc., which are important for isoflavone biosynthesis or the interconversion of aglycones and glycon; genes coding for peroxidase, FLS2, PBS1 and transcription factor MYC2, etc., which are important components in plant–pathogen interaction; and genes coding for GPAT and α-L-fucosidase, which are important in polyesters formation in cell membrane and the degradation of fucose-containing glycoproteins and glycolipids on the external surface of cell membrane, respectively. This analysis implied that AOA and sonication treatment not only relieved the structural membrane barriers of *Agrobacterium* entering into cells, but also hindered the perception of 'invasion' signal on cell membrane and intercellular signal transmission, thus effectively alleviated the defense responses and increased the cell susceptibility to *Agrobacterium* infection. All these factors benefit the transformation process; other measures should also be further explored to improve soybean transformation.

Keywords Soybean · Transformation · *Agrobacterium tumefaciens* · Gene expression profiles · α-Aminooxyacetic acid · Sonication

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Introduction

Soybean [*Glycine max* (L.) Merrill.] transformation via *Agrobacterium tumefaciens* mediated methods is difficult, although a few reports claimed high efficiency ranging

from 9.4 to 26.2 % (Olhoft et al. 2001, 2003; Dang and Wei 2007), these milepost workings in soybean genetic transformation were scarcely successfully replicated by other laboratory (Atif et al. 2013). Besides the strong dependence of transformation operation on personal practice, the lack of better understanding of transformation mechanism increased the difficulties of success. The mechanism underlying *Agrobacterium*-mediated transformation had been explored in both bacterium and plants (Gelvin 2010; Pitzschke and Hirt 2010; Liu et al. 2010; Lacroix and Citovsky 2013; Subramoni et al. 2014; Shi et al. 2014).

Agrobacterium infection is accompanied by pathogen defense of the host and the counter-defense launched by *Agrobacterium.* In the context of pathogen defense, the mitogen-activated protein kinase MPK3 has merited special attention in defense signaling pathway (Nakagami et al. 2005; Pitzschke et al. 2009). MPK3 phosphorylates the *Arabidopsis* VIP1 protein and thereby triggers the cyto-nuclear translocation of VIP1 protein, which increases T-DNA transfer and transformation efficiency (Djamei et al. 2007). The strategies utilized by *Agrobacterium* to transform the plant cell were well reviewed by Pitzschke (2013); while those for plant response to *Agrobacterium* were summarized by Gohlke and Deeken (2014). The host defense response is activated more or less strongly depending on the plant system and *Agrobacterium* genotype used for infection (Ditt et al. 2006; Lee et al. 2009; Gohlke and Deeken 2014), the timing and intensity of the microbe activated host defense reaction determine the success of transformation (Pitzschke 2013).

The development of full genome sequencing provides insights into *Agrobacterium*-induced host changes at the transcript level. *Agrobacterium* is capable of altering plant gene expression, specifically, the expression of plant defense related genes. Many *Agrobacterium* induced transcripts of *Ageratum conyzoides* plant cell cultures encoded putative defense factors (Ditt et al. 2001). *Agrobacterium* infection caused host defense response has also been reported by Veena Jiang et al. (2003), Zipfel et al. (2006) and Anand et al. (2008). The plant defense system has an important role in controlling infection and its gene expression level is negatively correlated with *Agrobacterium*-mediated transformation efficiency (Ditt et al. 2005). Deeken et al. (2006) found that the increased levels of anions, sugars and amino acids in metabolic solute of *Arabidopsis thaliana* tumors were correlated with changes in gene expression. The functional categories of up-regulated set of genes were very different from that of down-regulated (Ditt et al. 2006). Zhao et al. (2011) investigated the proteomic profile of grapevine embryogenic callus after co-cultivation with *A. tumefaciens* and found that the cellular reactive oxygen species (ROS) removal system, mitochondrial energy metabolism and the protein-degradation machinery for misfolded proteins were markedly inhibited by *Agrobacterium* transformation, while the apoptosis signaling pathway and hypersensitive response are strengthened. Zhou et al. (2013) analyzed the differentially expressed genes (DEGs) in wheat callus cells co-cultured with *Agrobacterium* and found that a big part of these DEGs was related to the process of stress or immunity response. We previously demonstrated that the high content of endogenous isoflavones is a serious obstacle in achieving high efficient *Agrobacterium*-mediated transformation in soybean (Zhang et al. 2015). Isoflavones are synthesized as part of the phenylpropanoid pathway. Phenylalanine ammonia-lyase (PAL) catalyzes the first step in the biosynthesis of phenylpropanoids. α-Aminooxyacetic acid (AOA), a specific inhibitor of PAL, had been successfully used in the inhibition of the isoflavonoid biosynthesis in cell suspension cultures of kudzu [*Pueraria lobata* (WILLD) Ohwi] (Li et al. 2009). Sonication treatment could disturb the synthesis of isoflavones by down regulating the expression of chalcone synthase (CHS) (Larkin 2001). Combined use of antagonist AOA and sonication treatments (novel method) greatly improved soybean's T-DNA delivery efficiency (Zhang et al. 2015). In this study, soybean gene expression profile analysis has been performed to elucidate the general response of soybean plant to *Agrobacterium* at an early stage of infection and to uncover the mechanism underlie this novel method toward developing an efficient transformation protocols for recalcitrant soybean plant.

Materials and methods

Plant explant preparation and transformation

Soybean [*Glycine max* (L.) Merrill.] seeds of Jidou17 were surface sterilized with chlorine gas for 24 h and subsequently soaked in sterile distilled water overnight. The cotyledons were separated by a longitudinal cut along the hilum and the hypocotyl, seed coat and embryonic axis were removed. EHA105 (pCAMBIA2201) was used as *A. tumefaciens* strain and the infection solution preparation was as described (Zhang et al. 2015). Soybean transformation was prepared in groups: 15–20 cotyledonary-node explants were placed in a 50 ml glass tube containing 25 ml of infection solution and sonicated for 0 or 15 s in a bath sonicator (KH2200B, Kunshan Hechuang, China) and then incubated for another 20 min at ambient temperature. The infected explants were blotted dry on sterile filter paper and then transferred onto co-culture medium, which had the same ingredients as the infection medium plus 1 mM dithiothreitol (DTT), 1 mM sodium thiosulfate,

3.3 mM cysteine, and 5.0 g L^{-1} agar, with or without 20 μM AOA. Three treatments were designed: (a) without *Agrobacterium* infection and cultured on medium free of AOA; (b) infected by conventional *Agrobacterium* transformation method and cultured on medium free of AOA; (c) infected by *Agrobacterium* along with sonication treatment at 40 kHz for 15 s and cultured on medium with 20 μM AOA. Fifteen explants were cultured on one plate with a minimum of ten replicates per treatment. Co-cultivation was conducted for 3 days at 25 °C in dark.

