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ORIGINAL PAPER

Inhibition of isoflavone biosynthesis enhanced T-DNA delivery in soybean by improving plant–*Agrobacterium tumefaciens* interaction

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Abstract Soybean is a difficult crop to manipulate through *Agrobacterium tumefaciens*-mediated genetic transformation. Plant–bacterium interaction plays an important role in the transformation process. Being rich with isoflavones, soybean may have an adverse effect on the *A. tumefaciens*-mediated genetic transformation. To investigate whether high content of endogenous isoflavones in soybean is a serious obstacle in achieving high efficient *Agrobacterium*-mediated transformation in soybean, a series of experiments on inhibition of isoflavone biosynthesis were conducted to improve upon soybean transformation efficiency. Results indicated that soybean isoflavones inhibited *A. tumefaciens* growth and respiration, the transformation efficiency [β-glucuronidase (GUS) transient expression] was negatively correlated with the phenylalanine ammonia-lyase activity and isoflavones content. The biosynthesis of soybean isoflavones was partially inhibited by sonication treatment and applying antagonists in co-culture medium and thereby decreased the adverse effects of isoflavones on *Agrobacterium* infection. A discernible improvement in transformation efficiency was achieved when sonication at 40 kHz for 3 min was applied along with *Agro*-infection and the explants were cultured on co-culture medium containing 20 μM α-aminooxyacetic acid (AOA), with the percentage of GUS transient expression as 41.4 %, being 3.6 times higher than that not sonicated and co-cultured on medium without AOA. Sonication was found not only to simply make micro-wounds for *Agrobacterium* to penetrate or releasing phenolic compounds for induced *Agrobacterium vir* gene expression; it disturbed the biosynthesis of isoflavones at the transcription level and decreased the adverse effects of isoflavones on soybean transformation, and thereby improving soybean transformation efficiency.

Keywords Soybean · Isoflavones · Inhibition · Transformation · *Agrobacterium tumefaciens*

Abbreviations

PAL	Phenylalanine ammonia-lyase
AOA	α-Aminooxyacetic acid
AS	Acetosyringone
SAAT	Sonication-assisted *Agrobacterium*-mediated transformation

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Introduction

Soybean [Glycine max (L.) Merrill.] is a dicotyledonous plant widely used for human and animal food and considered to be a compatible host for *Agrobacterium* (Pedersen et al. 1983), but difficult to transform via *Agrobacterium tumefaciens* mediated methods (Larkin 2001), although quite a few studies has been reported (Hinchee et al. 1988; Parrott et al. 1989; Meurer et al. 1998; Yan et al. 2000; Paz et al. 2006; Hong et al. 2007; Muhammad et al. 2010; Liu et al. 2014). In order to

enhance soybean transformation via *Agrobacterium* various parameters have been evaluated. These parameters include the use of the super-virulent *A. tumefaciens* strain (Meurer et al. 1998; Ko et al. 2003, 2004; Yukawa et al. 2007, 2008), employment of mutant bacteria that constitutively express their *vir* genes (Hansen et al. 1994; Benzle et al. 2014), adding the phenolic compound like acetosyringone during inoculation and/or co-culture medium to induce expression of *vir* genes (Godwin et al. 1991), adding antioxidant substances, such as L-cysteine and thiol compounds in co-culture medium, to prevent tissue browning and necrosis (Olhoft et al. 2001, 2003, 2004, 2007; Zeng et al. 2004; Paz et al. 2004, 2006), and screening soybean genotypes that were competent to *Agrobacterium* infection and regeneration (Meurer et al. 1998; Paz et al. 2004; Song et al. 2013a, b). Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) has also been used for transforming various tissue types and plant species to increase the number of infection sites (Trick and Finer 1997; Santarém et al. 1998; Meurer et al. 1998; Jiang et al. 2004; Zaragoza et al. 2004; Dutta et al. 2013; Teixeira da Silva and Dobránszki 2014). Those research works improved soybean transformation efficiency substantially either by strengthening the ability of *Agrobacterium* infection or by reducing tissue browning and necrosis to some extent. 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 works in soybean genetic transformation were scarcely successfully replicated by other laboratory. Currently available transformation protocols for soybean transformation remained inefficient in most studies, with efficiency ranging from 0.2 to 2 %, very rarely up to 5–6 % (Atif et al. 2013) and limited to a few cultivars (Trick and Finer 1997). Soybean transformation efficiency was affected by many factors, genotypes, tissue types, *A. tumefaciens* strain, medium component and culture condition, transformant selection, etc., even the skills of operator. These may be the main reason for the poor repeatability. To date it has not been fully understand why soybean transformation via *Agrobacterium* is inefficient in most studies. One of the limitations for efficient plant transformation is a lack of understanding of gene expression during the selection and regeneration processes, plant–bacterium interactions also may prohibit or negatively affect the transformation process (Mello-Farias and Chaves 2008).

The defense response is considered to be one of the most important governing plant–microbe interaction during the transformation process and may strongly influence transformation efficiency as well (Mello-Farias and Chaves 2008; Anndrea Pitzschke 2013). Plants produce signal molecules that induce resistance responses following wounding or pathogen invasion (Mello-Farias and Chaves 2008). Soybean isoflavones play diverse roles in plant–microbe interactions (Subramanian et al. 2004, 2005). A variety of genes involved in regulating plant defense responses have already been identified in soybean (Larkin 2001), including many genes in isoflavone kind of secondary metabolic pathway. Wingender et al. (1989) detected enhanced CHS expression in the roots of sterile-grown soybean seedlings infected with *Agrobacterium* C58. Silencing genes of isoflavone synthase (IFS) or chalcone reductase (CHR) in soybean roots led to a nearly complete (95 %) suppression of all isoflavone metabolites in roots (Graham et al. 2007) and enhanced susceptibility to *Phytophthora sojae* (Subramanian et al. 2005; Graham et al. 2007).

