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Construction and analysis of a suppression subtractive hybridization library of regeneration-related genes in soybean

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ABSTRACT. The development of a genetic transformation system is needed to address the problem of the low efficiency associated with soybean regeneration. To contribute to the enhancement of the soybean regenerative capacity, we explored the developmental mechanisms of soybean regeneration at the molecular level using a suppression subtractive hybridization cDNA library constructed from cotyledonary nodes of soybean cultivar DN50. A total of 918 positive clones were identified and screened, with most inserted fragments ranging from 100 to 750 bp. Of these, 411 differentially expressed functional expressed sequence tags were identified and annotated based on their similarity to orthologs and paralogs detected in GenBank using the nucleotide and translated nucleotide Basic Local Alignment Search Tools. Functional analysis revealed that the associated genes were involved in signal

transduction, synthesis, and metabolism of macromolecules, glucose and protein synthesis and metabolism, light and leaf morphogenesis, regulation of apoptosis, cell defense, cell wall differentiation, and a variety of hormone and cytokinin-mediated signaling pathways. The information uncovered in our study should serve as a foundation for the establishment of an efficient and stable genetic transformation system for soybean regeneration.

Key words: Soybean; Suppression subtractive hybridization (SSH); cDNA library; Soybean regeneration

INTRODUCTION

Following the success of the *Agrobacterium*-mediated DNA transfer method (Hinchee et al., 1988) and particle gun technology (McCabe et al., 1988), no further breakthroughs have occurred in the production of transgenic soybean (*Glycine max* [L.] Merrill.). The low frequency and poor reproducibility of soybean regeneration are recognized as a bottleneck in the transformation of this important crop species. Research on soybean regeneration systems has focused mainly on aspects such as explant type, genotype, hormone type, and culture conditions. Few studies have investigated the molecular basis of regeneration systems, especially the underlying mechanisms and the genes playing roles in the regeneration process. If the expression of soybean regeneration genes can be regulated, then the problem of soybean's low regeneration ability could possibly be addressed.

In recent years, somatic embryo and cotyledon node regeneration-related genes have been frequently studied. Li and Komatsu (2000) characterized the *CRO1* gene, which is involved in the regeneration process in rice cell-suspension cultures. In petunia, Zubko et al. (2002) identified the regeneration candidate gene *Sho* that increases the expression of isopentenyl transferase, a key enzyme in the cytokinin pathway. Komatsuda et al. (1993) obtained the shoot regeneration-related gene *Shd1* through quantitative trait locus mapping, and Zakizadeh et al. (2010) isolated the candidate regeneration gene *SERK* from rose.

In *Arabidopsis*, the regulation of the regeneration process involves a large number of genes. For example, the overexpression of the *ESR1* gene, which was detected in *Arabidopsis* by Banno et al. (2001), can induce the start of shoot regeneration, and the *ESR2* gene, which was cloned by Ikeda et al. (2006), can promote regeneration. *ESR1* and *ESR2* both have an AP2/EREBP transcription factor domain. Ikeda et al. (2006) reported that *ESR2* controls *Arabidopsis* regeneration by regulating the *CUC1* gene. In addition, Leibfried et al. (2005) found that the *WUSCHEL* gene family can control somatic embryogenesis by regulating cytokinin-responsive elements.

Suppression subtractive hybridization (SSH) is a powerful and widely used approach to generate subtracted cDNA libraries to identify differentially expressed genes. SSH has many outstanding advantages, such as a low false-positive rate, high sensitivity, a short screening cycle, and high efficiency. Using SSH, Low et al. (2001) identified the regeneration candidate gene *PKSF1* in *Paulownia kawakamii*. This gene encodes a leucine zipper transcription factor that promotes callus formation. We found that the cotyledon node of DN50 induced the cytokinin 6-benzyladenine (6-BA), and we used SSH to identify differentially expressed genes. Functional analysis of these genes revealed their roles in the studied regeneration system,

opening the door to further research into the molecular basis of soybean regeneration.