Sample collection, cDNA library preparation and sequencing

Explants from three replicates were collected at 5 h after infection and pooled into two different parts, respectively. One part was used in the digital gene expression library preparation and Ion-Proton sequencing, and the other was used for quantitative PCR analysis. All samples were rapidly frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

RNA was isolated from each sample using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The total RNA samples were first treated with DNase I (RNase Free) to degrade any possible DNA contamination and then purified by magnetic beads (Invitrogen, Carlsbad, CA, USA). After that, the mRNA was enriched using the oligo (dT) conjugated magnetic beads (for eukaryotes), and then fragmented into short fragments (about 200 bp) using divalent cations at high temperature. Then cDNA was synthesized using random hexamer-primed reverse transcription (Invitrogen, Carlsbad, CA, USA). The double strand cDNA was purified with magnetic beads and then adaptors were ligated to the ends of these fragments. Ligation products were selected by size and purified on TAE-agarose gel. Finally, the fragments were enriched by PCR amplification, purified by magnetic beads and dissolved in the appropriate amount of Epstein–Barr solution. Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA) was used to qualify and quantify the sample library. The qualified library was ready for sequencing via Ion Proton platform when necessary at BGI tech (Shenzhen, China).

Sequence analysis

Raw reads produced by the sequencer were transferred into clean data by removing some adaptor sequences and/or low quality reads present in the raw reads data. All high quality sequences were mapped to the reference genome (ftp://jgi-psf.org/pub/compgen/phytozome/v9.0/Gmax/assembly/Gmax_189.fa.gz) and reference gene set (ftp://jgi-psf.org/pub/compgen/phytozome/v9.0/Gmax/annotation/Gmax_189_transcript.fa.gz) using the short reads alignment software TMAP3.4.1 (https://github.com/iontorrent/TMAPreads).

The quantification of gene expression was performed by mapping reads to the reference gene set; reads that were not mapped to the reference gene set might be mapped to the reference genome, which were important for novel transcript prediction. The expression level for each gene was determined by the numbers of reads uniquely mapped to the specific gene and the total number of uniquely mapped reads in the sample and was calculated by RPKM method (Mortazavi et al. 2008). A rigorous algorithm was developed to identify DEGs between different treatments. False discovery rate (FDR) was used to determine the threshold of *P* value in multiple tests and analysis. We used an FDR of 0.001 and the absolute value of log2Ratio ≥1 (twofold change) as the threshold to judge the significance of the gene expression difference.

With nr annotation, Blast2GO program was used to get GO annotation of DEGs. The DEGs in the GO database (http://www.geneontology.org/) were mapped into significantly enriched GO terms (Bonferroni corrected *P* value ≤0.05) by Go enrichment analysis. WEGO software was used to do GO functional classification of DEGs. KEGG pathway enrichment analysis was performed using KEGG database (http://www.kegg.jp/kegg/pathway.html) to identify significantly enriched metabolic pathways or signal transduction pathways in DEGs comparing with the whole genome background.

Validation of DEGs by real-time quantitative PCR, enzyme activity and metabolite content

Soybean cotyledonary-node explants collected at the same time as those used for gene expression profile analysis were used for real-time quantitative PCR validation. RNA extraction and cDNA synthesis were as described in the RNAseq part. The real-time quantitative PCR was performed on the 7500/7500 Fast Real-Time PCR System (Applied Biosystems, USA) as described (Zhang et al. 2015). The relative quantification of gene expression was calculated as $2^{-\Delta\Delta C_T}$. A total of 40 DEGs were selected; the primers were designed using online tools IDT-PrimerQuest Input (http://www.idtdna.com/primerquest/Home/Index) and were synthesized by Sangon Biotech (Shanghai, China) (Table S1). Triplicate per gene was included in each plate. *Actin* was used as an endogenous parallel control.

Explants 24 h after *Agrobacterium* infection were sampled for assaying the activity of phenylalanine ammonia-lyase [EC: 4.3.1.24], β-glucosidase [EC: 3.2.1.21] and peroxidase [EC: 1.11.1.7] as well as isoflavones content. PAL activity and isoflavones content determination were as previously described (Zhang et al. 2015). Peroxidase activity and β-glucosidase activity were determined following the methods described in "*Guide for modern plant physiology experiments*"(eds. Institute of Shanghai plant physiology, Chinese academy of sciences, Shanghai plant physiology society (China) 1999).

Histochemical assay of infected explants

The explants 3 dpi were used to perform GUS histochemical assay as described by Jefferson (1987). Percentage response was determined as the number of cotyledonary nodes of staining blue with X-gluc (Gold Biotechnology, Inc., USA) at the regenerable area divided by the number of cotyledonary nodes assayed. All other chemicals not specially stated were supplied by Sangon Biotech (Shanghai, China).

Results

Agrobacterium infection stimulated host defense related gene expression

10,586,715, 11,770,754 and 11,876,728 total reads were generated from A, B and C samples, respectively, with 98.85 % above mapped to reference gene set and 99.84 % above mapped to reference genome.

Compared with non-transformed control A, 2158 DEGs were identified from the *Agrobacterium* infected explants B, including 1190 up-regulated and 968 down-regulated DEGs. Among 2158 DEGs, 1304 DEGs were with pathway annotation (118 pathways), the 3 biggest pathway categories were plant–pathogen interaction (115 DEGs, 8.82 %), plant hormone signal transduction (112 DEGs, 8.59 %), and phenylpropanoid biosynthesis (94 DEGs, 7.21 %). Comparing with the whole genome background, the top three significantly enriched pathways, taking RichFactor coefficient as accounts, were phenylpropanoid biosynthesis (94 DEGs, 7.21 %), flavonoid biosynthesis (77 DEGs, 5.9 %) and isoflavonoid biosynthesis (19 DEGs, 1.46 %) (Fig. S1). The DEGs in significantly enriched pathway by *Agrobacterium* infection (A-vs-B) are listed in Table S2. Among 115 DEGs in plant–pathogen interaction categorization, 95 genes were up-regulated and 20 genes were down-regulated. The genes encoding the bacterial flagellin flg22 related LRR receptor-like serine/threonine-protein kinase FLS2, brassinosteroid insensitive 1-associated receptor kinase 1 (BAK1), and bacterial EF-Tu related EFR genes were stimulated greatly by *Agrobacterium* infection. The number of DEGs up-regulated was also significantly higher than that of down-regulated in phenylpropanoid biosynthesis (74 up-vs-20 down), flavonoid biosynthesis (64 up-vs-13 down) and isoflavonoid biosynthesis (16 up-vs-3 down), their detailed information was sketched into maps (Figs. S2, S3, S4). Based on pathway enrichment analysis, the peroxidase [EC: 1.11.1.7], WRKY transcription factor, LRR receptor-like serine/threonine-protein kinase FLS2 [EC: 2.7.11.1], brassinosteroid insensitive 1-associated receptor kinase 1 (BAK1) [EC: 2.7.10.1, 2.7.11.1], flavonoid 3′-monooxygenase (F3′H) [EC: 1.14.13.21], and chalcone synthase (CHS) [EC: 2.3.1.74], etc., were the most up-regulated metabolites in significantly enriched pathway by *Agrobacterium* infection (Table 1).

