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ORIGINAL PAPER

PgWUS expression during somatic embryo development in a *Panax ginseng* 2c3 cell culture expressing the *rolC* oncogene

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Abstract It is well known that embryogenesis in plants is regulated by the *WOX* family of transcription factors (*WUS* homeobox); the *WUS* gene is the founding member of this family. *Panax ginseng* is one of the most valuable traditional Chinese medicinal herbs, and its reproduction must be maintained to fulfill production demands. A ginseng embryogenic cell culture 2c3 was previously obtained as a result of the transfer of a bacterial oncogene, *rolC*. This cell line has been used as a model system for studying plant somatic embryogenesis. Several genes that are homologous to the plant *WUS* genes have been shown to be expressed in the embryogenic 2c3 cell culture; no sequences homologous to *WOX* genes have been detected. We show that the expression levels of *PgWUSII-6*, *PgWUSII-5*, and *PgWUSII-8* are significantly higher in the early developmental stages of somatic embryogenesis compared to vector-transformed cells. During late stages of embryogenesis, *PgWUSI-3*, *PgWUSI-7*, and *PgWUSII-5* expression is activated. We represent the results suggesting that the initiation of *P. ginseng* somatic embryogenesis may correlate with significant activation of *PgWUS* expression.

Keywords *Panax ginseng* · *rolC* gene · Somatic embryogenesis · *WUS* gene

Abbreviations

CDPK	Calcium-dependent protein kinase
WOX	WUS homeobox

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Introduction

Over the last 20–30 years, much attention has been paid to finding genes that are expressed differently during the process of plant embryogenesis (Lin et al. 1996). Genes that have a key role in the induction of embryogenesis and whose expression is increased during the early stages of embryogenesis are quite interesting. Today some of these gene families are well known, e.g. SERK (Hecht et al. 2001), LEC (Braybrook and Harada 2008), WOX (Schoof et al. 2000; Zuo et al. 2002), etc. One example is the *WOX* (*WUS* homeobox) family of transcription factors, including the founding member, the *WUS* gene. For example, Arabidopsis WUS is the founding member of the WUS-related WOX (WUS homeobox) gene family of transcription factors comprising at least 15 members in the Arabidopsis genome (Haecker et al. 2004). They all share a characteristic nucleotide sequence, called the homeobox that encodes a WUS-type homeodomain, with a characteristic helix-turn-helix DNA-binding motif. Beside the homeodomain, many WUS/WOX proteins share the WUS-box, located downstream of the homeodomain. The function of the WUS-box is unknown (Nardmann and Werr 2006).

The expression of the homeobox-containing *WUS* gene in *Arabidopsis thaliana* is sufficient to support the required amount of stem cells for a shoot and flower meristem (Schoof et al. 2000). The *WUS* gene activates *CLAVATA* expression, while *CLAVATA* inhibits *WUS* expression. This self-regulated system allows for the maintenance of a constant pool of stem cells in the meristem (Lenhard and Laux 2003). The *WUS* gene also plays a key role in plant embryogenesis by inducing somatic cells reorganize into embryonic stem cells (Zuo et al. 2002). *WUS* is expressed under a strong promotor in *A. thaliana* and its expression initiates the process of making an embryo from somatic

cells (Zuo et al. 2002). It has been recently shown that *WUS* expression in Arabidopsis is regulated by a defined critical level of exogenous auxin that finally leads to somatic embryo induction (Su et al. 2009).

It has been shown by Gorpenchenko et al. (2006) that the moderate level of oncogene *rolC* expression from *Agrobacterium rhizogenes* causes the initiation of somatic embryogenesis in the *rolC*-transgenic cell cultures of *Panax ginseng*. Thus, *rolC* is the only gene to date that is not of plant origin but still capable of triggering plant embryogenesis. It is known the *rolC* gene that is contained in *A. rhizogenes* T-DNA causes the plant disease referred to as "hairy roots". The expression of *rolC* in plants causes significant morphological and biochemical changes (Faiss et al. 1996; Spena et al. 1987). The protein product of the *rolC* gene is able to stimulate root formation in cultured tobacco cells (Spena et al. 1987) and in leaf explants of *Atropa belladonna* (Bonhomme et al. 2000). The mechanism by which this gene triggers root formation and somatic embryogenesis is still unknown.