MATERIAL AND METHODS

Material

The soybean variety DN50, which has a high regeneration rate, was provided by the Soybean Institute, Northeast Agricultural University, Harbin, China. After chlorine sterilization, seeds were inoculated with the hilum down onto germination medium [4.43 g Murashige and Skoog basal medium (MS), 3% sucrose, and 0.7% agar, pH 5.8] and cultured for 5-7 days at 25°C under 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ white-light illumination and long-day conditions (16/8-h light/dark photoperiod). The seed coat was then removed, and most of the hypocotyl was excised, leaving only 3-5 mm of the hypocotyl on the cotyledon. The 2 cotyledons were separated vertically along the hypocotyl midline, and the terminal and axillary buds were removed to obtain the cotyledon explants. Some of the cotyledon explants were inoculated into liquid medium containing 4.43 g MS, 2 mg/L 6-BA, and 3% sucrose, pH 5.8, with samples taken 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h later for use as SSH tester samples. The remaining cotyledon explants were inoculated into liquid medium lacking 6-BA, and samples were taken 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h later as SSH driver samples. All samples were frozen in liquid nitrogen and stored at -80°C until SSH library construction.

Experimental methods

SSH library construction

Total RNA was extracted from soybean leaves using Trizol reagent (Takara, Dalian, China). Soybean total RNA quality was checked by 1% agarose gel electrophoresis. Bands corresponding to 18S and 28S RNA were clearly visible on the gels (Figure 1), indicating a high level of RNA integrity and purity. Total RNA was reverse-transcribed to cDNA using a SMARTer PCR cDNA Synthesis kit (Clontech/Takara, Mountain View, CA, USA) according to manufacturer instructions. Double-stranded cDNA was obtained using 32 amplification cycles, which was sufficient to achieve the logarithmic amplification phase (Figure 2). SSH was performed on cDNA from 6-BA-treated cotyledon nodes (SSH tester) and non-treated nodes (SSH driver) using a PCR-Select cDNA Subtraction kit (Clontech/Takara) according to manufacturer instructions. Tester cDNA was digested with the restriction endonuclease *Rsa*I and divided into 2 samples; the first sample was ligated to Adaptor 1, and the other was ligated to Adaptor 2R. The samples were stored overnight at 16°C. An initial hybridization with driver cDNA was performed by incubating each ligated tester cDNA at 98°C for 90 s, followed by hybridization at 68°C for 9 h. An excess of denatured driver cDNA was added to the resulting SSH mixture for a second hybridization at 68°C overnight. The nested polymerase chain reaction (PCR) amplifications were then performed twice. The SSH product was purified, recovered, and cloned into a pMD-T vector at 16°C overnight; then, it was transformed into *Escherichia coli* DH5α competent cells using a heat shock method. The transformed cells were cultured on agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, isopropyl β-D-1-thiogalactopyranoside, and ampicillin for blue-white spot screening. White colonies were picked and inoculated into liquid Luria-Bertani medium and incubated over-

night at 37°C. A single spot was picked after shaking the culture, and it was subjected to 2 rounds of nested PCR amplifications using primers Primer1F (5'-TCGAGCGGCCGCCCG GGCAGGT-3') and Primer2R (5'-AGCGTGGTCGCGGCCGAGGT-3'). PCRs were performed in 25-µL volumes consisting of 2 µL culture broth, 2.5 µL 10X PCR buffer, 0.5 µL 10 mM dNTP mix, 0.5 µL of each primer, 0.3 µL 5 U/L LA DNA polymerase, and 18.7 µL sterile water. PCR amplification cycles were as follows: 94°C for 5 min; 30 cycles of 94°C denaturation for 30 s, 68°C annealing for 30 s, and 72°C extension for 2 min; and a final extension of 72°C for 5 min. Colonies that were identified as positive clones were preserved by adding 30% glycerol and stored at -80°C.

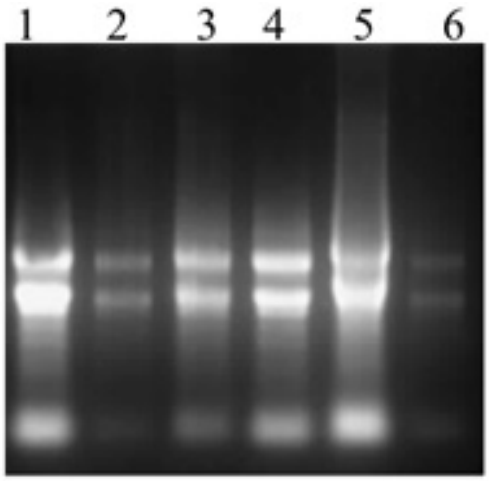

Figure 1. Total RNA extracted from soybean cotyledonary nodes. *Lanes 1* to *6* = total RNA of soybean cotyledonary nodes.