Exogenous chemicals can be used in the regulation of soybean isoflavones synthesis. Phenylalanine ammonia-lyase (PAL) is the first enzyme in the plant secondary metabolism pathway. The isoflavonoid biosynthesis in cell suspension cultures of kudzu [Pueraria Lobata (WILLD) Ohwi] was found to be significantly inhibited by α-aminooxyacetic acid (AOA), a specific inhibitor of phenylalanine ammonia lyase (Li et al. 2009). Actinomycin D and cycloheximide inhibited the increase of PAL activity in sweet potato [*Ipomoea batatas* (Lam.) L.] (Wang and Xue 1981).

SAAT method was used in the transformation of various tissue types and plant species (genotypes), especially, that are considered to be difficult to transform (Bakshi et al. 2011; Subramanyam et al. 2011; King et al. 2014). It was believed that the micro-wounds produced by sonication treatment, allowing *Agrobacterium* to infect deeper within the plant tissue, were the main reason for the improvement in transformation efficiency by SAAT method (Trick and Finer 1997; Meurer et al. 1998). It was speculated that the micro-wound in plant tissues due to SAAT treatment released compounds that facilitated the growth and accumulation of bacteria under aerobic conditions so enhances transformation efficiency (Finer and Finer 2000). The candidate compounds were focused on phenolic compounds (Song et al. 2013a, b). However, decreased CHS expression following sonication treatment was detected (Larkin 2001), indicating that sonication may disturb the synthesis of isoflavones at the transcription level, rather than simply provide micro-wounds for *Agrobacterium* to penetrate into or release phenolic compounds for induced *Agrobacterium vir* gene expression.

From the above review of literature, we speculate that the low efficiency of soybean transformation is partially due to the inhibitory effects of isoflavones, a type of secondary metabolites, available in plenty in soybean, upon *Agrobacterium* infection. To our knowledge, literature on blocking isoflavone synthesis to improve soybean transformation has not been reported. The objectives of this study were to investigate whether endogenous soybean

Table 1 Treatment and preparation for evaluating the effects of daidzein on growth of *Agrobacterium tumefaciens*

Treatment	Content
Positive control	99 ml YEP medium plus 1 ml initial *Agrobacterium* solution
Solvent control 1 (0.17 % (v/v) DMSO)	99 ml YEP medium plus 1 ml initial *Agrobacterium* solution and 167 µl DMSO (for 1.0 mM daidzein treatment)
Solvent control 2 (0.33 % (v/v) DMSO)	99 ml YEP medium plus 1 ml initial *Agrobacterium* solution and 334 µl DMSO (for 2.0 mM daidzein treatment)
1.0 mM daidzein	99 ml YEP medium plus 1 ml initial *Agrobacterium* solution and 167 µl 600 mM daidzein stock solution
2.0 mM daidzein	99 ml YEP medium plus 1 ml initial *Agrobacterium* solution and 334 µl 600 mM daidzein stock solution
Blank solution 1	YEP medium (used in OD600 measurement for positive control, solvent control 1 and solvent control 2)
Blank solution 2	10 ml YEP plus 17 µl 600 mM daidzein stock solution (used in OD600 measurement for 1.0 mM daidzein treatment)
Blank solution 3	10 ml YEP plus 34 µl 600 mM daidzein stock solution (used in OD600 measurement for 2.0 mM daidzein treatment)

The *Agrobacterium tumefaciens* strain was EHA105 harboring a binary vector pCAMBIA2201. The initial *Agrobacterium* solution was prepared by inoculating a single colony in 30 ml of YEP medium containing 25 mg l^{-1} Rif and 50 mg l^{-1} kanamycin for 4 h with 200 rpm shaking at 28 °C. Daidzein was dissolved in DMSO to form a 600 mM stock and then added to YEP medium at low dosages

isoflavones were the obstacles to high efficient genetic transformation of soybean via the *A. tumefaciens*-mediated method and whether the inhibition of isoflavone biosynthesis by physical and chemical measures could decrease or eliminate its adverse effect on soybean genetic transformation and thereby improve upon transformation efficiency.

Materials and methods

Soybean cultivar Jihuang 13 was used as plant material. *A. tumefaciens* strain EHA105 (Hood et al. 1993) harboring a binary vector pCAMBIA2201 (GenBank: AF234314.1), containing both a GUS-intron gene and an NPTIIselectable marker, was used as the tested bacterium.

Effects of isoflavone on *Agrobacterium tumefaciens* growth

Agrobacterium tumefaciens strain EHA105::pCAMBIA2201 from a glycerol stock stored at −70 °C was incubated for 2–3 d at 28 °C on solid YEP medium (yeast extract 10 g l^{-1}, peptone 10 g l^{-1}, NaCl 5 g l^{-1}, pH 7.2) containing 50 mg l^{-1} kanamycin and 25 mg l^{-1} rifampicin until colonies appeared. A single colony was inoculated in 30 ml of liquid YEP medium containing 50 mg l^{-1} kanamycin and 25 mg l^{-1} rifampicin for 4 h with 200 rpm shaking at 28 °C. These *A. tumefaciens* cultures were used as the initial solution to evaluate the effects of isoflavones on *Agrobacterium* growth. Treatments and their preparation were as listed in Table 1. The *Agrobacterium* solutions with different concentration of daidzein were cultured with 200 rpm shaking at 28 °C and their absorbance at 600 nm were detected using a spectrophotometer (UV-3200, Mapada Instruments, China) for 38 h at 2 h intervals. YEP medium containing different concentrations of daidzein was used as blank in the OD_{600nm} measurements to eliminate the influence of daidzein on luminosity. Logistic equation of *Agrobacterium* growth was obtained by nonlinear regression analysis with SPSS11.0 software.