We have previously observed the expression of several *P. ginseng WUS* genes in embryogenic cell cultures expressing *rolC* (Kiselev and Tchernoded 2009). All of the *P. ginseng WUS* genes characterized by the homeobox nucleotide sequence are more similar to the *Arabidopsis* and *Oryza WUS* genes. The represented results suggest that the initiation of *P. ginseng* somatic embryogenesis may correlate with significant activation of *PgWUSII-6* and *PgWUSII-8* expression. Further development of embryogenesis is connected with the significant expression of *PgWUSI-3* and *PgWUSII-5*.

Materials and methods

P. ginseng cell cultures

The generation of the *rolC* transgenic cell cultures has been established previously (Gorpenchenko et al. 2006) by transformation of the 1c calli with *A. tumefaciens* strains GV3101/pMP90RK containing the plasmid vector pPCV002-CaMVC (Spena et al. 1987). The expression of *rolC* was controlled by the cauliflower mosaic virus (CaMV) 35S promoter (Spena et al. 1987). The 1c-vector culture (designated GV) was obtained by co-cultivation of the 1c calli with *A. tumefaciens* empty vector GV3101/pPCV002 (Gorpenchenko et al. 2006). The appearance of hairy roots was registered within a year after transformation. The CII hairy root cell culture was obtained by excision the adventitious roots from the 1c-CII tumors and placing into liquid medium (Gorpenchenko et al. 2006). The 2cR2 callus cell culture was obtained from the root explants of the CII cell culture. Two callus cultures (2c2 and 2c3) were established from the non-root-forming 1c-CII calli by selection of vigorously-growing cell clusters. After 11-month cultivation, the 2c3 calli spontaneously began to form single leaf-like and embyo-like structures while the 2c2 and 2cR2 cultures continued to grow as undifferentiated calli. While cultivating *P. ginseng* cells without hormones, the quantity of somatic embryos in *P. ginseng* cell culture 2c3 did not decrease. Therefore, it is reasonable to propose that the somatic embryos grow independently from exogenous hormones. While 1c, GV, 2c2, 2cR2, and 2c3 cell cultures were cultivated on W_{4CPA} solid medium (Gorpenchenko et al. 2006), supplemented with 0.4 mg/ml p-chlorophenoxyacetic acid (4-CPA) in the dark at 25°C with 30-day subculture intervals. We cultivated *P. ginseng* cell cultures on W_{4CPA} because it is optimal growth medium for all used cell cultures.

It is significant that the control cell culture, GV (Fig. 1a) did not show any signs of morphological differentiation. In contrast, the *rolC*-transgenic ginseng cell cultures developed roots and somatic embryos, likely as a consequence of the *rolC* gene expression. Cell culture 2c3 with moderate *rolC* expression (Gorpenchenko et al. 2006) naturally presents heterogeneous callus containing several aggregations with varying degrees of differentiation. The 2c3 cell culture was divided into groups of cells according to stage of somatic embryogenesis:

2c3-DD: friable dedifferentiated callus presenting as small meristematic cells (Fig. 1b) with a typical structure described earlier (Gorpenchenko et al. 2006). In 2c3-DD cells the *rolC* expression was decreased by 1.7-fold as compared to 2c3 (Fig. 2a). We do not know exact reasons for the significant differences in *rolC* expression between the 2c3 and 2c3-DD cell cultures. It has been recently shown that expression of a gene which is under control of the 35S promoter may be regulated differently in various plant species and tissues (Benfey and Chua 1990). Presumably, the reason for these differences is varying activity of CaMV35S promoter in the different tissues derived from the 2c3 culture of *P. ginseng*.

2c3-ES: early stages of the somatic embryogenesis. In 2c3-ES cells the *rolC* expression was increased by 1.1-fold as compared to 2c3 (Fig. 2a). Somatic embryos in the early developmental stages having several forms: globule, heart-shaped and torpedo-shaped constructions, as described earlier (Gorpenchenko et al. 2006) (Fig. 1c).