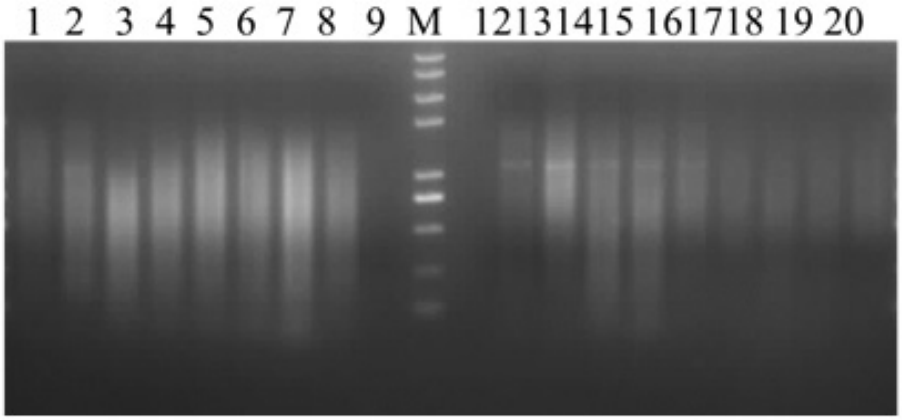

Figure 2. Polymerase chain reaction amplification of tester and driver cDNA. *Lane M*: DL15000 DNA marker; *lanes 1-9*: cDNA amplification of tester cDNA with 15, 18, 21, 24, 27, 30, 33, 36, and 39 cycles; *lanes 12-20*: cDNA amplification of driver cDNA with 15, 18, 21, 24, 27, 30, 33, 36, and 39 cycles.

DNA sequencing and sequence analysis

We used real-time reverse transcription (RT)-PCR to confirm the relative expression of 4 differentially expressed genes that were identified in the SSH library. To obtain material for analysis, cotyledon nodes were generated by culturing DN50 seeds on MS medium for 6 days. The cultures were then shaken in liquid MS medium with or without 6-BA, and samples were taken at 1, 2, 4, 6, 8, 12, 24, and 48 h. Total RNA was extracted with Trizol reagent, reverse-transcribed into cDNA, and stored at -80°C until use.

PCR primers were designed based on sequences in GenBank and are listed in Table 1. A SYBR(R) Ex Script RT-PCR kit (Takara) was used according to manufacturer instructions for the amplification reactions. The soybean housekeeping gene actin 4 was used as an internal reference.

Real-time RT-PCR mixtures comprised 1.6 µL first-strand cDNA, 1.6 µL upstream and downstream primers, 10 µL SYBR qPCR Mix, and 6.8 µL deionized water to give a total volume of 20 µL. PCR amplification cycles were as follows: 95°C denaturation for 30 s, followed by 40 cycles of 95°C denaturation for 5 s, 60°C annealing for 20 s, and 72°C extension for 20 s. PCRs were repeated 3 times for each sample.

The Opticon Monitor 3.1 software was used to analyze the data and calculate the relative gene expression.

Table 1. Primers used for fluorescence real-time reverse transcription-polymerase chain reaction.

Gene (accession number)	Amplification size	Oligonucleotide sequence 5'→3'
Actin 4 (AF049106)	214 bp	F: GTGTCAGCCATACTGTCCCCATTT R: GTTTCAAGCTCTTGCTCGTAATCA
1F: *Glycine max* acid phosphatase (NM_001249251.1)	126 bp	F: TCGACCTTGTATGATGAATGGGTT R: TTGCTTCTGTTACGGCCTGTTTG
2F: *Glycine max* vspA (M76981.1)	148 bp	F: TCGACCTTGTATGATGAATGGGTT R: TTGCTTCTGTTACGGCCTGTTTG
3F: 16S ribosomal (AF537102.1)	102 bp	F: TTATGCTAGTGAACGCGAAGTCC R: GGTTTCATTAAATTCCTCCACCC
4F: *Glycine max* porphobilinogen deaminase (NM_001253959.2)	125 bp	F: GCTTAGTAGTGCCAATGCAAGTT R: AGCGATGGAAGATCACCGAATAT
3R: *Glycine max MET2* (NM001248577.1)	82 bp	F: TGTATGTGATTGTTGTGACTCGTTC R: AGTTACCACCGCAGCAAGACATTTT

RESULTS

Analysis of PCR amplifications after subtractive hybridization

PCR amplification of mixtures obtained following 2 rounds of subtractive hybridization yielded uniform, smeared DNA fragments of 200-1500 bp. The average fragment length was between 400 and 700 bp (Figure 3).