Respiration assay: *Agrobacterium* cultured overnight in YEP medium with approximately 0.8 OD_{600nm} was centrifuged at 5,000 rpm and 4 °C for 10 min. The pellet was washed with 0.9 % NaCl solution once and then re-suspended in physiological saline solution to reach a final concentration of 10 g l^{-1}. *Agrobacterium* respiration was assayed using an Oxygraph (Hansatech Oxygraph, Hansatech Instruments Ltd, Pentney, Norfolk, United Kingdom). The reaction systems include 1.8 ml of 0.1 M phosphate buffer, pH 7.2, 0.2 ml of 10 g l^{-1} glucose, and 0.5 ml of 10 g l^{-1}*Agrobacterium* suspension. Different concentration of daidzein was added to evaluate the effect of isoflavones on *Agrobacterium* respiration.

Plant explants preparation

Seeds were sterilized in a sealed desiccator with chlorine gas generated by the reaction between 100 ml of chlorine bleach and 4 ml 12 N HCl for 24 h and subsequently germinated on B5 medium (Gamborg et al. 1968) at 25 °C under 16 h/8 h light/dark photoperiod for 4 days. The cotyledonary node explants were prepared by removing the majority of the hypocotyl tissue approximately 0.5 cm below the cotyledonary node and removing the epicotyl and axillary buds about 1 mm above the cotyledonary node as described by Hong et al. (2007).

Soybean cotyledonary node transformation

Recovery from −70 °C glycerol stock and propagation of *A. tumefaciens* strain EHA105::pCAMBIA2201 in liquid YEP

medium were described above. When the OD_{600nm} had reached to 0.8, the *A. tumefaciens* cultures were centrifuged for 5 min at 5,000 rpm and re-suspended in infection medium, i.e., 1/10 liquid MS salt with B5 vitamins medium containing 2 g l^{-1} 2-(*N*-morpholine)-ethane sulphonic acid (MES), 0.05 mg l^{-1} benzylaminopurine (BA), 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g l^{-1} sucrose, and 40 mg l^{-1} acetosyringone (AS), adjusting OD_{600nm} to approx. 0.2–0.3. Soybean cotyledonary node transformations were prepared in groups: 15–20 explants were placed in a 50 ml glass tube containing 25 ml of infection medium and sonicated for 0 or 3 min in a bath sonicator KH2200B, then incubated for another 15 min at ambient temperature. Parallel experiments without *Agrobacterium* infection were conducted for evaluating the effects of antagonists of isoflavone synthesis and sonication on PAL activity and isoflavone accumulation. The explants were blotted dry on sterile Whatman paper to remove excess water before incubating for 3 days in darkness at 25 °C on solid 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. Fifteen explants were cultured on one plate per treatment with a minimum of three replicates. After co-culture, the explants were rinsed twice with sterile water and maintained on FNL medium (Samoylov et al. 1998) supplemented with 660 mg asparagine, 2 g l^{-1} MES, 0.2 mg l^{-1} BA, 0.1 mg l^{-1} Thidiazuron (TDZ), 0.2 mg l^{-1} 2,4-D, 30 g l^{-1} sucrose, and 4.5 g l^{-1} agar, and 500 mg l^{-1} cefotaxime pH 5.8 for 7 days at 25 °C under 16 h/8 h light/dark photoperiod. Shoot induction and rooting followed the methods described by Hong et al. (2007). 100 mg l^{-1} kanamycin were used to select the transformant. GUS activity in explants 3 days after transformation and newly regenerated plantlets were assayed histochemically as described by Jefferson (1987). Percentage response was determined as the number of cotyledonary nodes of staining blue with X-gluc at the regenerable area divided by the number of cotyledonary nodes assayed.

Effects of antagonists of isoflavone synthesis and sonication on PAL activity and isoflavone accumulation

Explants at different time points after infection (24 h for *Agrobacterium* infected explants, and multiple time points between 0 and 30 h for those not infected by *Agrobacterium*) were sampled for the assay of PAL activity and determination of isoflavones content. The PAL activity assay followed the method described in "Guide for modern plant physiology experiments" [eds. Institute of Shanghai plant physiology, Chinese academy of sciences, Shanghai plant physiology society (China) 1999]. Ten explants per treatment was ground into homogenate in 10 ml 0.1 M Boric acid buffer solution (pH 8.8) on ice, the homogenate was filtrated through double-layer gauze and centrifuged for 30 min at 13,000 rpm and 4 °C condition, the supernatant was used as raw enzyme extracts. The reaction was performed in a 5 ml volume with 0.01 M Boric acid buffer (pH 8.8), 0.1 M Phenylalanine and 100 μl enzyme extracts at 37 °C for 1 h. Parallel control without 0.1 M phenylalanine was included for each treatments. Their absorbance at 290 nm was determined with a spectrophotometer (UV-3200, Mapada Instruments, China). The enzyme unit was defined as the difference between A290 nm in test tube and that in control tube divided by 1,000, the PAL activity was calculated as enzyme unit per hour per mg protein. Isoflavones were extracted with 80 % ethanol and sonicated at 40 kHz for 30 min at 50 °C in a bath sonicator (KH2200B, Kunshan Hechuang, China). Its absorbance at 260 nm was detected with a spectrophotometer (UV-3200, Mapada Instruments, China) to calculate the content of isoflavones according to the standard curve made from genistin as the standard isoflavone.

