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Improving crops genome through genetic engineering of the key metabolic pathways

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ABSTRACT. Soybean loss due to pests and pathogens is a serious problem worldwide. Soybean producers have few options to manage diseases caused by general pathogens where major genes for full resistance have not been discovered. The innate defense of soybean plants could be enhanced by improving content and composition of lignin by genetic engineering of the phenylpropanoid pathway. We used a novel technique of germ-line genetic transformation of soybean plants via natural pollen tubes as vectors. This technique uses *Agrobacterium tumefaciens* to mediate transfer of genes of interest to the zygote to introduce the key lignification genes (PtMYB4, PAL5, F5H, CAD1) into soybean genome. We observed 5.6% average transformation efficiency in the first generation of transgenic plants and in the second generation the presence of the transgene constructs was confirmed in more than 50% (for CsVMV/PtMYB4sens, 35SVTM/PAL5, C4H/F5H, CsVMV/CAD1 constructs) transgenic soybean lines. We confirmed the expression of the introduced genes at transcriptional level using RT-PCR and Northern blot. Functional analysis using lignin content determination and the activity of PAL5 and CAD1 enzymes demonstrated that the transgenes perform their function *in planta*. The proposed technique is effective and inexpensive and can be used to create novel stress and disease resistant soybean genotypes.

Keywords: germ-line genetic transformation; lignification; soybean; biotic stress resistance.

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Introduction

The world-wide soybean (*Glycine max*, L.) production has increased several fold starting from 1960s' and currently soybean is one of the largest source of vegetable oil and animal feed (Pagano & Miransari, 2016). The demand for soybean will increase in future due to the rising population and the increased demand in both animal feed and products for human consumption. Growing of soybean meets a number of challenges such as biotic stress (micropathogens, pests and weeds). It is estimated that only in the US soybean diseases decrease the yield by up to 11% (Hartman et al., 2016).

Currently, there are no individual soybean genes related to the resistance to single micropathogenes, although the search for the quantitative trait loci associated with the resistance continues (Kandel et al., 2018; Zatybekov, Abugalieva, Didorenko, Rsaliyev, & Turuspekov, 2018). A large number of genes are associated with abiotic and biotic stress resistance in plants (Cabane, Afif, & Hawkins, 2012; Pérez-Clemente et al., 2013; Tran & Mochida, 2010). They are related to the various metabolic and signaling pathways including reactive oxygen species signaling (Baxter, Mittler, & Suzuki, 2014), plant hormones, inorganic ion fluxes (Kissoudis, Van de Wiel, Visser, & Van der Linden, 2014) as well as transcription factors (Birkenbihl, Liu, & Somssich, 2017). To date, genetic manipulation of a single or a few genes involved in signaling/regulatory pathways are used to improve environmental stress resistance of soybean plants (Homrich, Strohm, Weber, & Zanettini, 2012).

Lignin is a phenolic compound providing strength to the secondary cell wall in vascular plants, that is assigned to a broad range of physiological processes participating in plant growth, providing the rigidity to the cell walls, the natural mechanical barrier and defense against pathogen penetration. It acts as a barrier against pathogen penetration as well as a toxins produced by the pathogens, promoting plant immunity

(Lee et al., 2019; Sattler & Harris, 2013). Lignin is produced by phenylpropanoid pathway that provides monolignol compounds such as p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol which are precursors of p-hydroxyphenyl (H), guaiacyl (G), syringyl (S) monomeric units of lignin, respectively. Lignin composition was shown to change as a response to abiotic and biotic stresses (Liu, Luo, & Zheng, 2018; Moura, Bonine, Viana, Dornelas, & Mazzafera, 2010). The molecular mechanisms of lignin biosynthesis are well studied in model organisms such as arabidopsis (*Arabidopsis thaliana*) and poplar (*Populus* spp.) (Bonawitz & Chapple, 2010; Vanholme et al., 2012) as well as in some agricultural plants: rice (*Oryza sativa*) (Gui, Shen, & Li, 2011), grapevine (*Vitis vinifera*) (Cavallini et al., 2015) and others. The first step of lignin synthesis is deamination of phenylalanine by PAL enzymes to produce cinnamic acid – a step that is common between all metabolites of the phenylpropanoid pathway. Cinnamic acid is further converted in p-coumaric acid by C4H (cinnamate 4-hydroxylase) enzyme. Intermediate products are processed by other enzymes such as 4CL (4-coumarate-CoA ligase), COMT (caffeic acid methyltransferase), F5H (ferulate 5-hydroxylase) to synthesize aldehyde precursors of coniferyl and synapil alcohols. These are further reduced to the respective alcohols by CAD enzymes (cinnamyl alcohol dehydrogenases) (reviewed in Barros, Serk, Granlund, & Pesquet, 2015). Lignin synthesis is regulated by a number of transcription factors such as R2R3-MYB family (Bonawitz & Chapple, 2010). In *Arabidopsis thaliana* MYB46 and MYB83 were shown to regulate the key lignin synthesis genes (Kim, Kim, Ko, Kang, & Han, 2014). Similar functions are observed for *Pinus taeda* MYB46 homologs (Patzlaff et al., 2003).