PCR amplification of inserted fragments in the SSH cDNA library

The cDNA fragments that were generated from the SSH cDNA library were transformed into *E. coli* DH5α. According to the blue and white spot screening, 917 positive clones were randomly chosen. PCR amplification confirmed that the length of most inserted cloned fragments was between 100 and 750 bp, with an average length of about 500 bp (Figure 4).

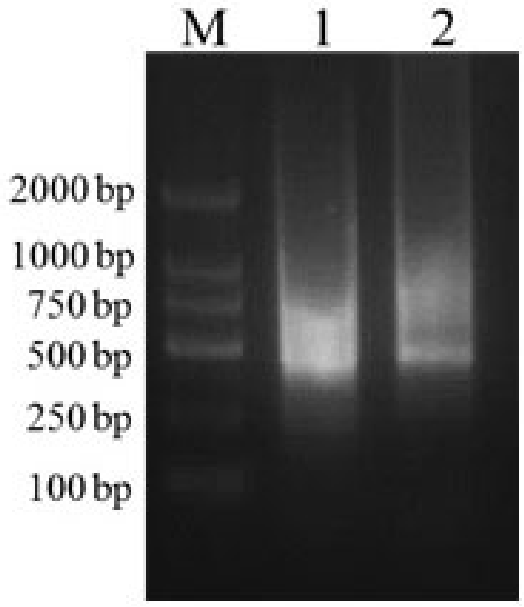

Figure 3. PCR hybridization products after subtraction. *Lane M* = DL2000 DNA marker; *lane 1* = tester PCR products after subtraction; *lane 2* = driver PCR products after subtraction.

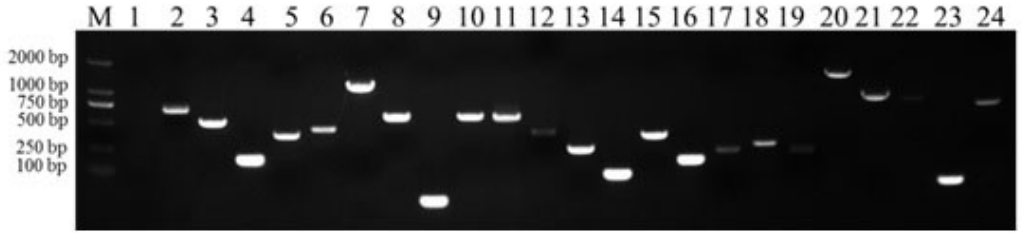

Figure 4. Screening of the inserts in clones of the suppression subtractive hybridization (SSH) cDNA library. *Lane M* = DL2000 DNA marker; *lanes 1-24* = inserts.

Expressed sequence tag (EST) sequences and homology search results

A total of 917 clones were randomly obtained and sequenced, and 411 (44.8%) of the resulting ESTs were high quality. These sequences were searched against the GenBank database using the nucleotide and translated nucleotide Basic Local Alignment Search Tools (BLAST). The sequences were matched to annotated genes involved in signal transduction, glucose and protein synthesis and metabolism, light and leaf morphogenesis, regulation of apoptosis, cell defense, cell wall differentiation, and a variety of hormone and cytokinin-mediated signaling pathways (Figure 5 and Table 2).