Expression of genes important for isoflavone biosynthesis

Quantitative RT-PCR assays for *PAL*, *CHS*, *IFS1* and *IFS2* were performed involving *Agrobacterium*-infected cotyledonary node explants incubated for 5 h on co-culture medium. Five explants were frozen immediately, ground in liquid nitrogen, then total RNA was extracted using 2 ml RNArose LS Reagent (Watson Biotechnologies, China) as described by Liu et al. (2011). The RNA was digested with DNase I (RNase Free) for 30 min to remove the contamination of genomic DNA. The cDNA was converted from RNA by performing reverse transcription PCR (RT-PCR) with RNA PCR Kit (AMV) Ver. 3.0 according to the manufactures introduction (Takara, Dalian, China) and was used as template in real-time quantitative PCR. Two-step real-time quantitative PCR was performed using the comparative Ct method on the 7500/7500 Fast Real-Time PCR System (Applied Biosystems, USA) according to the manufacturer's instructions. The reactions were performed in a 20 μl volume with 0.25 μM primers. The nucleotides dNTP, *Taq* DNA polymerase, Mg^{2+}, SYBR Green fluorescent dye, ROX reference dye and PCR buffer were included in the 2× RealStar Power SYBR Mixture (GenStar Biosolutions, Beijing, China). The cycle condition was preheating at 95 °C for 30 s; 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The final dissociation stage was automatically run by the equipment to generate a melting curve and consequently verify the specificity of the amplification products. The relative quantification of gene expression was calculated as $2^{-\Delta\Delta C_T}$. The primer sequences were as

follows: PAL forward: 5′-ATTATGGATTCAAGGGAGCT-3′, PAL reverse: 5′-AATGAGGAAAGTGGAGGACA-3′; IFS1 forward: 5′-GGCCACCTTCACCTCTTAAA-3′, IFS1 reverse: 5′-AGCCGAAGGAGAGAGAGAATA-3′; IFS2 forward: 5′-CCCTTCATAGGACACCTTCATC-3′, IFS2 reverse: 5′-CATGGAGCCAAAGTAGAGAGAG-3′; CHS forward: 5′-GGTCAACCCAAGTCCAAGAT-3′, CHS reverse: 5′-GGCGAAGGCCTAATAGTTTAGT-3′; Actin forward: 5′-GTGTCAGCCATACTGTCCCCATTT-3′, Actin reverse: 5′-GTTTCAAGCTCTTGCTCGTAATCA-3′. Actin was used as the endogenous control gene.

Results

Isoflavones inhibited *Agrobacterium* growth

The maximum OD_{600nm} after 36 h of culture was decreased by 68.5 and 82.2 % for 1.0 and 2.0 mM daidzein treatments, respectively, compared to 0 mM daidzein treatment; lower concentration (0.17–0.34 % (v/v)) of solvent DMSO in YEP medium had negligible influence on *Agrobacterium* growth (Fig. 1a). The maximum velocity (V_{max}) of *Agrobacterium* growth was decreased by 81.8 and 92.6 %, compared to 0 mM daidzein treatment, for 1.0 and 2.0 mM daidzein treatments, respectively; the appearance time of V_{max} (T_{max}) was delayed by about 5 h (Table 2). The respiration rate of *Agrobacterium* was inhibited by 29.7 and 36.9 % for 1 and 2 mM daidzein treatments, respectively, relative to their solvent control (Table 3).

Antagonists of isoflavone synthesis and sonication interfered with isoflavone biosynthesis

Agrobacterium infection stimulated the expression of genes important for isoflavone biosynthesis (Fig. 2). The relative quantification (RQ) of gene expression after 5 h of infection, averaged between treatments with and without AOA addition, was increased by 56.8, 148.1, 70.6 and 118.3 %, for genes *PAL*, *IFS1*, *IFS2* and *CHS*, respectively, when compared with that not transformed control. Antagonists and sonication decreased the expression of genes important for isoflavone biosynthesis (Figs. 2, 3). Antagonists of 20 μM AOA treatments decreased the relative expression of the PAL gene by 31.1 %, the IFS1 gene by 43.8 %, the IFS2 gene by 14.3 %, and the CHS gene by 47.0 %, relative to that of on medium without AOA. Sonication at 40 kHz for 3 min along with *Agrobacterium* infection decreased the relative gene expression by 32.6, 35.3, 56.5,

Table 2 Effects of daidzein on the parameters of logistic equation of *Agrobacterium tumefaciens* growth

Parameters	0 mM daidzein	1 mM daidzein	2 mM daidzein
K	2.101	0.719	0.380
a	771.353	92.802	38.483
b	0.388	0.204	0.161
R^2	0.996	0.993	0.924
T_{max}	17.134	22.208	22.672
T_1	13.74	15.75	14.49
T_2	20.53	28.66	30.85
V_{max}	0.204	0.037	0.015
V_{lag}	0.032	0.010	0.006
V_{log}	0.179	0.032	0.013
V_{sta}	0.030	0.013	0.008

Logistic equation ($Y = K/(1 + ae^{-bt})$) was obtained by nonlinear regression analysis with SPSS11.0 software

V_{max} and T_{max} represent maximum velocity of bacterium growth and time point respectively. T_1 and T_2 represent the time points of beginning and end of rapid growth respectively. V_{lag}, V_{log} and V_{sta} represent the average velocity of bacterium growth at lag, logarithmic and stationary phase, respectively