Antagonists and sonication changed the gene expression profile

Compared with explants B transformed with conventional method, the number (ratio) of down-regulated DEGs was greatly increased in explants C transformed with the novel method of antagonist AOA combined with sonication treatment (3524 DEGs down-regulated, being 69.6 % of the total 5062 DEGs). Among 5062 DEGs, 3018 DEGs were with pathway annotation (123 pathways), the four biggest pathway categories were plant hormone signal transduction (303 DEGs, 10.04 %), plant–pathogen interaction (285 DEGs, 9.44 %), starch and sucrose metabolism (143 DEGs, 4.74 %) and phenylpropanoid biosynthesis (89 DEGs, 2.95 %). From the view of whole genome background, the most significantly enriched pathway was fatty acid biosynthesis, flavone and flavonol biosynthesis, other glycan degradation, starch and sucrose metabolism (Fig. S5). The DEGs in significantly enriched pathway by antagonists and sonication treatment (B-vs-C) are listed in Table S3. Among 303 DEGs in plant hormone signal transduction, 196 DEGs were down-regulated, being 83 % higher than that of up-regulated, which was different from the situation in A-vs-B, where the number of up-regulated DEGs was similar to that of down-regulated (Table S2). Similar situation happened in many pathways such as plant–pathogen interaction (151 down-vs-134 up), phenylpropanoid biosynthesis (55 down-vs-34 up), isoflavonoid biosynthesis (14 down-vs-4 up), etc. Antagonist and sonication treatment alleviated the defense response elicited by *Agrobacterium* infection. Genes coding for peroxidase [EC: 1.11.1.7], β-glucosidase [EC: 3.2.1.21], flavonoid 3′-monooxygenase (F3′H) [EC: 1.14.13.21], shikimate *O*-hydroxycinnamoyltransferase (HCT) [EC: 2.3.1.133], isoflavone 7-*O*-glucosyltransferase (IF7GT) [EC: 2.4.1.170], LRR receptor-like serine/threonine-protein kinase FLS2 [EC: 2.7.11.1], serine/threonine-protein kinase PBS1 [EC: 2.7.11.1] and transcription factor MYC2, etc., which are important for isoflavone biosynthesis and plant–pathogen interaction, were all significantly down-regulated (Table 2).

Validation of enriched pathway by qPCR, enzyme activity and metabolite content

To evaluate the validity of gene expression profile results using high-throughput sequencing, we performed

Table 1 The most up-regulated metabolites in significantly enriched pathway by *Agrobacterium* infection (A-vs-B)

Name	No. DEGs up-regulated
3,9-Dihydroxypterocarpan 6a-monooxygenase (CYP93A1)	6
Chalcone synthase [EC:2.3.1.74]	11
Flavonol synthase [EC:1.14.11.23]	5
Leucoanthocyanidin reductase [EC:1.17.1.3]	6
Leucoanthocyanidin dioxygenase [EC:1.14.11.19]	9
Flavonoid 3′-monooxygenase [EC:1.14.13.21]	10
Caffeoyl-CoA *O*-methyltransferase [EC:2.1.1.104]	5
Chalcone isomerase [EC:5.5.1.6]	6
Phenylalanine ammonia-lyase [E4.3.1.24]	5
4-Coumarate–CoA ligase [EC:6.2.1.12]	6
Beta-glucosidase [EC:3.2.1.21]	5
Shikimate *O*-hydroxycinnamoyltransferase [EC:2.3.1.133]	7
Peroxidase [EC:1.11.1.7]	28
LRR receptor-like serine/threonine-protein kinase FLS2 [EC:2.7.11.1]	12
Brassinosteroid insensitive 1-associated receptor kinase 1 [EC:2.7.10.1, 2.7.11.1]	15
WRKY transcription factor 25	14
WRKY transcription factor 29	16
Jasmonate ZIM domain-containing protein	5
Transcription factor MYC2	6

A represents explants without *Agrobacterium* infection and cultured on medium free of AOA. B represents explants infected by conventional *Agrobacterium* transformation method and cultured on medium free of AOA. In a pairwise comparison of A-vs-B, the former one (A) is considered as the control, and the latter one (B) is considered as the treatment

Table 2 The most down-regulated metabolites in significantly enriched pathway by antagonist and sonication treatment (B-vs-C)

Name	No. DEGs down-regulated
Beta-glucosidase [EC:3.2.1.21]	14
Shikimate *O*-hydroxycinnamoyltransferase [EC:2.3.1.133]	11
Peroxidase [EC:1.11.1.7]	13
Flavonoid 3′-monooxygenase [EC:1.14.13.21]	10
Isoflavone 7-*O*-glucosyltransferase [EC:2.4.1.170](IF7GT)	6
Flavonol 3-*O*-methyltransferase [EC:2.1.1.76]	6
Calmodulin	5
LRR receptor-like serine/threonine-protein kinase FLS2 [EC:2.7.11.1]	22
Brassinosteroid insensitive 1-associated receptor kinase 1 [EC:2.7.10.12.7.11.1]	5
WRKY transcription factor 25	5
Pathogenesis-related protein 1	5
Disease resistance protein RPM1	5
Serine/threonine-protein kinase PBS1 [EC:2.7.11.1]	27
Heat shock protein 90 kDa beta	7
Jasmonate ZIM domain-containing protein	6
Transcription factor MYC2	27