2c3-LS: late stages of the somatic embryogenesis. In 2c3-LS cells the *rolC* expression was the highest from analyzed cells, significantly increased by 1.3-fold as compared to 2c3 (Fig. 2a). Usually this process does not reach late stages in the 2c3 culture when cultivated on solid W_{4CPA} medium. With transplantation onto

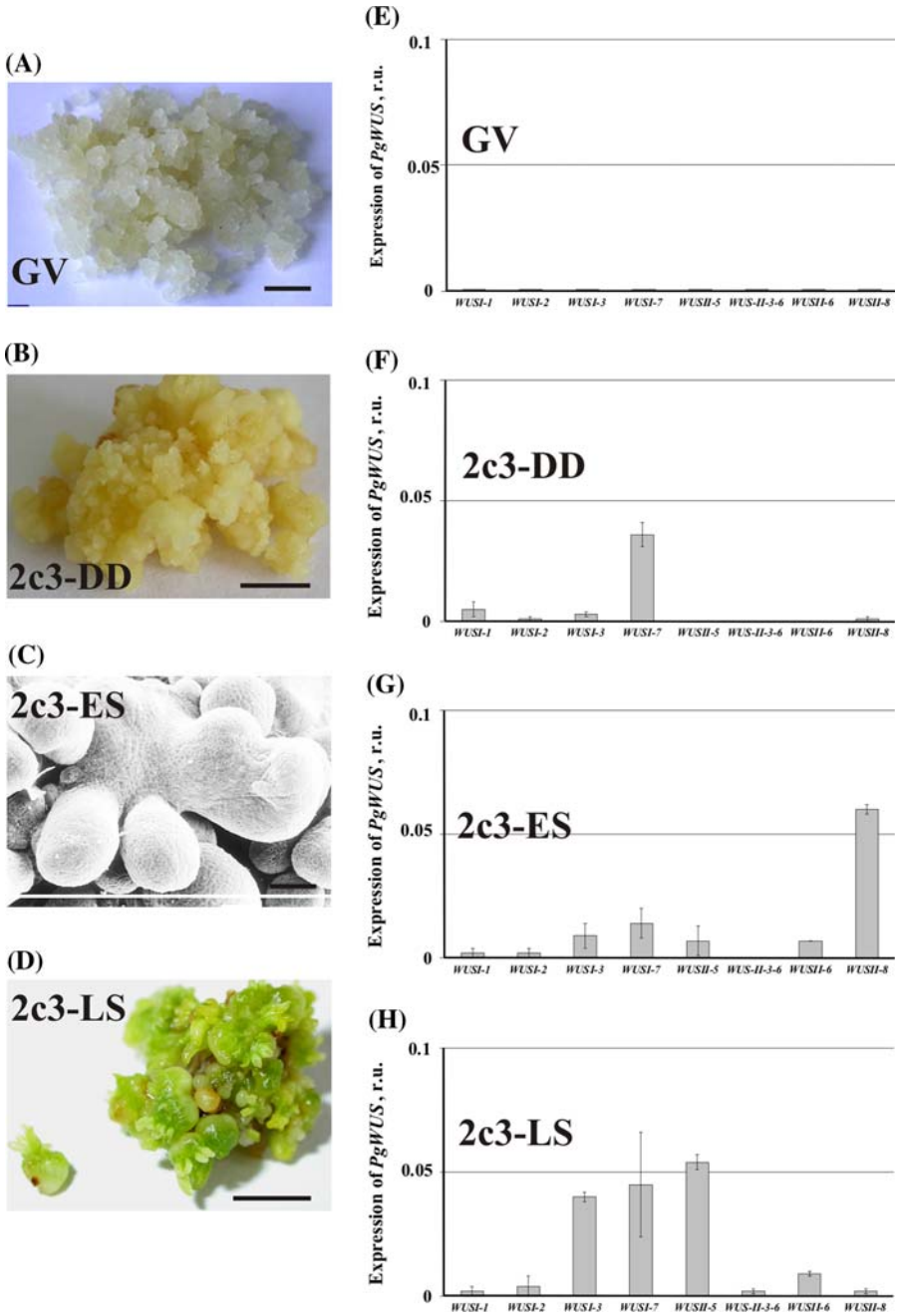

Fig. 1 *WUS* expression in *P. ginseng* cell cultures. A typical view of cultures of GV (control) (**a**), 2c3-DD (**b**), 2c3-ES (**c**), and 2c3-LS (**d**). The expression of the *PgWUS* genes in the ginseng control cell culture GV (**e**), 2c3-DD (**f**), 2c3-ES (**g**), and 2c3-LS (**h**) cultures. Please see the text for further explanation. *Bars* 10 mm (a, b, d) or 100 μM (c)

initiating medium (Junaid et al. 2007) and light (16 h light/8 h night periods) with white light illumination (120 μmol photons/m^2 s) we could stimulate the development of the late stages of embryogenesis (Fig. 1d).