Additionally, phenylpropanoid pathway is known to produce a large number of secondary metabolites that include flavanols, isoflavanoids, anthocyanins (Vogt, 2010). Some of these metabolites are demonstrated to be components of plant innate immunity, preventing the spread of the pathogens (Piasecka, Rey, & Bednarek, 2015). Genetic manipulation of the phenylpropanoid pathway may not only alter lignin synthesis but also enhance plant innate immunity. Therefore one of the strategies to increase the resistance of soybean plants is to increase the expression of genes involved in lignin biosynthesis (Lozovaya et al., 2007; Lozovaya, Lygin, Zernova, & Widholm, 2005; Lygin et al., 2013, 2010; Lygin, Rahman, Ulanov, Widholm, & Lozovaya, 2012). This approach aims to produce biotech products of second generation (Pérez-Clemente et al., 2013). These plants be more resistant to stress and could provide an increased yield, in comparison with biotech crops of first generation, such as Roundup Ready crops.

The most common techniques for soybean genetic transformation are particle bombardment (McCabe, Swain, Martinell, & Christou, 1988) and transformation mediated by *Agrobacterium tumefaciens* (Yin & Zhang, 2010). Transformation of mature cotyledonary node or cotyledonary explants derived from mature soybean seed is currently regarded as the most effective methods (Li et al., 2017; Paz, Martinez, Kalvig, Fonger, & Wang, 2006). However, these methods have some critical disadvantages: they are genotype specific and include an expensive tissue culture step and plant regeneration *in vitro*, which takes about one year before transgenic first generation plants (T1) are recovered and in most cases zero generation (T0) plants are weak and require careful handling.

Several approaches are used to omit the complicated tissue culture step. One of them is floral dip method primarily used for *A. thaliana* transformation (Clough & Bent, 1998). In this method developing floral tissues are dipped into a solution containing *A. tumefaciens,* as well as surfactant and sucrose. However, this method is not suitable for legumes due to the flower structure. An alternative method is pollen-tube pathway transformation technique which is based on the removal of the recipient plant's stigma shortly after pollination and introduction of an exogenous DNA solution into the severed style of the recipient plant (Ali, Bang, Chung, & Staub, 2015). Pollen-tube pathway soybean transformation was described by a number of authors however, it seems to be not efficient and irreducible (Liu, Su, An, & Yang, 2009; Li & Wu, 2007; Li, Nelson, Widholm, & Bent, 2002; Shou, Palmer, & Wang, 2002).

In this paper we propose a novel technique of germ-line genetic transformation via natural pollen tubes using *A. tumefaciens* mediated DNA transfer. We aim to use this method to target lignin content and composition in soybean plants which can significantly improve basic resistance to soybean pathogens. The ultimate goal of this work is to produce new strains of soybean for breeders and biodiversity with improved biotic, abiotic stress resistance and yield enhancement.

Material and methods

Plant material

Three cultivars of USA breeding: Jack and Jack x 4 (resistant to biotic stress), and Spencer (sensitive to micropathogens infection), 7 varieties of Kazakhstan breeding (Kazakhstan, Eureka, Vita, Tazhan, Zara, Perizat, Dannaya) were used for introduction of lignification genes. Ten biological replicates of each homozygous transgene and wild-type were grown simultaneously in a random block design, in the same environment in greenhouse. Plants were grown under long-day conditions (16 hours light, 28°C, and 65% humidity) to allow the development of health soybean plants.

Molecular vectors

Germ-line transformation was performed using *A. tumefaciens*, strain EHA 105 transformed with following vectors (Table 1).

Table 1. Vectors used for genetic transformation

Construct name	Gene of interest	Origin of the gene of interest	Promoter	Backbone	Source
CsVMV/PtMYB4sens*	PtMYB4	*Pinus taeda*	CsVMV	pILTAB357	(Patzlaff et al., 2003)
35SVTM/PAL5*	PAL5	*Phaseolus vulgaris*	35SVTM	pBI121, pBI 101	(Ritter & Schulz, 2004; Wanner, Li, Ware, Somssich, & Davis, 1995)
C4H/F5H*	F5H	*Arabidopsis thaliana*	C4H	pBI121	(Meyer, Cusumano, Somerville, & Chapple, 1996; Meyer, Shirley, Cusumano, Lelong, & Chapple, 1998)
CsVMV/CAD1*	CAD1	*Arabidopsis thaliana*	CsVMV	pBI121 and pBI101	(Raes, Rohde, Christensen, Van De Peer, & Boerjan, 2003)

*The genetic constructs of lignification genes were kindly presented by Professor J.M. Widholm laboratory, Crop Science Dep., UIUC, USA.

Transformation in *A. tumefaciens* or *E. coli* (for glycerol plasmid stocks and plasmid propagation) was performed using electroporation.

Preparation of *Agrobacterium tumefaciens* suspension for flower stigma pipetting

Single colony of transformed *A. tumefaciens* was transferred from Petri dishes to 3 mL of LB medium with 100 mg L^{-1} kanamycin (Sigma Aldrich), and incubated for 1 – 1.5 days at 28°C on a shaker at 150 rpm. Cells were harvested by centrifugation at 4500 g for 10 minutes and resuspended in LB medium without antibiotics. Pipetting of flower stigma was performed with the bacterial suspension 10^{10} cells mL^{-1} (equal OD=1 when measured at ƛ=600 nm using Beckman Coulter DU 800 spectrophotometer (USA)). Surfactant Pluronic F68 (0.001%) (Thermo Fisher) was added to bacterial suspension to activate vir-zone of T-plasmid and enhance transformation efficiency.