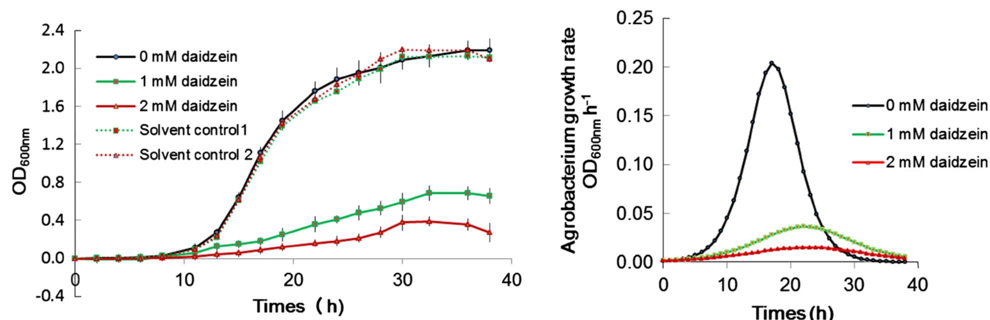

Fig. 1 The time course of *Agrobacterium* growth in YEP medium supplemented with different concentration of daidzein. *Left* demonstrate change of OD_{600nm} with time course; *right* demonstrate change of *Agrobacterium* growth rate with time course. Daidzein was dissolved in DMSO to form a 600 mM stock and then added to YEP medium at low dosages. The *Agrobacterium* solution with different concentrations of daidzein were cultured with 200 rpm shaking at 28 °C. YEP medium containing different concentrations of daidzein was used as blank in OD_{600nm} measurement. The data of OD_{600nm} were collected from three replications and the *whiskers* represent the standard deviation about the mean. The *Agrobacterium* growth rate was obtained by the first order derivative of logistic regression equation

Table 3 Effects of daidzein on the respiration rate of *Agrobacterium tumefaciens*

	Rate of respiration (μmol O_2 g^{-1} min^{-1})	Ratio of inhibition (%)
Positive control (0 mM daidzein)	2.96 ± 0.01[a]	
Solvent control 1(0.17 % (v/v) DMSO)	2.96 ± 0.22[a]	
Solvent control 2 (0.24 % (v/v) DMSO)	2.95 ± 0.18[a]	
1 mM daidzein treatment	2.08 ± 0.13[b]	29.7
2 mM daidzein treatment	1.86 ± 0.16[b]	37.2

Daidzein was dissolved in DMSO to prepare a 600 mM daidzein stock solution and then added to the measure systems at low dosages. The same dosage of DMSO (0.17 % (v/v) to 0.34 % (v/v) as daidzein treatment was used as solvent control. Data for respiration were collected from three replications and presented as the mean ± SD. Superscript letters indicate the significant differences ($P < 0.05$) according to the Fisher's Least Significant Difference (LSD) test

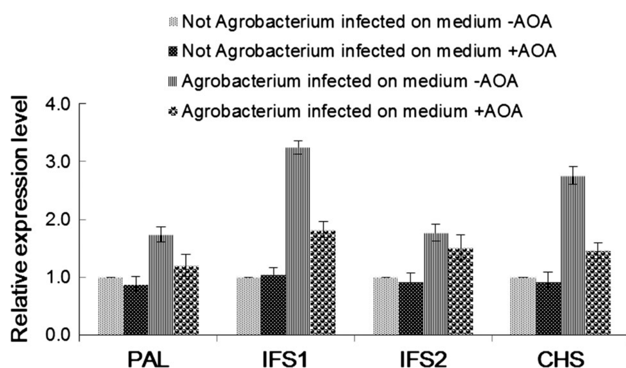

Fig. 2 Effects of α-aminooxyacetic acid (AOA) on expression of genes important for isoflavone biosynthesis. Samples were collected from three biological replications after Agrobacterium infection and incubated for 5 h on co-culture medium. Real-time quantitative PCR was performed using the comparative Ct method on the 7500/7500 Fast Real-Time PCR System (Applied Biosystems, USA) according to the manufacturer's instructions. Actin was used as the endogenous gene control. *Bars* represent the mean data of relative quantification and *whiskers* represent the SD

and 21.8 %, for the genes *PAL*, *IFS1*, *IFS2* and *CHS*, respectively, relative to that of *Agrobacterium* infection only.

Antagonists of isoflavone synthesis AOA and sonication treatment decreased PAL activity relative to the control (Table 4). The highest PAL activity of control plants was at 12 h after treatment and was 44.1 % higher than that of at 0 h. The peak of PAL activity was not obvious for 20 μM AOA treatments, with only a 5.8 % increase at 6 h after treatment, relative to that of at 0 h; then the PAL activity decreased gradually to 83.1 % (at 24 h) and 73.9 % (at 30 h) of that at 0 h, respectively. The PAL activity of sonication treatment remained lower level and no obvious peak appeared throughout the tested period. *Agrobacterium*

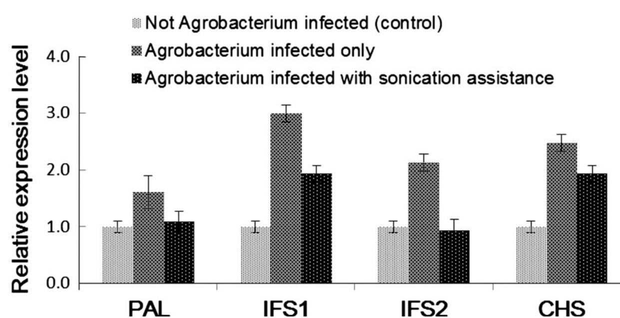

Fig. 3 Effects of sonication on expression of genes important for isoflavone biosynthesis. Samples were collected from three biological replications after Agrobacterium infection and Incubated for 5 h on co-culture medium. Real-time quantitative PCR was performed using the comparative Ct method on the 7500/7500 Fast Real-Time PCR System (Applied Biosystems, USA) according to the manufacturer's instructions. Actin was used as the endogenous gene control. *Bars* represent the mean data of relative quantification and *whiskers* represent the SD

infection stimulated the increment of PAL activity by 62.6 % and increased isoflavone content by 4.7 % after 24 h of treatment, averaged among treatments of with (without) AOA and sonication, compared to that not infected with *Agrobacterium* (Table 5). Antagonists 20 μM AOA treatment decreased the PAL activity by 25.6 % and isoflavone content by 5.9 % on average compared to that without AOA treatment. Sonication at 40 kHz for 3 min decreased the PAL activity by 4.0 % and isoflavone content by 22.8 % on average compared to that without sonication treatment.