B represents explants infected by conventional *Agrobacterium* transformation method and cultured on medium free of AOA. C represents explants infected by *Agrobacterium* along with sonication treatment at 40 kHz for 15 s and cultured on medium with 20 μM AOA. In a pairwise comparison of B-vs-C, the former one (B) is considered as the control, and the latter one (C) is considered as the treatment

quantitative real-time PCR for 40 genes. Results of real-time PCR analysis verified the Ion Proton sequencing data (Table 3). Furthermore, the enzyme activity and metabolite content in soybean coty-node explants were detected 24 h after *Agrobacterium* inoculation. Compared with treatment A, the PAL activity, isoflavones content and peroxidase

Table 3 Validation of DEGs by real-time PCR

Gene ID	Annotation	KEGG pathway	log2Ratio (B/A)	RQ by real-time PCR
Glyma20g24810.1	2-Hydroxyisoflavanone synthase [EC:1.14.13.136]	K00487	2.3	2.2
Glyma02g40290.1	2-Hydroxyisoflavanone synthase [EC:1.14.13.136]		1.2	8.6
Glyma08g11620.1	Naringenin-chalcone synthase [EC:2.3.1.74]	K00660	4	16.6
Glyma08g11520.1	Naringenin-chalcone synthase [EC:2.3.1.74]		3.1	14.1
Glyma01g22880.1	Naringenin-chalcone synthase [EC:2.3.1.74]		2.7	8.2
Glyma20g38580.1	Chalcone isomerase [EC:5.5.1.6]	K01859	2.5	30.8
Glyma10g43850.1	Chalcone isomerase [EC:5.5.1.6]		1.9	7.6
Glyma02g42470.1	Flavonol synthase [EC:1.14.11.23]	K05278	10.3	7
Glyma03g34510.1	Flavonol synthase [EC:1.14.11.23]		4.6	8.1
Glyma15g16490.1	Flavonol synthase [EC:1.14.11.23]		−2	2
Glyma08g10950.1	Flavonoid 3′-monooxygenase [EC:1.14.13.21]	K05280	2.2	2
Glyma20g33090.1	Flavonoid 3′-monooxygenase [EC:1.14.13.21]		−5	0.6
Glyma03g33890.1	Phenylalanine ammonia-lyase [EC:4.3.1.24]	K10775	2.3	5.8
Glyma10g06600.1	Phenylalanine ammonia-lyase [EC:4.3.1.24]		1.9	6.3
Glyma09g03460.1	4-Coumarate–CoA ligase [EC:6.2.1.12]	K01904	4.7	21.6
Glyma11g09710.2	4-Coumarate–CoA ligase [EC:6.2.1.12]		3.8	9.1
Glyma15g00600.2	Cinnamoyl-CoA reductase [EC:1.2.1.44]	K09753	2	3.4
Glyma08g41930.1	Shikimate *O*-hydroxycinnamoyltransferase [EC:2.3.1.133]	K13065	12	69.4
Glyma12g32650.1	Shikimate *O*-hydroxycinnamoyltransferase [EC:2.3.1.133]		4	47.6
Glyma19g03730.1	Shikimate *O*-hydroxycinnamoyltransferase [EC:2.3.1.133]		2	2.3
Glyma10g35400.1	Shikimate *O*-hydroxycinnamoyltransferase [EC:2.3.1.133]		−4.2	1.1
Glyma05g28530.1	Shikimate *O*-hydroxycinnamoyltransferase [EC:2.3.1.133]		−2.5	3.5
Glyma13g34010.1	Ferulate-5-hydroxylase [EC:1.14.-.-]	K09755	11.5	174.5
Glyma20g33090.1	Ferulate-5-hydroxylase [EC:1.14.-.-]		−5	35.6
Glyma10g34460.1	Ferulate-5-hydroxylase [EC:1.14.-.-]		−1.9	0.4
Glyma08g10950.1	Flavonoid 3′-monooxygenase [EC:1.14.13.21]	K05280	2.2	2.1
Glyma20g33090.1	Flavonoid 3′-monooxygenase [EC:1.14.13.21]		−5	9.1
Glyma09g12440.1	Flavonol 3-*O*-methyltransferase [EC:2.1.1.76]	K05279	3.4	13
Glyma10g35980.1	Flavonol 3-*O*-methyltransferase [EC:2.1.1.76]		3.1	5.5
Glyma20g35630.1	Flavonol 3-*O*-methyltransferase [EC:2.1.1.76]		2.3	10.7
Glyma03g29790.1	2-Hydroxyisoflavanone synthase [EC:1.14.13.136]	K13257	2.5	9.6
Glyma13g24200.1	2-Hydroxyisoflavanone synthase [EC:1.14.13.136]		2.3	16.7
Glyma02g17720.1	Flavonoid 6-hydroxylase [EC:1.14.13.-]	K13267	2.7	16.2
Glyma01g38610.1	Flavonoid 6-hydroxylase [EC:1.14.13.-]		1.8	11.7
Glyma11g06690.1	Flavonoid 6-hydroxylase [EC:1.14.13.-]		1.7	3.3
Glyma11g06660.1	Flavonoid 6-hydroxylase [EC:1.14.13.-]		1.7	1.9
Glyma01g45020.1	2-Hydroxyisoflavanone dehydratase [EC:4.2.1.105]	K13258	2.2	2.4
Glyma10g39610.1	2-Hydroxyisoflavanone dehydratase [EC:4.2.1.105]		1.2	1.3
Glyma02g15130.1	2-Hydroxyisoflavanone dehydratase [EC:4.2.1.105]		−2.6	1
Glyma09g23720.1	Isoflavone 7-*O*-glucosyltransferase [EC:2.4.1.170]	K13263	1.2	1.9
Glyma09g23750.1	Isoflavone 7-*O*-glucosyltransferase [EC:2.4.1.170]		1.1	1

A in log2Ratio (B/A) represents explants without *Agrobacterium* infection and cultured on medium free of AOA. B in log2Ratio (B/A) represents explants infected by conventional *Agrobacterium* transformation method and cultured on medium free of AOA

activity for treatment B were increased by 50, 7.5 and 37.2 %, respectively, which generally verified the sequencing data; but the β-glucosidase activity of treatment B was decreased by 14.8 % when compared with treatment A, showing a different trend from that of RNAseq analysis (Table 4). Antagonist AOA and sonication treatment greatly decreased the activity of above enzymes and isoflavones content, consisting with the sequencing results.