Agrobacterium tumefaciens suspension pipetting in soybean flower stigma

Soybean flower petals were separated and flower was cut in the carpel remove 1/3 of stigma with small scissors. About 2 µL of *A. tumefaciens* suspension transformed with a vector containing a gene of interest was placed in the center of the open flower to the stigma by a microsyringe or a micropippete (Figure 1a). After 30 minutes flowers were inspected and if the suspension was not enough the second pipetting was performed. Treated flowers were marked by color threads correspondent to the genes of interest (Figure 1b). Pipetting time was optimized to introduce transformed *A. tumefaciens* at 3-4 stages of soybean flower development, when the anthers were fully formed and pollen tubes are growing into mature, but not yet dividing zygote (Figure 1c). To synchronize pipetting time all soybean flowers opened the day before were removed at 8:00. It was determined that the stigma allocation of substances that stimulate the germination of pollen starts at 9:00, at around 9:15 pollen is attached to the stigma and the pollen germination begins and continues till 9:30 (Figure 1d-f). Therefore, the best time for pipetting in a greenhouse conditions (at 28°C) was 9:00 am – 12:00 pm. In the field the time range can be 11:00 – 13:00 the same day, when the flowers have opened and the resulting pollen tubes are able to pass the desired genetic material (at the time of the pollen activity maximum). The timing of pipetting in the field may be dependent on temperature and solar radiation.

Figure 1. *Agrobacterium tumefaciens*-mediated soybean germ-line genetic transformation. a The process of soybean flower pipetting with *A. tumefaciens* - suspension containing constructs with the genes of interest. b Marking of treated flowers by colored threads. c Scheme of stages of soybean flower and stigma development, adapted from https://www.agrodialog.com.ua/biologiya-cveteniya-soi.html. d - f – anatomy of soybean pollination (magnification 400x): d - stigma allocation of substances that stimulate the germination of pollen, 9:00 am; e - pollen attachment to the stigma and the beginning of pollen germination, 9:15 am; f - germination of the pollen tube, 9:30 am

Molecular analysis

Plasmid DNA was extracted from an overnight culture by using QIAprep Spin Mini prep Kit QIAGEN (Qiagen, Valencia, CA, USA). Genomic DNA was extracted from fresh mature but young transgenic soybean leaves using the method (Weigel & Glazebrook, 2009) and was used in PCR. Following primers were used (Table 2).

Table 2. PCR primers used for the study

Target sequence (gene)	Primers	Fragment length
PtMYB4	forward 5'- AGG ATA CAA CAG AGA – 3' reverse 5'- GGA TCC TCA CAG AAG CCG TGG AAG ATA - 3'	1,000 bp
PAL5	forward 5'- TGG CAG ACA TCA CTT CAG ACA GCA CAG C -3 reverse 5'- AAG CCG CTC ATT TCA GTT CCA T - 3'	1,500 bp
F5H	forward 5'- CCA TTA TAG TTT GTG TAT CCG – 3', reverse 5'- CTT ACA AGA AAT TTT CGA -3'	1,000 bp
NPTII	forward 5'- ATC TCA CCT TGC TCC TGC – 3', reverse 5'- ATA CCG TAA AGC ACG AGG - 3'	1,000 bp
LicB reporter	forward 5'- GTC GTA AAT ACG GCC TTT TGT T GCA – 3' reverse 5'- GTT AGG ATA GTA TTT TAC ATAT TCG - 3'	700 bp
T-nos terminator	forward 5'- CGA TAA TTT ATC CTA GTT TGC GCG – 3', reverse 5'- TGA ATC CTG TTG CCG GTC TTC - 3'	200 bp

Transgene presence in plant leaves tissues was confirmed by the PCR reaction using Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA) with denaturation at 94 °C (1 min.), annealing at 48°C or 54°C (40 s) for PtMYB4., PAL5, F5H, CAD1 constructs and extension at 72°C (90 s), for 35 cycles.

Multiplex PCR was performed similarly, with the exception of the temperature of the annealing stage (60°C).

RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) was used for extraction of total RNA from examples. DNA was digested with DNase I, using the TURBO DNA-free kit (Ambion/Life technologies, Carlsbad, CA, USA). cDNA was synthesized using oligo (dT) primer and reverse transcriptase enzyme, Promega GoScript kit, (Madison, WI, USA) for positive control or excluding reverse transcriptase enzyme as a negative control for genomic DNA contamination. To amplify PtMYB4, PAL5, F5H and CAD1 transcripts from cDNA same primers as for genomic DNA detection were used.

Southern and northern blot

Total genomic DNA of transgenic plants was digested with *Bam*HI endonuclease (Fermentas, ThermoFisher, USA). Fragment size was 1,5 kB for PAL5 transformants and 1,2 kb for CAD1 transformants. DNA was precipitated in isopropanol and sodium acetate solution, precipitate was washed with ethanol and dissolved in water. After DNA electrophoresis DNA was transferred to a nylon membrane (Amersham, UK) using denaturation alkaline buffer (1 N NaOH + 0.4 M NaOH + 0.6 M NaCl). Membrane was dried for 30 min., DNA was fixed in UV-light (265 nm) for 5 minutes. Membrane was used for subsequent hybridization with P^{32}-labeled probes prepared from PAL5 and GUS PCR products (as described in molecular analysis section) using NEBlot® Kit (New England Biolabs, Ipswich, MA, USA). After a prehybridization at 50°C hybridization was performed for 16h at 50°C in hybridization oven (Amersham, UK). Membrane was washed with 2 times in 1X SSC and 0.1% SDS at 50°C for 15 minutes and 3 times in 0.2X SSC and 0.1% SDS at 58°C. The intensity of radioactive label was measured using radioactivity counter. Exposure of the membrane with the X-ray film (Hyperfilm TMMP, Amersham, UK) was carried out with an amplifying screen. Same membrane was hybridized with the *gus*-probe.