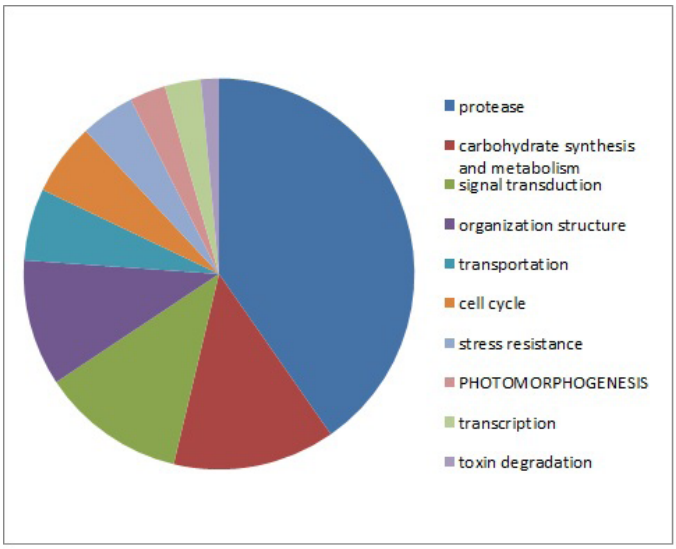

Figure 5. Classification of the expressed sequence tags in the SSH library.

Table 2. Similarity between expressed sequence tags in the suppression subtractive hybridization library and genes in GenBank.

Gene No.	Accession No.	Gene homology	Gene name
F14_12_RV_M_D02_1110082547J	NM_001251530.2	Glycine max 28-kDa protein (VSPA), mRNA	VSPA
F14_19_21_RV_M_A11_1110082616J	AB118810.1	*Phaseolus vulgaris* PSP gene for pod storage protein, complete CDS	*PSP*
F14_19_32_RV_M_C12_1110082627J	AF537102.1	16S ribosomal RNA gene, partial sequence	16S ribosomal
F14_57_RV_M_A08_1110082592J	NM_001249251.1	*Glycine max* acid phosphatase (LOC547669)	acid phosphatase
F173_RV_M_H02_1111083379J	DQ455592.1	*Phaseolus vulgaris* SGT1-like protein mRNA, partial CDS	*SGT1*
F180_RV_M_B11_1111083386J	AB242267.2	*Sesbania rostrata SrGLU5* mRNA for beta-1,3-glucanase	β-1,3-glucanase
F4_120_RV_M_B01_1111080367J	NM_001255715.2	*Glycine max* porphobilinogen deaminase, chloroplastic-like	*PBGD*
F4_81_RV_M_A11_1111080328J	JN033738.2	*Chilo suppressalis* serine protease inhibitor 012 mRNA	serine protease inhibitors
F4_91_RV_M_C10_1111080338J	DQ317523.1	*Glycine max* cultivar PI 437654 chloroplast, complete genome	chloroplast
F_315_RV_M_B03_1111083667J	EF464674.1	*Gossypium hirsutum* GDP-mannose pyrophosphorylase mRNA, partial CDS	*GMPPB*
F_396_RV_M_B11_1111083748J	JQ711538.1	*Olea europaea* xylose isomerase mRNA, partial CDS	xylose isomerase
F_567_RV_M_B08_1111083919J	AJ223716.1	*Squalus acanthias* mRNA for *sgk-2* serine-threonine protein kinase	serine-threonine protein kinase
F_576_RV_M_C09_1111083928J	NM_001250642.1	*Glycine max* catalase (*CAT4*), mRNA	*CAT4*
R19_17_RV_M_A04_1110082740J	NM_001251763.1	*Glycine max* lipoxygenase	*LOXB1*
R2_39_RV_M_E08_1111080692J	EU683445.1	*Malpighia glabra* L-galactono-1, -lactone dehydrogenase mRNA, complete CDS	L-galactono-1,4-lactone dehydrogenase
R400_RV_M_B04_1111083521J	U39567.1	*Glycine max* ribulose-1,5-bisphosphate carboxylase small subunit mRNA	ribulose-1,5-bisphosphate carboxylase
R4_23_RV_M_B06_1111080445J	NM_001248577.1	*Glycine max* metallothionein type 2	*MET2*
R4_36_RV_M_G03_1111080458J	J05208.1	Soybean (*Glycine max*) proline-rich cell wall protein gene	*SbPRP2*

Relative expression analysis of candidate cDNA fragments by real-time quantitative PCR

The results of the real-time RT-PCR analysis of 4 genes that were differentially expressed between the 6-BA treatment and control conditions in cotyledon nodes are shown in Figure 6. The expression of the acid phosphatase (*ASP*) gene was steady for the first 4 h, increased at 6 h, and then decreased sharply after 12 h, indicating that this gene is negatively regulated by 6-BA. The *VSPA* gene expression was stable for the first 6 h, rapidly increased at 8 h, reached a peak at 12 h, and then decreased rapidly. The expression of the 16S ribosomal gene following 6-BA treatment stabilized before 8 h and then reached its highest expression level at 12 h, suggesting that the 16S ribosomal gene may play a downstream role in the cytokinin pathway. The porphobilinogen deaminase gene expression increased suddenly at 2 h, when it reached its peak at 4 h, and then it stabilized, suggesting that this gene may play an upstream role in the cytokinin pathway (Figure 6).