Table 4 Validation of DEGs by enzyme activity and metabolite content

	Treatment A	Treatment B	Treatment C
PAL activity (U mg^{-1} protein h^{-1})	40.6 ± 5.1^{b}	60.9 ± 8.7^{a}	41.9 ± 6.2^{b}
Isoflavones content (mg kg^{-1} DW)	5.3 ± 0.5^{a}	5.7 ± 0.9^{a}	4.6 ± 0.6^{b}
POD activity (U mg^{-1} protein min^{-1})	17.2 ± 0.9^{b}	23.6 ± 0.6^{a}	18.3 ± 0.7^{b}
β-Glucosidase activity (nmol mg^{-1} protein h^{-1})	82.8 ± 2.6^{a}	70.6 ± 1.8^{b}	59.3 ± 1.7^{c}

Treatment A: without *Agrobacterium* infection and cultured on medium free of AOA. Treatment B: infected by conventional *Agrobacterium* transformation method and cultured on medium free of AOA. Treatment C: infected by *Agrobacterium* along with sonication treatment at 40 kHz for 15 s and cultured on medium with 20 μM AOA. Data were collected 24 h after *Agrobacterium* infection from three to five replicates and presented as the mean ± SD. Superscript letters indicate significant differences ($P < 0.05$) according to the Fisher's least significant difference (LSD) test

Antagonists and sonication made soybean easy to be transformed by *Agrobacterium*

As illustrated in Table 5, sonication at 40 kHz for 15 s along with *Agrobacterium* infection and cultured on medium of 20 μM AOA dramatically promoted the efficiency of T-DNA delivery in soybean, with the mean percentage of GUS transient expression as 54.2 %, significantly higher than that of not sonicated and co-cultured on AOA free medium (11.0 %). The ratio of coty-nodes with dark blue was greater in treatment C than that in treatment B (Fig. 1).

Discussion

Defense response in *Agrobacterium* infected soybean

Plant–pathogen interaction and plant hormone signal transduction are the two biggest categorized pathways by *Agrobacterium* infection when compared with non-transformed control. Some genes involved in both plant–pathogen interaction and plant hormone signal transduction, such as BAK1, PR1 (pathogenesis-related protein 1), JAZ (jasmonate ZIM domain-containing protein) and transcription factor MYC2. It was generally believed that the phytohormone salicylic acid (SA), jasmonic acid, and ethylene participate in plant defense regulation (Grant and Lamb 2006; Anand et al. 2008), but we failed to detect DEGs in SA metabolic pathway, this was in consistence with the results in barley where bacteria-triggered systemic immunity was associated with WRKY and ethylene responsive factors but not with salicylic acid (Dey et al. 2014). However, the expression of genes related to brassinosteroid metabolism was greatly changed in soybean (Table S2). Brassinosteroids are endogenous plant-growth regulators that modulate cell elongation and division (Clouse et al. 1996). BAK1 is a typical leucine-rich repeat sequence receptor kinase (LRR-RK) and was initially identified as a dual co-receptor of BRI1 and FLS2, which mediate BRs signaling and PAMP-triggered immunity (PTI) in plant, respectively (Tian et al. 2014). Subsequent works proved that BAK1 could associate with ligand-binding LRR-RLKs including BRI1, flagellin sensitive 2 (FLS2), EF-Tu receptor (EFR), receptors of BRs,

Table 5 Transient expression of GUS in infected cotyledonary nodes

Batch	Treatment	No. explants infected	No. explants with GUS focus	Percent of GUS transient expression*
1	B	50	8	16.0
	C	84	51	60.7
2	B	37	3	8.1
	C	42	22	53.4
3	B	67	6	8.9
	C	74	36	48.6
Total	B	154	17	11.0 ± 4.3^{B}
	C	200	109	54.2 ± 6.1^{A}

Treatment A: without *Agrobacterium* infection and cultured on medium free of AOA. Treatment B: infected by conventional *Agrobacterium* transformation method and cultured on medium free of AOA. Treatment C: infected by *Agrobacterium* along with sonication treatment at 40 kHz for 15 s and cultured on medium with 20 μM AOA

* The percent of transient expression was determined as the number of cotyledonary nodes of staining blue with X-gluc at the re-generable area divided by the number of cotyledonary nodes assayed. The percentage data were arcsine square root transformed prior to one-way ANOVA and presented as the mean ± SD. Superscript letters indicate the significant differences ($P < 0.01$) according to the Fisher's LSD test

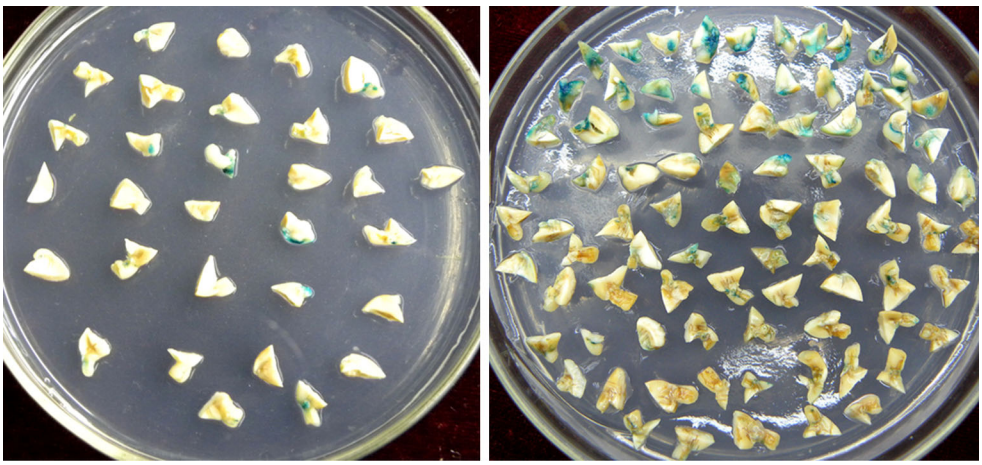

Fig. 1 Transient expression of GUS in soybean cotyledonary nodes 3 days after their co-cultivation with *Agrobacterium* strain EHA105 (pCAMBIA2201). *Notes Left photograph* shows GUS stain in cotynodes from treatment B. *Right photograph* shows GUS stain in cotynodes from treatment C

bacterial flagellin, and bacterial elongation factor Tu (EF-Tu), respectively, to form receptor complexes, initiating corresponding phosphorylation cascades and eventually regulating downstream target gene expressions (reviewed in He et al. 2013).

Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) was recognized as the first line of defense in plant and could be elicited by PRRs (Pattern Recognition Receptors) through specific cell surface located proteins, such as FLS2 and EFR, which recognize the oligopeptides flagellin and EF-Tu, respectively (Chinchilla et al. 2007). Each kind of PAMPs can be recognized by different PRRs with a very similar response pattern in plant cell. It was speculated that there are some common molecules to contact these signals and BAK1 was the central regulator of innate immunity (Heese et al. 2007). In addition to be a co-receptor of many PRRs to regulate PTI, BAK1 also is the targeted protein of many effectors to regulate ETI reaction (Chen and Zhou 2013), so BAK1 plays a vital role in plant pathogen defense and immunity reaction. RNAseq results indicated that *Agrobacterium* infection greatly stimulated the PAMP-triggered immunity in soybean, the genes encoding for BRI1, BAK1, BZR1, the bacterial flagellin flg22 related FLS2, as well as bacterial EF-Tu related EFR genes were stimulated greatly by *Agrobacterium* infection. Effector-triggered immunity was also elicited, for example, disease resistance protein RPM1, RPS2, RPS5, RPM1-interacting protein 4 (RIN4), and serine/threonine-protein kinase PBS1, were all up-regulated by twofold, even though not so much as that in PTI (data not shown). The overexpression of BAK1 benefits the activation of defense associated MPK and WRKY transcription factors in the MAPK pathway and hence to regulate immunity through cascade amplification of MAPK signal (Asai et al. 2002; Yang et al. 2012). In this study, WRKY25, WRKY29, MEKK1P, MKK4/5P and MYC2 were significantly up-regulated by *Agrobacterium* infection, implying that soybean explants were sensitive to *Agrobacterium* infection and effective in signal perception and signal amplification to defense this kind of invasion. As a result of defense response, genes related to respiratory burst oxidase (RBOH) and peroxidase were up-regulated to eliminate ROS (Table S2).

Isoflavonoid metabolite is another reason for lower efficiency of *Agrobacterium* infection

The DEGs caused by *Agrobacterium* infection were most significantly enriched in phenylpropanoid, flavonoid and isoflavonoid biosynthesis in this study, this was different from the results in wheat (Zhou et al. 2013), where, although a big part of DEGs was categorized in the secondary metabolites and phenylpropanoid biosynthesis, but not in flavonoid and isoflavonoid biosynthesis. Phenylpropanoids may undergo different branches of metabolic pathway in wheat and soybean. Phenylalanine ammonia-lyase (PAL) catalyzes the first step in the biosynthesis of phenylpropanoids, 5 DEGs coding for PAL were found up-regulated with log2Ratio $\geq$1.5 and PAL activity increased by about 50 %, compared to that of non-infection control. Many genes coding for 4-coumarate-CoA ligase (4CL), ferulate-5-hydroxylase (F5H), HCT, CHS, F3′H, flavonol synthase (FLS), leucoanthocyanidin dioxygenase (LDOX), and 3,9-dihydroxypterocarpan 6a-monooxygenase (CYP93A1), important for flavonoid and isoflavonoid biosynthesis, were dramatically up-regulated in soybean by conventional *Agrobacterium* infection (Table S2). Soybean isoflavones play diverse roles in

plant–microbe interactions; it significantly influenced soybean rhizosphere bacterial diversity (White et al. 2015). Many genes in isoflavone secondary metabolism are involved in plant defense responses. Soybean isoflavone includes two kinds of compounds, isoflavone aglycones and their glucosides, the latter accounts for about 97–98 % of the total isoflavones content and be of biological functions only in the situation that they turned into aglycones type through hydrolysis; β-glucosidase plays a role in this process (Sun et al. 2007). β-Glucosidase was up-regulated 5 h after *Agrobacterium* infection as revealed by RNAseq results, implying that soybean intended to increase its defense response to *Agrobacterium* invasion in virtue of active component ascension, but this phenomenon was not validated when *p*-nitrophenyl-β-D-glucopyranoside was used as substrate to determine the β-glucosidase activity (Table 5). It is also regretted that only total isoflavones content, not each isoflavone components, was determined in this study because of the constrain of experimental condition. Nevertheless, it does not interfere with the conclusions that isoflavones is an important factor in *Agrobacterium* infection elicited soybean defense response, which was in accordance with our previous conclusion (Zhang et al. 2015).

Improvement of soybean transformation by regulating plant defense response

Intense host defense response is often associated with reduced transformation efficiency, and thus, the attenuation of these responses by external measures can improve transformation efficiency (Pitzschke 2013). The improvement of transformation by manipulating the plant's immune system to optimize plant–microbe interactions has recently been reported. AvrPto is an effector protein that suppresses plant immunity by interfering with plant immune receptors. Great improvement of transformation was achieved when *AvrPto* transgenic *Arabidopsis* plants were infected with *Agrobacterium* (Tsuda et al. 2012). Zhang et al. (2013) alleviated the defense responses by omission of myo-inositol in culture medium, combined with cold treatment before infection, which promoted *Agrobacterium* binding to the cell surface and inhibited the ROS after *Agrobacterium* infection, and ultimately improved *Agrobacterium* mediated transformation efficiency in perennial ryegrass (*Lolium perenne* L.). Salicylic acid is another important metabolin in plant defense regulation. To demonstrate the role of SA in *Agrobacterium* infectivity, Anand et al. (2008) used transgenic tomato plants overexpressing salicylate hydroxylase (NahG), which degrades SA to catechol, to perform GUS activity assays at 2 dpi, results indicated that NahG-expressing plants were more susceptible to *Agrobacterium* infection (Anand et al. 2008).

Soybean isoflavones had inhibitory effects on *A. tumefaciens* growth and respiration and was negatively correlated with T-DNA delivery efficiency (Zhang et al. 2015), which was consistent with the reports where silencing genes of isoflavone synthase (IFS) or chalcone reductase (CHR) led to a nearly complete (95 %) suppression of all isoflavone metabolites in soybean roots (Graham et al. 2007) and enhanced susceptibility to *Phytophthora sojae* (Subramanian et al. 2005; Graham et al. 2007). In this study, antagonist AOA and sonication treatment along with *Agrobacterium* infection dramatically decreased the expression of genes coding for F3′H, HCT, β-glucosidase and IF7GT, important for isoflavone biosynthesis (Figs. 2, 3, 4); isoflavones content was ultimately decreased by about 19.3 %. IF7GT converts the flavonoid aglycones into glycon in the last step of isoflavonoid biosynthesis. The down-regulation of IF7GT at transcription level maybe caused by the reduction of aglycones substrate, because the biosynthesis of isoflavone aglycon had been disturbed in the upper steps, such as F3′H, HCT and flavonol 3-*O*-methyltransferase [EC:2.1.1.76]. Genes coding for peroxidase, LRR receptor-like serine/threonine-protein kinase FLS2, serine/threonine-protein kinase PBS1 and transcription factor MYC2, etc., which are important components in plant–pathogen interaction, were also significantly down-regulated. All these factors benefit the transformation process.