For Northern blot total RNA was extracted from the same plants using guanidinium thiocyanate method. 5 ug RNA was subjected to agarose gel electrophoresis and was transferred to a nylon membrane (Amersham, UK). Membrane was prepared similarly to the membrane used for Southern blotting. Membrane was used for subsequent hybridization with P^{32}-labeled probes. Probes were prepared from PCR products (as described in the molecular analysis section) using NEBlot® Kit (New England Biolabs, Ipswich, MA, USA). Membrane was washed with 0.2X SSC and 1% SDS at 58°C for 1 hour.

Lignin analyses

Lignin content and composition was performed as described in (Van Acker et al., 2013). The lignin composition was investigated with thioacidolysis (Robinson & Mansfield, 2009). The monomers involved in β–O–4-ether bonds, released upon thioacidolysis, were detected with gas chromatography (GC) as their trimethylsilyl (TMS) ether derivatives on a Hewlett-Packard HP 6890 Series GC system (Agilent, Santa Clara, CA, USA) coupled with a HP-5973 mass-selective detector. The GC conditions were as described in the protocol. The quantitative evaluation was carried out based on the specific prominent ions for each compound. Response factors for H (hydroxyphenyl), G (guaiacyl), and S (syringyl) units were taken from. The sum of H, G, and S is a good estimate of the total thioacidolysis yield and, thus, the condensation degree of the lignin polymer.

Enzyme assays

PAL activity was determined by the conversion of L-phenylalanine to trans-cinnamic acid essentually as described previously (Edwards & Kessman, 1992) with a few modifications: 300 mg of fresh leaves were ground in liquid nitrogen and extracted with 1200 µL of the extraction buffer, containing 0.05 M TrisHCl (pH-8.8), 0.5% ascorbate, 10% glycerol and 10 mM 13-mercaptoethanol. 100 µL of crude enzyme extract was combined with 400 µL of 0.05 M Tris buffer (pH 8.8), containing 0.2 mM phenylalanine as substrate. The reaction mixture was incubated for 30, 60, 120 and 180 min. at 37°C. The reaction was stopped by adding of 100 µL 0.5M HCl. Cinnamic acid was extracted with 1 mL of toluene and the OD was measured at 290 nm, with toluene used as a blank. The calibration curve was obtained using trans-cinnamic acid from Sigma. Total protein was measured using Bio-Rad Protein Assay (Bradford method).

For CAD1 enzyme activity determination plant tissue (300 mg) was ground in liquid nitrogen and crude extract was prepared using the extraction buffer containing Tris HCL 0.05M, pH 8.8, 10% glycerol and 10 mM β-mercaptoethanol. The CAD assay was performed according to (Mansell, Gross, Stöckigt, Franke, & Zenk, 1974) with a few modifications. The enzyme activity was determined by the conversion of alcohol to

aldehyde (reverse reaction). The formation of hydroxycinnamaldehyde at 37°C was monitored at 400 nm using the following molar extinction coefficients: 1.78 x 10^4 M^{-1} cm^{-1} for coniferyl aldehyde and 14.7 x 10^3 M^{-1} cm^{-1} for sinapyl aldehyde (Hawkins & Boudet, 1994). The reaction mixture contained in a total volume of 500 µL : 25 mM Tris- HCl, pH 8.8, 200 µl NADP (2mM), 200 µL (2mM) coniferyl alcohol/sinapyl alcohol and 100 µL crude extract. The same mixture, except 100 µL of TrisHCl buffer instead of the crude extract, was used as a blank. Protein concentrations were determined by the Bradford dye-binding assay.

Results

Genetic transformation of soybean plants by *Agrobacterium tumefaciens* pipetting for introduction of genes of lignification

We performed *A. tumefaciens*-mediated transformation of 1070 flowers of 3 USA and 7 established and zoned in Kazakhstan soybean varieties. Overall we obtained 833 pods (78% efficiency) containing 2000 putative transgenic soybean seeds of zero generation (T_0), expressing the genes of interest (PtMYB4, PAL5, F5H, and CAD1), respectively. Seeds forming efficiency on average was 187%. We did not observe any significant differences by size, shape or mass of pods and seeds between T_0 and control plants (data is not shown). The average number of seeds in a pod at T_0 was 2.5 seeds. T_0 seeds were planted to obtain plants of the first generation (T_1) for the further molecular analysis.

Molecular analysis of T_1 transgenic soybean plants

We analyzed the presence of the transgenes in the DNA and the presence of the transgene transcripts in T_1 soybean plants with PCR using genomic DNA and RT-PCR respectively. As a positive control for PCR of transgenic soybean plants, we used vector plasmid DNA. As a negative control for PCR, genomic DNA of untransformed wild parental plants was used.

The results of the standard PCR analysis of PtMYB4 gene insertion into soybean genome of T_1 plants shown in Figure 2a, b, and *PAL5* gene insertion into genome of T_1 plants – shown in Figure 2c. RT-PCR analysis of transgenic soybean lines of first generation demonstrated expression of PtMYB4 gene in RNA level (Figure 2 d, e).