Inhibition of isoflavone biosynthesis improved soybean transformation

The inhibition of isoflavone biosynthesis by either antagonists or sonication increased the T-DNA delivery

Table 4 PAL activity of soybean cotyledonary nodes at different time point after antagonist or sonication treatment

Times (h)	PAL activity (U mg^{-1} protein h^{-1})		
	Control	20 μM AOA	Sonication (40 kHz, 3 min)
0	121.2 ± 22.1[c]	121.2 ± 22.1[a]	121.2 ± 22.1[a]
6	151.6 ± 11.4[b]	128.2 ± 12.5[a]	110.6 ± 7.0[b]
12	174.5 ± 10.5[a]	113.7 ± 9.9[b]	109.8 ± 6.9[b]
24	146.5 ± 6.1[b]	100.7 ± 7.8[c]	113.2 ± 6.4[b]
30	126.3 ± 7.8[c]	89.6 ± 6.4[c]	106.1 ± 5.6[b]

The explants were not infected by *Agrobacterium* and were cultured in darkness on medium containing 1/10 MS salt plus B5 vitamins with or without antagonists AOA. Data were collected from three 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

Table 5 Effects of *Agrobacterium* infection, antagonists of isoflavone biosynthesis and sonication treatment on soybean PAL activity and isoflavones accumulation

Treatment		PAL activity (U mg^{-1} protein h^{-1})	Isoflavones content (mg kg^{-1} DW)
Not infected with *Agrobacterium*	−AOA −sonication	146.5 ± 6.1^{c}	3.7 ± 0.1^{ab}
	+AOA −sonication	100.7 ± 7.8^{de}	3.6 ± 0.0^{b}
	−AOA +sonication	113.2 ± 6.4^{d}	2.8 ± 0.1^{c}
	+AOA +sonication	93.4 ± 5.3^{e}	2.8 ± 0.1^{c}
Infected with *Agrobacterium*	−AOA −sonication	230.4 ± 11.8^{a}	4.0 ± 0.0^{a}
	+AOA −sonication	163.1 ± 9.6^{c}	3.6 ± 0.3^{b}
	−AOA +sonication	193.2 ± 16.4^{b}	3.1 ± 0.2^{c}
	+AOA +sonication	151.2 ± 13.2^{c}	2.8 ± 0.2^{c}

−AOA means no AOA was added in the infection and co-culture medium. +AOA means 20 μM AOA was added in the infection and co-culture medium. −sonication means no sonication was used during *Agrobacterium* infection. +sonication means there was sonication along with *Agrobacterium* infection at 40 kHz for 3 min. Data were collected 24 h after treatment from three 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

efficiency as evidenced by transient GUS expression (Table 6). When the cotyledonary nodes were infected with *Agro* strain EHA105::pCAMBIA2201 without sonication and co-cultured on AOA deficient medium, an average of 9.0 % explants displayed GUS activity. The percentage of GUS transient expression for 20 μM AOA treatment was 2.1-fold that of without AOA treatment, averaged between treatments with and without sonication; the data for sonication treatment was averagely 1.9-fold that of no sonication treatment. A spectacular enhancement of GUS expression was observed when sonication was applied along with *Agrobacterium* infection and the explants were cultured on co-culture medium containing 20 μM AOA, with the percentage of GUS transient expression as 41.41 %, being 3.6 times higher than that without sonication and AOA treatment. Different effects on following tissue proliferation and transformant selection were observed for sonication and antagonists AOA treatment, respectively. Sonication treatment at 40 kHz for 3 min resulted in about 30 % explants loss of regenerative ability due to sonication, while, 20 μM AOA treatment increased the percentage of explants with regenerated shoot buds by about ten percentage points (Table 6). Results of GUS staining indicated that GUS gene was not only transiently expressed in explants but also stably expressed in regenerated shoot buds (Fig. 4). The effects of antagonists and sonication on stable transformation need to be further evaluated.

Discussion

Transgenic soybean plants were produced via both *Agrobacterium*-mediated genetic transformation (Hinchee et al. 1988) and particle bombardment (McCabe et al. 1988). Nevertheless, works aimed to improve upon soybean transformation efficiency did not bring major breakthroughs during the following two decades (Yamada et al. 2010, 2012; Wiebke et al. 2011; Mariashibu et al. 2012). This status forced us to think about the mechanism underline the recalcitrant transformation of soybean. It was speculated that plant–bacterium interactions might prohibit or negatively affect the transformation process (Mello-Farias and Chaves 2008), and better understanding the ongoing molecular battle between agrobacteria and attacked hosts provide a breakthrough toward developing transformation protocols for recalcitrant plant species (Andrea Pitzschke 2013).