UDP-glycosyltransferase had been confirmed to participate in the response to pathogens (von Saint Paul et al. 2011). Transgenic *A. thaliana* expressing a barley UDP-glucosyltransferase exhibited resistance to the mycotoxin deoxynivalenol (Shin et al. 2012). *Arabidopsis hat* mutant over-expressing a UDP-glucosyltransferase gene was found to be resistant to *Agrobacterium*-mediated transformation (Gelvin 2010). Zhou et al. (2013) found UDP-glucosyltransferase were up-regulated at the level of transcription after infection by *Agrobacterium* and inferred that saccharide metabolism might affect the infection process. In this study, the DEGs were also significantly enriched in starch and sucrose metabolism and other glycan degradation in B-vs-C compare set (Table S3; Fig. 5). Glycerol-3-phosphate 1-*O*-acyltransferase (GPAT) [EC: 3.2.1.15], β-galactosidase [EC:3.2.1.23], alpha-L-fucosidase [EC:3.2.1.51], endoglucanase [EC:3.2.1.4], and β-glucosidase [EC:3.2.1.21], etc., were remarkably down-regulated

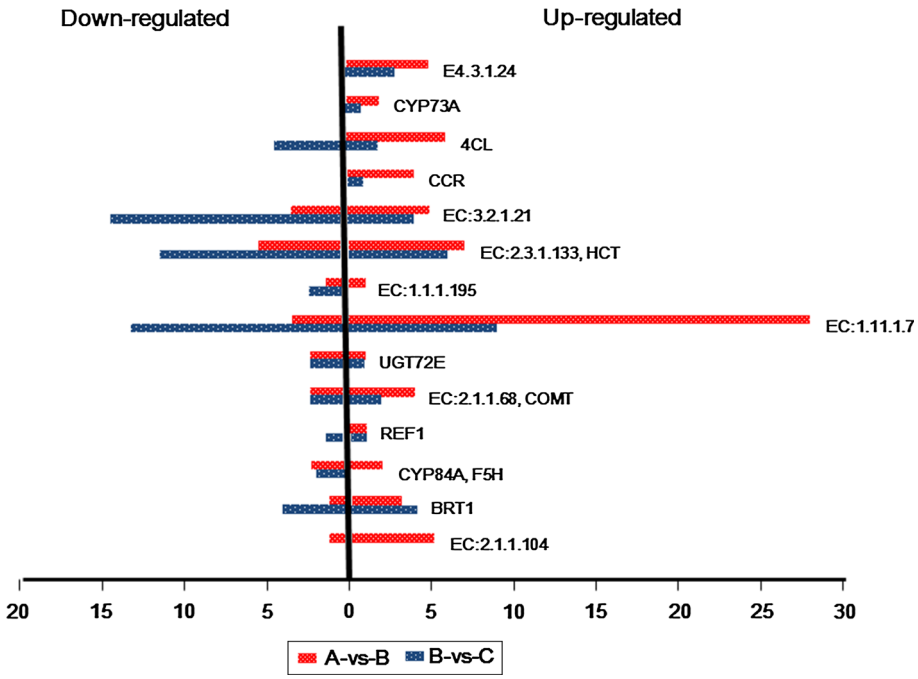

Fig. 2 Functional categorization of DEGs in phenylpropanoid biosynthesis pathway. *Notes* In a pairwise comparison (denote as A-vs-B for example), the former one (A) is considered as the control, and the latter one (B) is considered as the treatment, the same hereinafter. Each *bar* represents a functional group of transcripts with up-regulated (*right bar*) or down-regulated (*left bar*) expression in soybean tissue 5 h after *Agrobacterium* infection, the axis of abscissa represents the number of DEGs in that group

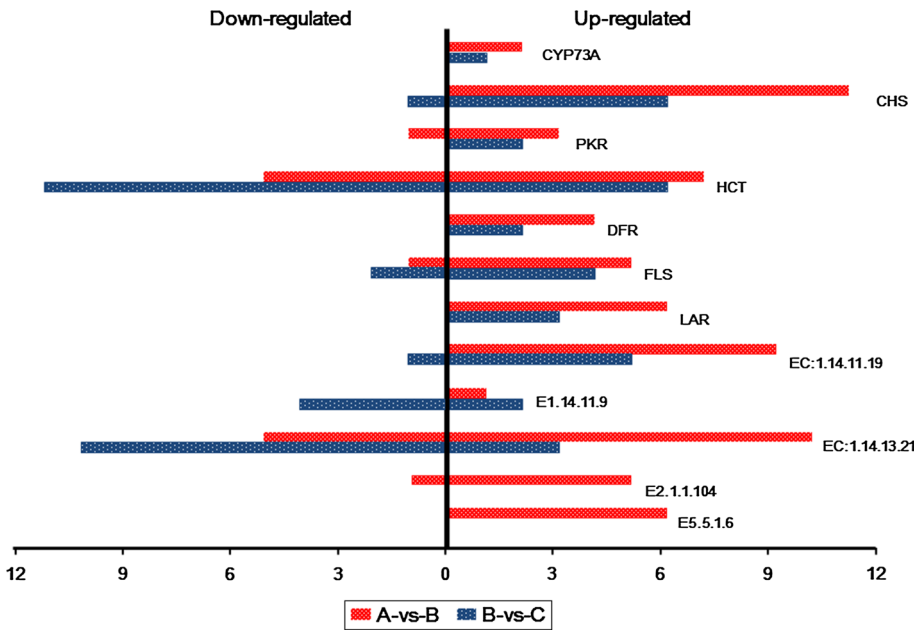

Fig. 3 Functional categorization of DEGs in flavonoid biosynthesis pathway. *Notes* In a pairwise comparison (denote as A-vs-B for example), the former one (A) is considered as the control, and the latter one (B) is considered as the treatment, the same hereinafter. Each *bar* represents a functional group of transcripts with up-regulated (*right bar*) or down-regulated (*left bar*) expression in soybean tissue 5 h after *Agrobacterium* infection, the axis of abscissa represents the number of DEGs in that group

as revealed by log2Ratio (B/C), but UDP-glucosyltransferase was not significantly enriched in these metabolic pathways. GPAT is a key enzyme in the triacylglycerol biosynthetic pathway, catalyzing triacylglycerol, chitin, suberin and other lipid biosynthesis (Shockey et al. 2015). Chitin and suberin are two important polyesters in plant, which constitute a protective guard to prevent pathogen invasion (Kolattukudy 1980), thus the down-regulation of GPAT may benefit *Agrobacterium* infection. 29 DEGs coding for α-L-fucosidase were down-regulated by AOA and sonication treatment (Table S3). α-L-Fucosidase is involved in the degradation of fucose-containing glycoproteins and glycolipids, which are located on the external surface of cell membrane and involved in the cell recognition, intercellular communication and immunity. The down-regulation of β-glucosidase impeded the conversion