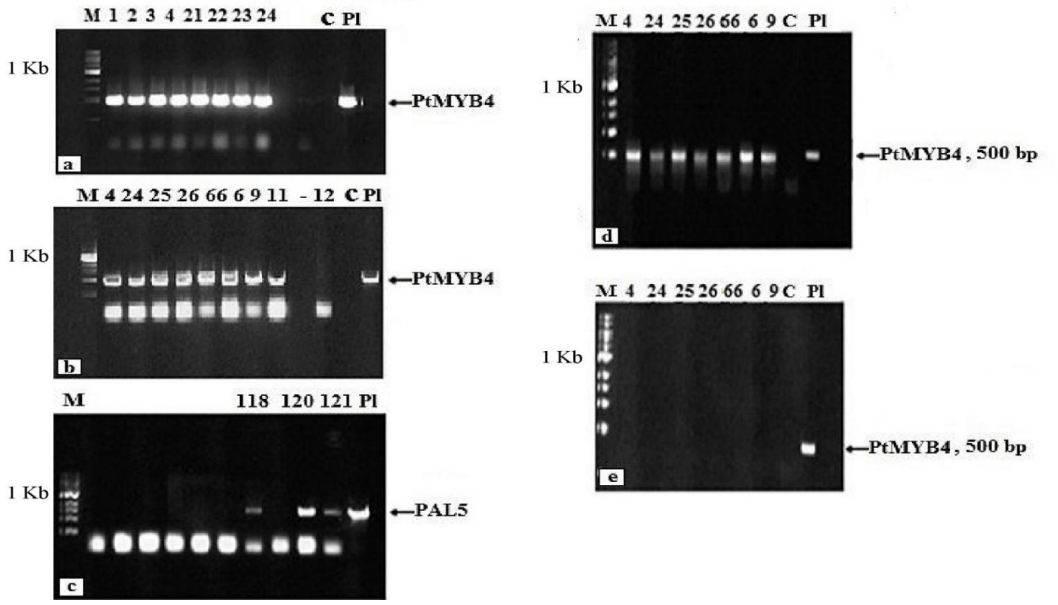

Figure 2. PCR and RT-PCR analyses of T_1 transgenic soybean plants. M – marker, Pl + plasmid, positive control; – wild type, negative control. a: PCR analysis of transgenic lines 1, 2, 3, 4, 21, 22, 23, 24 of Jack cultivar demonstrate PtMYB4 introduction into genome. b: PCR analysis of transgenic lines 4, 24, 25, 66, 6, 9, 11, 12 of Vita cultivar demonstrate PtMYB4 introduction into genome. c: PCR analysis of transgenic lines 118, 120, 121 of Tazhan cultivar demonstrate PAL5 introduction into genome. d: RT-PCR with reverse transcriptase of transgenic lines 4, 24, 25, 26, 66, 6, 9 demonstrate *PtMYB4* expression at the RNA level. e: RT-PCR without reverse transcriptase of transgenic lines 4, 24, 25, 26, 66, 6, 9.

Overall, the presence of PtMYB4 gene insertion into genome was confirmed in 45 transgenic soybean lines, presence of *PAL5* – in 30 transgenic lines, *F5H* – in 17 lines, *CAD1* – in 20 transgenic lines. From 2000

analyzed putative transgenic soybean seeds we generated 112 T_1 transgenic plants with average efficiency of transformation 5,6 %. Importantly we did not observe any significant difference between the varieties in term of transformation efficiency, which proves that the developed method of germ-line transformation is not genome-specific. Data for other lignification genes is not shown.

Seeds of T_1 transgenic soybean plants were planted to obtain of second-generation plants (T_2) for confirmation of stable transformation by molecular and biochemical analyses.

PCR molecular detection of T_2 transgenic soybean plants

About 1000 T_2 transgenic soybean plants, obtained from confirmed by PCR T_1 transgenic plants, were selected randomly and screened by PCR and multiplex PCR (Figure 3). As shown in Figure 3 a-c, a large number of T_2 transgenic soybean lines demonstrates the presence of the gene of interest (*PtMYB4* and *PAL5)* (Figure 3 a, b), and marker gene nptII (Figure 3c). PCR of soybean plants of second generation T_2 have confirmed stable insertion of valuable PtMYB4 and PAL5 genes and marker gene nptII (Figure 3 c) into soybean genome.

As shown in Figure 3d, all genetic elements of CsVMV/PtMYB4sens. construct: the valuable gene, reporter LicB - (lichenase), marker nptII, T-nos terminator confirmed to be inserted into soybean genome in second generation (T_2) of transgenic plants. Multiplex PCR shows the main genetic elements included in gene construction through alignment at annealing temperature 48°C for all genetic elements (valuable gene, reporter, marker, t-nos terminator) of the CsVMV/PtMYB4sens, 35SVTM/PAL5, C4H/F5H, CsVMV/CAD1 constructs. Multiplex PCR demonstrated the integration of the whole transgenic construction into genome of transgenic plant. This confirms the stable insertion of whole genetic construction containing the genes of interest into genome of soybean transgenic lines. Overall, we confirmed the stable integration of all elements of genetic constructions of CsVMV/PtMYB4sens., 35S/PAL5, C4H/F5H, Cs/CAD1 genes into soybean genome with the efficiency more than 50%, in second generation T_2 of transgenic soybean lines.