Many genes in isoflavone secondary metabolism are involved in plant defense responses (Larkin 2001), the best-characterized plant defense genes are members of the CHS family. Wounding and *Agrobacterium* infection could enhance the isoflavone metabolic pathway. Wingender et al. (1989) once detected enhanced CHS expression in sterile soybean roots infected with *Agrobacterium* C58 with maximum expression at 4–8 h after infection. In the present study, we detected enhanced expression of *PAL*, *IFS1*, *IFS2*, and *CHS* gene important for isoflavone biosynthesis due to wounding and *Agrobacterium* infection. The enhanced expression of defensive genes may well explain the low efficiency in soybean transformation. Soybean isoflavones play diverse roles in plant–microbe interactions (Subramanian et al. 2005). Isoflavones inhibited *A. tumefaciens* growth and respiration, the V_{max} of *Agrobacterium* growth was decreased by more than 95 % and *Agrobacterium* respiration was inhibited by 35 % above with 2 mM daidzein treatment. The T_{max} was delayed about 5 h by 1–2 mM daidzein treatments, implying that extending the co-culture time to three more days may further improve the efficiency of transformation. Whether the inhibition of isoflavone biosynthesis and by what could decrease or eliminate its adverse effect on soybean transformation? Silencing the isoflavone synthase (IFS) gene or chalcone reductase (CHR) in soybean roots led to a nearly complete (95 %) suppression of all isoflavone metabolites in roots (Graham et al. 2007), and enhanced susceptibility to *Phytophthora sojae*

Table 6 Effects of antagonists of isoflavone synthesis and sonication on plant regeneration and transformation efficiency

Treatment	No. explants infected	No. explants with GUS focus	Percent of GUS transient expression[a]	No. explants infected	No. explants regenerated shoot buds	Percent of explants regenerated shoot buds[b]	No. shoot buds with GUS focus*
−AOA −sonication	134	12	9.0 ± 2.9^{c}	119	83	69.7 ± 5.8^{b}	0 (20)
−AOA +sonication	132	30	22.5 ± 2.1^{b}	80	35	43.8 ± 4.3^{c}	3 (20)
+AOA −sonication	159	40	25.2 ± 2.3^{b}	110	94	85.5 ± 6.4^{a}	7 (26)
+AOA +sonication	169	68	41.4 ± 2.1^{a}	139	70	50.4 ± 3.9^{c}	11 (30)

−AOA means no AOA was added in the infection and co-culture media. +AOA means 20 μM AOA was added in the infection and co-culture medium. −sonication means no sonication was used during *Agrobacterium* infection. +sonication means sonication at 40 kHz for 3 min along with *Agrobacterium* infection. a: 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. b: the data was collected after 1 month selection on medium containing 100 mg l^{-1} Kanamycin. 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 ($P < 0.05$) according to the Fisher's Least Significant Difference (LSD) test (n = 3). * data in bracket represent the number of buds assayed

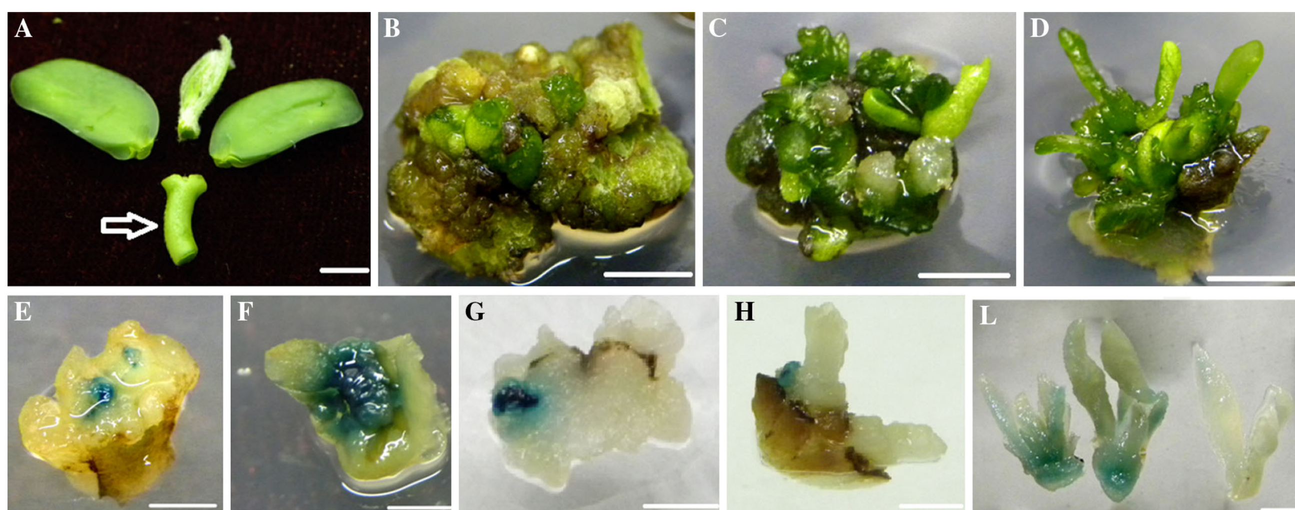

Fig. 4 Transformation of soybean cotyledonary nodes with Agrobacterium strain EHA105::pCAMBIA2201. **a** Explant preparation, *arrow* indicates explant. **b–d** Shoot induction on selection medium. **e**, **f** Transient expression of GUS after 3 days co-culture. **g**, **h** and **l** Stable expression of GUS in regenerated new buds (the *right* one in **l** was from not transformed control). *Bars* represent 3 mm

(Subramanian et al. 2005; Graham et al. 2007). Both physical (sonication) and chemical (antagonists) measures were adopted in this study to inhibit soybean isoflavone biosynthesis. The results showed that sonication and antagonists of isoflavone synthesis AOA were all found to be effective in decreasing the expression of genes governing isoflavone biosynthesis and PAL activity, also significantly decreased isoflavones accumulation. The isoflavone content was decreased by 5.9 % with 20 μM AOA treatment and 22.8 % with sonication at 40 kHz for 3 min treatment. Significant negative correlations were observed between the percentage of GUS transient expression and the relative quantification of gene expression as well as the PAL activity (Table 7), the isoflavone content was also negatively correlated with the transformation efficiency but didn't achieved significant level (r = −0.8819, n = 4). The results implied that decreasing the intensity of defense reaction during *Agrobacterium* infection is more important than lowering isoflavone content itself.