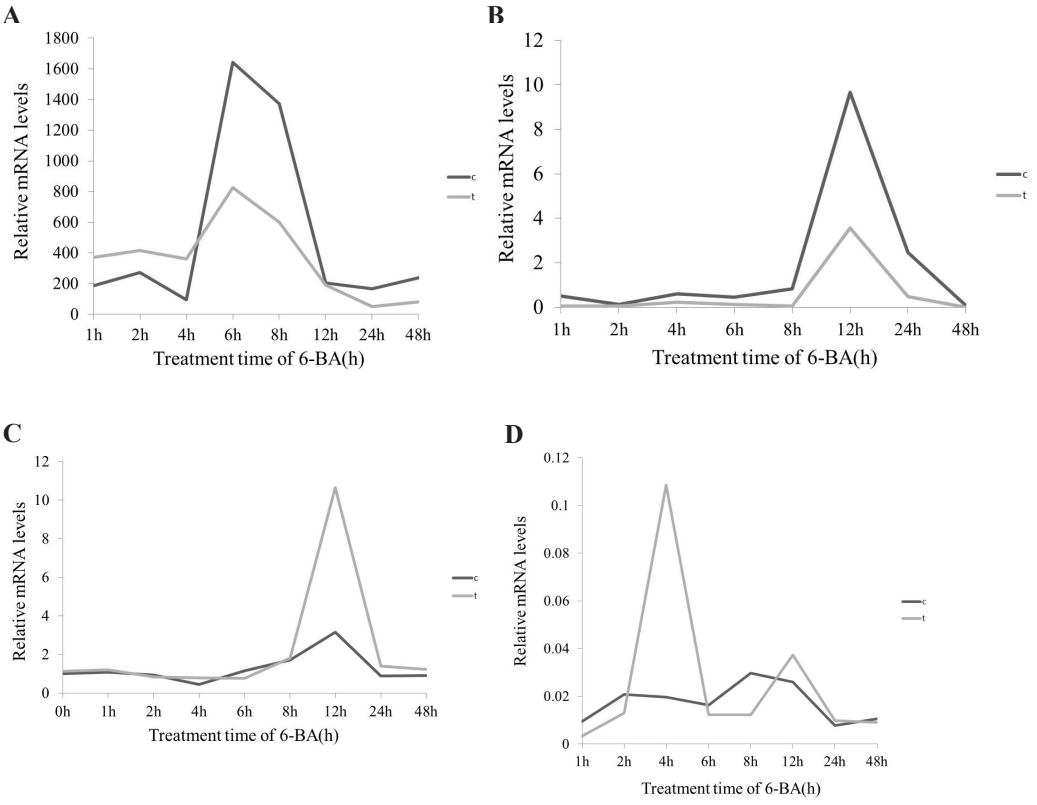

Figure 6. Expression of genes with 6-benzyladenine (6-BA) treatment. **A.** Expression of *Glycine max* acid phosphatase with 6-BA treatment. **B.** Expression of VSPA with 6-BA treatment. **C.** Expression of 16S ribosomal with 6-BA treatment. **D.** Expression of *Glycine max* porphobilinogen deaminase with 6-BA treatment. c = control; t = treatment.

DISCUSSION

Our study is the first reported construction of a cDNA library and isolation of differentially expressed genes from cytokinin-induced soybean cotyledonary nodes using SSH. Our data should contribute to research into soybean organogenesis regeneration pathways and can be used to screen soybean regeneration candidate genes.

The plant regeneration process generally involves 3 main steps (Huang et al., 2007): 1) induction by a hormone, 2) specific cell division, and 3) adventitious bud regeneration. Cytokinin, the hormone that is most frequently involved in the natural formation of adventitious buds, plays a vital role in root and stem cell division and many other plant developmental processes (Howell et al., 2003). During adventitious bud induction, cytokinin can act alone or in synergy with auxin. To respond to cytokinin, cells require receptors for signal perception (Kakimoto, 1998; Stock et al., 2000; Lohrmann and Harter, 2002; Riefler et al., 2006).