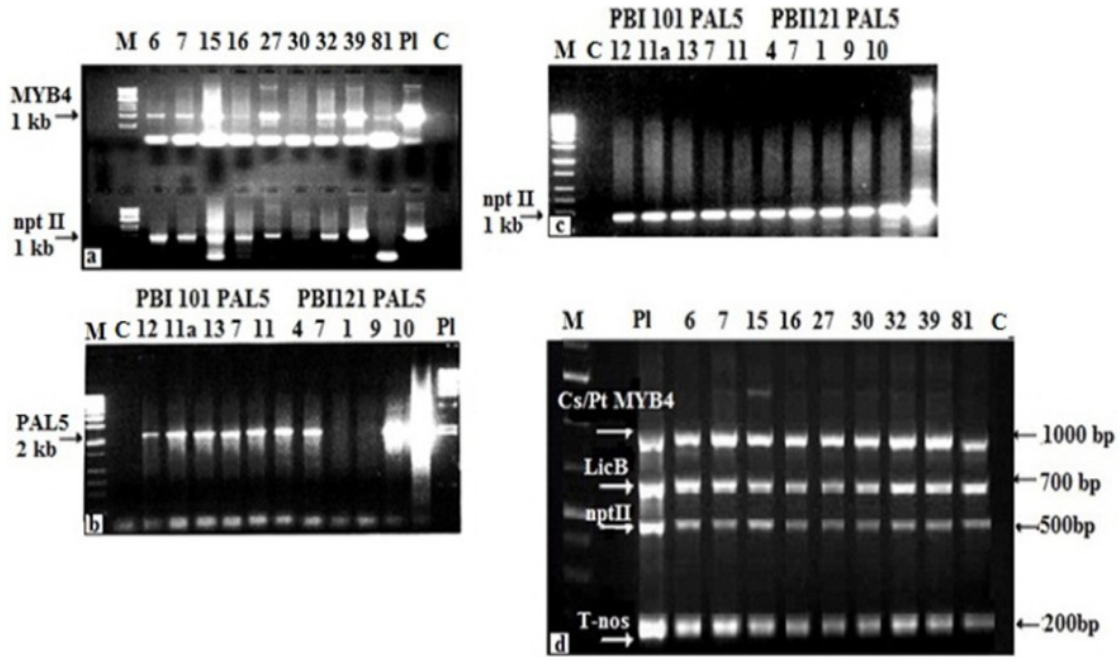

Figure 3. PCR-analysis (a-c) and Multiplex PCR-analysis (d) of T_2 soybean transgenic plants. a: Samples 6, 7, 15, 16, 27, 30, 32, 39, 81 - transgenic lines T2, confirmed introduction into genome of PtMYB4 gene (top part of fig.) and marker gene nptII (bottom part of fig.). b: samples 12, 11a, 13, 7, 11, 4, 7, 1 ,9, 10 - transgenic lines T2, confirmed introduction into genome of the PAL5 gene, and c: marker gene nptII. d: Samples 6, 7, 15, 16, 27, 30, 32, 39, 81 – T_2 transgenic lines, confirmed introduction of the elements of CsVMV/PtMYB4 construct: gene of interest (1000 bp), reporter gene LicB (700 bp), marker gene nptII (500 bp), T-nos terminator (200 bp).

Southern and northern blot analyses of genomic DNA and total RNA

The same putative transgenic plant examples were subjected to the Southern and Northern blot analysis (Figure 4). Total genomic DNA digested with BamHI. Hybridization with the PAL-probe confirmed the presence of the transgenes in all tested lines except PBI 121 PAL5 #1 and #3, which is in agreement with the

PCR data (Figure 4a). Further we hybridized the same membrane with the gus-probe allowed seeing the pattern of gene insertion and estimating the transgene copy number (Figure 4b). All tested lines have multiple copies of the transgenes. The fact that bands are much wider in PBI 101 PAL5 line suggests the presence of a tandem repeats. There were no bands in negative control lines.

The same transgenic lines were used for total genomic RNA isolation and a Northern blot analysis. Northern blot with total genomic RNA of PBI 101 PAL5 and PBI 121 PAL5 lines was performed with untreated samples and samples treated with water (Figure 4 c). Hybridization with the *pal* probe revealed the presence of the genomic PAL in all samples, including control and the presence of transgenic band in PBI 121 PAL5 line #4 (Figure 4 c). Hybridization of the same membrane with the *gus* probe resulted in a band at the same place, suggesting the presence of bisictronic mRNA due the expression of a transgene at the transcriptional level (Figure 4 d). This band appears much weaker in untreated samples due to the significantly lower amount of RNA. Hybridization with the nptII probe revealed the presence of the nptII band in all samples, except the control as expected (Figure 4 e). Thus, the soybean transgenic lines are capable of the expression of introduced genes at the transcriptional level.