Sonication has been used to enhance *Agrobacterium*-mediated transformation in a few different plant species (Trick and Finer 1997; Meurer et al. 1998; Bakshi et al. 2011; Subramanyam et al. 2011; Dutta et al. 2013; Teixeira da Silva and Dobránszki 2014; King et al. 2014). It was

Table 7 The coefficient of correlation between the transformation efficiency and the relative quantification of genes expression and isoflavone content

	PAL RQ	*IF1* RQ	*IF2* RQ	*CHS* RQ	PAL activity	Isoflavone content	Transformation efficiency
PAL RQ	1	0.9792**	0.8108	0.9549**	0.9547**	0.7342	−0.9674**
IF1 RQ		1	0.9096*	0.9132*	0.9421*	0.8568	−0.9985***
IF2 RQ			1	0.7506	0.7546	0.9839**	−0.9309*
CHS RQ				1	0.8259	0.6326	−0.9023*
PAL activity					1	0.7207	−0.9266*
Isoflavone content						1	−0.8819
Transformation efficiency							1

The transformation efficiency was defined as the percentage of explants with GUS activity. The relative quantification of genes expression was assayed 5 h after treatment. The PAL activity and isoflavone content was determined 24 h after treatment. * Significant at $P < 0.10$; ** Significant at $P < 0.05$; *** Significant at $P < 0.01$ (n = 4)

generally agreed that enhanced transformation from using SAAT most likely results from the micro-wounds both on the surface of and deep within the target tissue, which permit *Agrobacterium* to penetrate deep within the plant tissue and infect a large number of plant cells (Santarém et al. 1998; Finer and Finer 2000). The micro-wounds may aid in releasing compounds to facilitate growth and the accumulation of bacteria (Finer and Finer 2000). The candidate metabolites were focused on phenolic compounds (Song et al. 2013a, b). SAAT treatment was not effective at post co-cultivation period with decreased shoot proliferation from cotyledonary node of some soybean genotypes (Meurer et al. 1998). This might attribute to a not appropriate ultrasound intensity and duration. The appropriate intensity and duration depend on species and tissue types. Sonication at 50 kHz for more than 10 s resulted in tissue turned white and death a few days after sonication in immature pods of soybean (Santarém et al. 1998). Increased GUS-stained spots were observed, but few explants survived to 10 days when the cotyledonary nodes of mature soybean seeds were sonicated for 600 s (Meurer et al. 1998). The standardized sonication time was determined as 2 s for immature cotyledons (Santarém et al. 1998) and 6 s for mature cotyledons (Meurer et al. 1998) based on the transient GUS expression and continued tissue proliferation. The sonication treatment was implemented at 40 kHz for 3 min in this study and resulted in a 1.9-fold increase of transient GUS expression and about 30 % explants loss of regenerative ability due to sonication at the same time (Table 6), a rather longer sonication duration and lighter tissue damage than that previously reported (Santarém et al. 1998). This may be attributed to the different intensity and direction of the ultrasound wave. The waterbath sonicator used in this study was 40 kHz with only vertical wave from the bottom; its ultrasound is weaker than that of sonicator (Model PC5) which was 55 kHz with both vertical and horizontal wave output (Santarém et al. 1998; Meurer et al. 1998; Subramanyam et al. 2011). The tissue type may also affect the sonication effects and need further experimentation. Sonication treatment decreased the enhanced expression of the *PAL*, *IFS1*, *IFS2* and *CHS* genes, PAL activity, and isoflavones accumulation owing to wounding and *Agrobacterium* infection and promoted the transient GUS expression, indicating that sonication may not simply provide micro-wounds for *Agrobacterium* to penetrate or release phenolic compounds to induce *A. vir* gene expression, but it may disturb the synthesis of isoflavones at the transcription level, which decreases the adverse effects of isoflavones on soybean transformation. Expression profiling by RNA-Seq (Quantification) analysis indicated that 3,524 (1,538) genes were down-regulated (up-regulated) by inhibition of isoflavone biosynthesis. These differentially expressed genes involved into three GO categories: cellular component, molecular function, and biological process. KEGG Pathway enrichment analysis showed that the DEGs were enriched in fatty acid biosynthesis, flavone and flavonol biosynthesis, flavonoid biosynthesis, phenylpropanoid biosynthesis, propanoate metabolism, pentose and glucuronate interconversion, DNA replication, etc. (unpublished data). These results were generally in consistent with the view points of the present study that sonication and antagonists interfered with isoflavone biosynthesis. Sonication may also involve the regulation of circadian rhythm-plant (unpublished data).

In summary, higher content of isoflavones in soybean was the major obstacles to achieve high efficient *Agrobacterium*-mediated soybean transformation and negatively correlated with the percentage of GUS transient expression. Blocking the pathway of isoflavone synthesis decreased its adverse effects on soybean transformation. The most effective measure was sonication at 40 kHz for 3 min along with *Agrobacterium* infection and 20 μM AOA antagonists was added in the infection and co-culture medium. A synergistic effect was observed by the combined use of sonication and antagonists AOA, with the

percentage of GUS transient expression as 41.4 %, being 3.6 times higher than that not sonicated and co-cultured on AOA deficient medium. Sonication was found not simply created micro-wounds for *Agrobacterium* to penetrate or releasing phenolic compounds to induce *A. vir* expression, but rather it disrupted the synthesis of isoflavones at transcription level and decreased the adverse effects of isoflavones on soybean transformation, and thereby improved soybean transformation efficiency.

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

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