Cytokinins can cause cell division, induce the formation of buds, and promote bud growth and development. Cytokinins cause biological effects by signal transduction. The functions of the receptors of cytokinin signaling pathways, such as CRE1, CKI1, AHP, and ARR, have been investigated (Haberer and Kieber, 2002; Hutchison and Kieber, 2002; Ferreira and Kieber, 2005). Skoog and Miller proposed the auxin-cytokinin hypothesis in plant morphogenesis, in which an appropriate cytokinin and auxin play a key role in the process of growth after the formation of plant morphogenesis and roots. In this hypothesis, cytokinins are important in the regulation of plant morphogenesis (Kakimoto, 1996; Hwang and Sheen, 2001; Che et al., 2002; Sun et al., 2003).

Soybean contains 2 vegetative storage proteins (VSPs) that function as temporary storage reserves and are also closely related to plant ASPs from the haloacid dehalogenase superfamily. When ASP is reduced, VSPs can prevent the storage protein from functioning (Leelapon et al., 2004).

Some genes in the library have been studied. Differences in the quantity and cleavage efficiency of reactive oxygen species (ROS) are major causal factors in observed variations in the ability of plants to regenerate (Cutler et al., 1991). Papadakis and Roubelakis-Angelakis (2002) found that protoplast totipotency was positively correlated with antioxidant activity properties of cells in tobacco and grape, and negative correlations were observed between totipotency and ROS levels in the medium. Zhang et al. (2010) found that the amount of H_2O_2 in immature larch embryos increased and then decreased during somatic embryogenesis, thereby maintaining their volatility. They also reported that catalase activity was stable during prophase and elevated in middle and later periods, and then it decreased. Superoxide dismutase activity increased during prophase, and then it decreased rapidly, whereas APX activity fluctuated during prophase and increased during anaphase. During callus differentiation in strawberry tissue culture, Tian et al. (2003) observed that superoxide dismutase activity first increased and then decreased, catalase activity continually decreased, and peroxidase activity first decreased and then increased. They also reported that high O_2^- and low H_2O_2 content were related to organ formation. Superoxide dismutase activity is usually detected in callus only during regeneration, and exogenous H_2O_2 can promote bud regeneration in the callus (Tian et al., 2003). Thus, oxidative stress can induce the synthesis of the antioxidant enzyme, thereby promoting regeneration. On the other hand, the occurrence of oxidative stress in cells changes the cell membrane permeability, leading to cell death (Ye et al., 2012).

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is an important CO_2-

fixing enzyme in plant photosynthesis. It is also involved in plant photorespiration metabolic pathways, although its oxygenation of ribulose biphosphate leads to net photosynthetic efficiency losses of up to 50% (Lundqvist and Schneider, 1991; Xiong et al., 2003; Ashida et al., 2005). Therefore, the regulation of Rubisco activity is important to improve photosynthetic efficiency. Jiang et al. (2006) studied the effect of 6-BA and abscisic acid (ABA) on the decline of leaf photosynthetic function in different genotypes of tobacco varieties NC89 and JY H. They found that 6-BA treatment significantly improved the instantaneous photosynthetic rate, chlorophyll content, chloroplast electron transport activity, and Rubisco activity in tobacco leaves, and it prolonged photosynthetic function. It also improved the ratio of photosynthetic electron transport to carbon assimilation, thereby delaying photosynthetic function decline. The opposite results were obtained upon ABA treatment.

BLAST of other ESTs in the National Center for Biotechnology Information database indicated that genes that were induced by 6-BA could be functionally classified into categories associated with hormone signal transduction pathways; seed germination and flowering time; negative regulation of programmed cell death; leaf senescence; pollen tube growth; anabolic metabolism of glucose, proteins, and other macromolecules; photosynthesis; ribosomes; transport proteins; and catalase and acid phosphatase activity. We also uncovered many genes that were related to stress, such as cold, heat, drought, high salt, and mechanical damage, and some genes of unknown function. These genes were differentially expressed in the presence of cytokinin, indicating the importance of future studies that focus on soybean regeneration genes.

ACKNOWLEDGMENTS

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