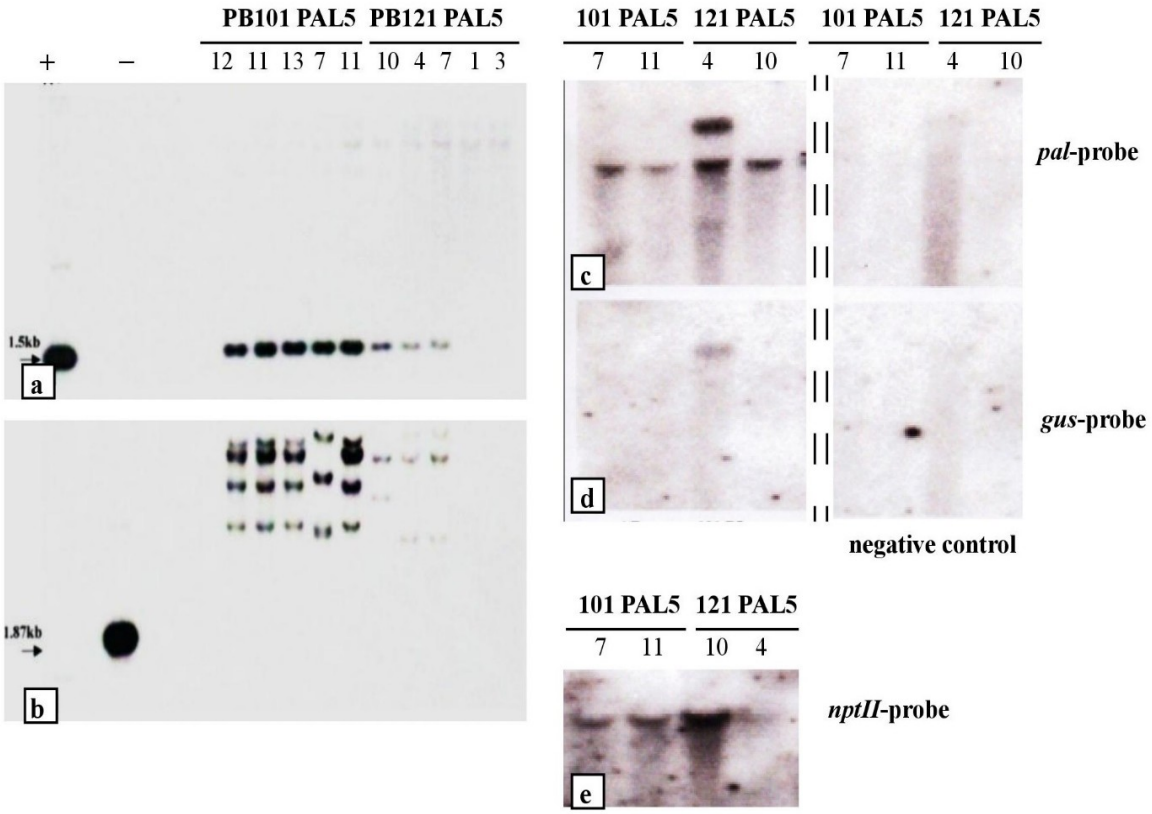

Figure 4. Southern (a, b) and Northern (c, d, e) blot analyses, using genomic DNA from transgenic soybean plants, transformed with PBI101PAL5 or PBI121PAL5, digested with BamHI, and hybridized with: *pal* probe (a, c), *gus*-probe (b, d), nptII probe (d). Dashed line in the blots c and d represents a cut in the membrane.

Lignin content and composition

Further we analyzed the content of lignin in the cell wall in CAD1 and PAL5 transformants. Lignin content in control lines was 23.17% of the dry weight, 28.8% in Cs/CAD1 transformants and 29.99% in 35S/PAL5 transgenic lines. This confirms that lignin content is increased in the presence of the target lignification genes expression.

Furthermore, we analyzed lignin composition in these transgenic lines. We observed an overall increase of lignin monomer content in 35S/PAL5 transgenic lines compared to the control lines. In the Cs/CAD1 transformants we observed an increase of S-monomers content compared to the control lines (Figure 5 a, b).

Additionally, we analyzed the activity of PAL and CAD enzymes expressed in the transgene plants to convert L-phenylalanine to trans-cinnamic acid and the conversion of coniferyl and synapil alcohols to the corresponding aldehydes, respectively. We observed a significant increase of PAL5 activity in PBI 101 PAL5 line #11 (Figure 5 c) as well as an increase of CAD1 activity in PBI 101 CAD1 line #5 (Figure 5 d). Correspondent

with the transgene expression this proves the functional activity of the inserted genes of interest. All tested lines have multiple copies of the transgenes.