of isoflavones glycon into aglycones, thereby remitting the inhibitory effects of aglycones on *Agrobacterium* infection. Above analysis implied that AOA and sonication treatment not only relieved the structural membrane barriers of *Agrobacterium* entering into cells, but also hindered the perception of 'invasion' signal on cell membrane and intercellular signal transmission, making the plant unable to establish the corresponding defense system before transformation complement. This may best explain why the T-DNA delivery efficiency was greatly promoted by this novel method. The inhibitors of protein kinase K252a, actinomycin D and cycloheximide were also effective in inhibiting the increase of PAL activity and isoflavones biosynthesis in soybean (data not shown). The shortage of this paper was that the separate effects of antagonist AOA and sonication could not be distinguished owning to the problems in experimental design.

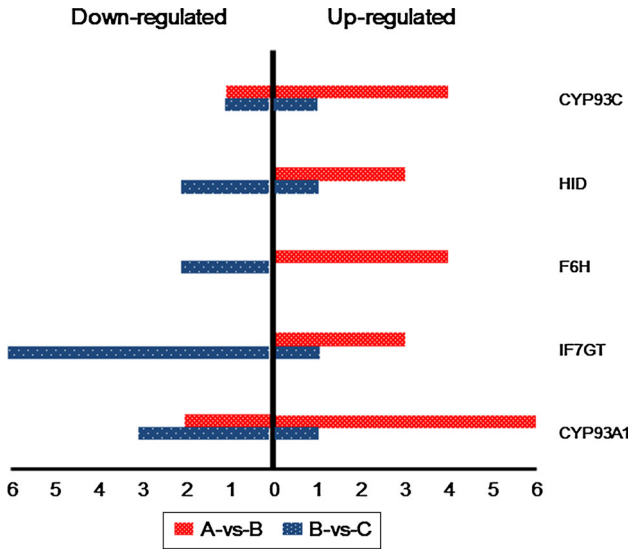

Fig. 4 Functional categorization of DEGs in isoflavonoid biosynthesis pathway. *Notes* In a pairwise comparison (denote as A-vs-B for example), the former one (A) is considered as the control, and the latter one (B) is considered as the treatment, the same hereinafter. Each *bar* represents a functional group of transcripts with up-regulated (*right bar*) or down-regulated (*left bar*) expression in soybean tissue 5 h after *Agrobacterium* infection, the axis of abscissa represents the number of DEGs in that group

In summary, the high content of endogenous isoflavones and fierce defense response to *Agrobacterium* infection result in the inefficiency of soybean transformation via *Agrobacterium* methods. Further improvement of the transformation efficiency lies in the manipulation of the plant itself to reduce the intensity of defense reaction or ignore this invasion signal, hence to increase the susceptibility of plant cells to *Agrobacterium* infection. This gene expression profile analysis has provided candidate external measures for improving soybean transformation efficiency. In addition to the presence of thiol compounds in co-culture medium, inhibition of isoflavones biosynthesis by sonication treatment and applying antagonists in co-culture medium relieved the structural barriers of *Agrobacterium* entering into cells and hindered the perception of 'invasion' signal on cell membrane and intercellular signal transmission, thus effectively alleviated the defense responses and increased the susceptibility of *Agrobacterium* infection. The antagonist used in this study was located in the forefront of isoflavones metabolic pathway; inhibitors for enzymes in the downstream of isoflavone biosynthesis pathway should be explored to reduce their

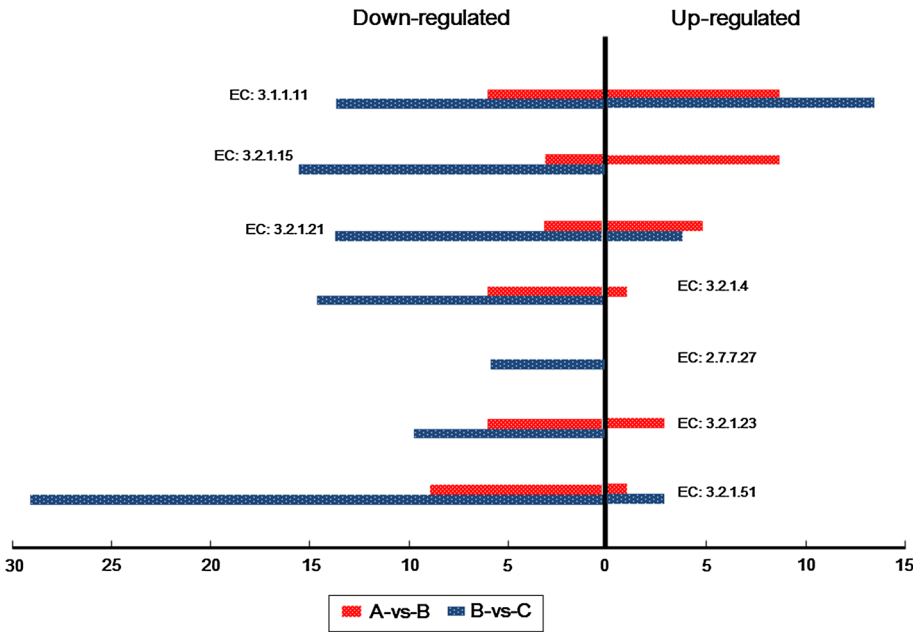

Fig. 5 Functional categorization of DEGs in starch and sucrose metabolism and other glycan degradation. *Notes* In a pairwise comparison (denote as A-vs-B for example), the former one (A) is considered as the control, and the latter one (B) is considered as the treatment, the same hereinafter. Each *bar* represents a functional group of transcripts with up-regulated (*right bar*) or down-regulated (*left bar*) expression in soybean tissue 5 h after *Agrobacterium* infection, the axis of abscissa represents the number of DEGs in that group

impacts on other metabolic pathways, other measures should also be studied.

Author contribution statement Z.Y.M. conceived, designed research and wrote paper. L.Z.H., Y.R.J., L.G.L. and G.X.L. conducted experiments. Z.H.N., Z.H.M. and D.R. analyzed data. Z.Q.S. contributed analytical tools. Z.M.C. and Z.H.M. provided capital. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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