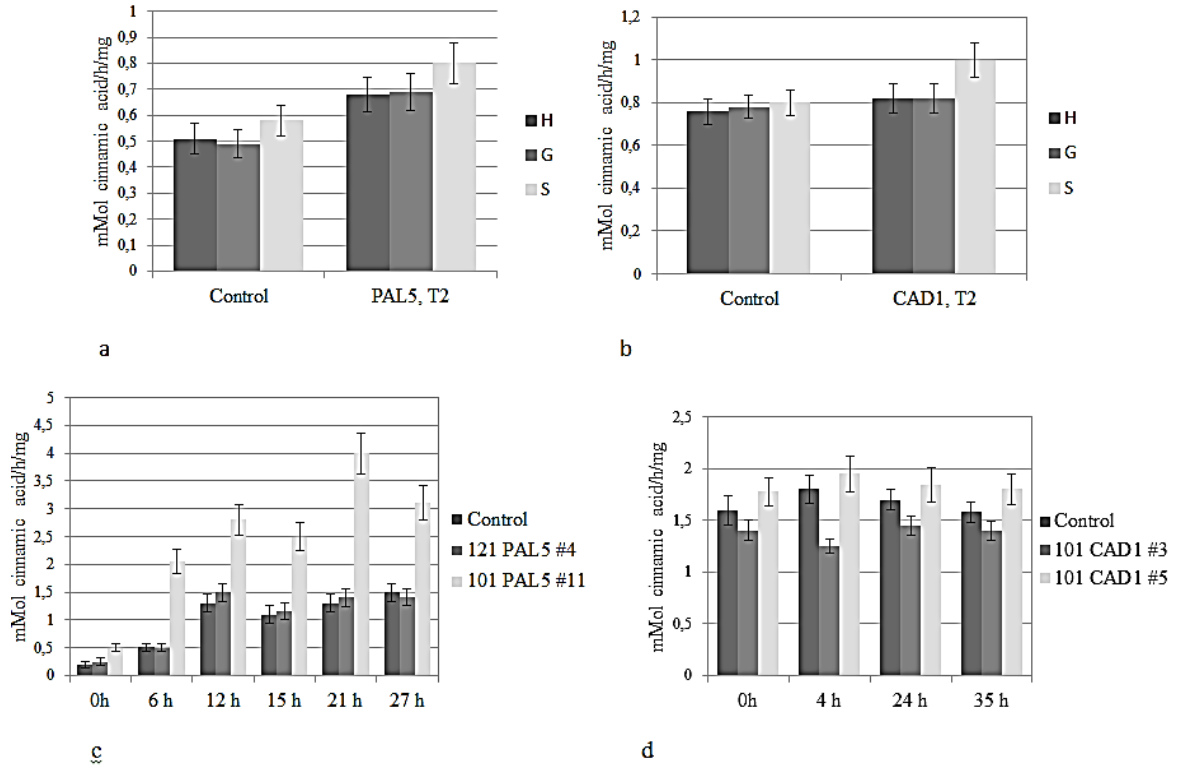

Figure 5. Lignin content and enzyme activity in T_2 transgenic lines with introduced lignification genes. a - The lignin content in control and T_2 soybean transgenic plants expressing PAL5 gene. b - The lignin content in control and T_2 soybean transgenic plants expressing CAD1 gene. H - hydroxyphenyl, G - guaiacyl, and S – syringyl monomers of lignin. c - Changes over time of enzyme PAL5 activity in transgenic lines expressing PAL5 gene. d – Changes over time of enzyme CAD activity in transgenic lines expressing CAD1 gene. Error bars represent (n=8) (Weissgerber, Milic, Winham, Garovic, 2015)

Discussion

We developed a novel germ-line technique for soybean genetic transformation using natural pollen tubes to introduce the key genes of lignification into mature but not yet divided zygote. This technique was used to perform a genetic transformation of 1070 flowers of 3 USA and 7 established and zoned in Kazakhstan soybean varieties. We obtained 833 pods (78% efficiency) with 2000 putative transgenic soybean seeds of zero generation (T_0). Seeds forming efficiency on average was 187%. We confirmed the expression of the key lignification genes as well as the expression of all elements in the constructs (CsVMV/PtMYB4sens., 35S/PAL5, C4H/F5H, Cs/CAD1) using PCR, RT-PCR, multiplex PCR in all 10 soybean varieties with the average efficiency of 5.6%. In the second generation of transgenic plants T_2 we observed a stable integration of all elements of genetic constructions of these genes into soybean genome with the efficiency more than 50%. All tested lines of PAL5 transformants have multiple copies of the transgenes as verified by Southern Blot and confirmed expression of transformed genes into soybean genome. Northern Blot analysis demonstrated expression of introduced genes (PAL, *gus*, nptII) at the transcriptional level. We observed the increase of lignin content from 23.17% of the dry weight in control to 28.8% in *Cs/CAD1* transformants and 29.99% in *PAL5* transformants. Additionally, PAL5 transformants demonstrated elevated levels of all lignin monomers (H-, G-, and S), and observed an increase of S-monomers in *CAD1*-transgenic lines compared to the control.

So, we have developed a novel soybean germ-line genetic transformation technique using natural pollen tubes as vectors for the *Agrobacterium*-mediated introduction of target transgenes into soybean genome. This method is based on previously described method of pollen-tube pathway transformation which is successfully used for crops like cotton or rice (Ali et al., 2015), however seem to be not efficient and irreducible in soybean (Shou et al., 2002). The major difference of the used method is using *A. tumefaciens* to introduce the vectors containing the genes of interest into the newly fertilized but not yet dividing zygote cell via pollen tubes.

The method has a high efficiency of T_1 plants generation containing the genes of interest (5.6% on average, compared to 2.8% dependent on genotype in transformation in cotyledonary nodes (Jia et al., 2015), or 3.8 (1.4–8.7%) (Paz et al., 2006). We confirmed the presence of the transgenes using standard molecular biology techniques such as PCR, RT-PCR and Southern and Northern blotting. One of the important findings is that the transformation efficiency seems to be independent on the genotype which is a big advantage of the method. However, further investigations on the efficiency of *A. tumefaciens*-mediated transduction using natural pollen tubes should be performed, including the selection of the most transductable genotypes and more rigorous analysis of the selected plants. We found several important advantages of the suggested method. Firstly, this method is similar with natural pollen hybridization and transgenic plants are generated within a short period of time (90–120 days). No tissue culture and plant regeneration steps are required which makes this technique much less expensive and simpler compared to the conventional approaches. The plants derived in this study are able to produce normal health seeds, comparable with control plants. We observed that the transgenic plants are healthy and are very similar to the plants derived using conventional breeding. Also, due to the transformation of the germ-line these plants are less likely chimeric therefore the genes of interest are transmitted to the next generation.

Importantly, as the method uses only single and simple transformation step it can be performed in greenhouses or in the field. This can greatly reduce the soybean transformation expenses on tissue culture and recovering steps thus the overall cost of transgenic plant production.

The described method was used to produce soybean plants with altered lignin concentrations by introduction of the genes related to the lignin synthesis. We created a number of transgenic cell lines stably expressing PtMYB4, PAL5, F5H, and CAD1. Importantly, we observed the an increased amount of lignin in the plant lines harboring PAL5 and CAD1 genes which are required for proper lignin formation. This demonstrates that the used technique is capable of producing transgenic lines with the desired phenotype. The next step is to determine if the changes in lignin content and lignin composition will provide the resistance to various pathogenes. Earlier it was shown that soybean plants with low stem lignin concentrations are more resistant to sclerotinia stem rot – one of the most common soybean fungal diseases (Peltier, Hatfield, & Grau, 2009). Therefore more studies should be performed to characterized the resistance profile of the derived transgenic plant lines. Other important line of the further studies is to identify the transgenic constructs that will increase soybean resistance and will not compromise plant qualities such as yield and nutritional value.

Conclusion

In summary, we developed a novel simple and effective germ-line transformation method for soybean genetic transformation and were able to introduce genes related to lignification which may be important for soybean biotic stress and disease resistance improvement. This simple and effective genetic transformation technique is expected to be beneficial for the Kazakhstan and US soybean growers and also world-wild and will help to generate great variation in phenotype, increase of biodiversity and establishment of different valuable soybean genotypes with enhanced stress tolerance and productivity for breeders.

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