Scanning electronic microscopic analysis

Samples were fixed, dehydrated, dried, and coated with gold as described previously (Gorpenchenko et al. 2006). The samples were examined with a JSM scanning electron microscope.

RT–PCR

The isolation of total RNA and semiquantitative RT–PCR analysis of the *WUS* transcripts was performed as described previously (Bulgakov et al. 2005; Kiselev et al. 2007). To amplify sequences corresponding to the *WUS* genes, the degenerate primers 5′-AARGAACTYTACTACAACA ATG and 5′-CTCTTCTTCTGTCTYTCACGRGC were

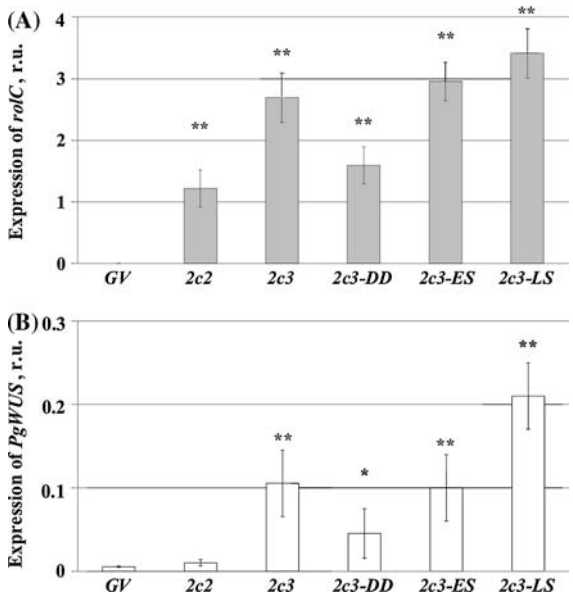

Fig. 2 Total *rolC* (**a**) and *PgWUS* (**b**) expression in GV, 2c2, 2c3, 2c3-DD, 2c3-ES, and 2c3-LS. The data were obtained from three independent experiments and averaged as mean ± SE. See the text for comments, ** denotes the statistical significance ($P < 0.01$) between the mean of the expression in 2c2, 2c3, 2c3-DD, 2c3-ES, and 2c3-LS cell cultures from expression in GV; * denotes a $P<0.05$

designed according to GenBank amino acid sequences of plant WUS (Kiselev and Tchernoded 2009). These primers were used to amplify a 150 bp *WUS* transcript with a Ta = 53°C and an elongation time of 10 s. To amplify sequences corresponding to the *rolC* gene, the primers 5′-TAACATGGCTGAAGACGACCTGT and 5′-TGCAA ACTTGCACTCGCCATGCC were designed according to *rolC* sequence (Kiselev et al. 2006).

The linearity of the PCR was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) and found to be between 40 and 45 cycles for the *WUS* genes, and between 30 and 35 cycles for the *rolC* gene. Quantitative analysis of mRNAs was performed using microchip technology with a DNA 1000 LabChip® kit and the Agilent 2100 Bioanalyzer, following the manufacturer's recommendations. The data are presented as relative fluorescent units normalized to the expression of the corresponding *P. ginseng* actin gene (Kiselev et al. 2006).

Screening of WUS clones

The cDNAs obtained after reverse transcription of mRNA in the RT–PCR reactions were isolated from the gel and subcloned into a pTZ57R/T plasmid as described (Kiselev et al. 2008). The clones were amplified with M13 primers and sequenced (Kiselev et al. 2006) at the Instrumental Centre of IBSS FEBRAS using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, USA). The BLAST search program was used for sequence analysis. Multiple sequence alignments were performed using the BioEdit 7.0.8 program (www.mbio.ncsu.edu/BioEdit/bioedit.html).

The amino acid sequences of the ginseng *WUS* genes were deduced from the nucleotide sequences using the Gene runner 3.05 program and compared with the known WUS sequences of other organisms using the BioEdit 7.0.8 and BLAST software programs. For each cell culture 52–58 clones of *WUS* were sequenced. These results and data on the total *WUS* expression assayed with the degenerate primers were used to estimate the expression level (in relative units) for each of the *WUS* genes. The relative expression was estimated as the total expression normalized to the expression of the ginseng actin gene × % clones of each gene/100 (Kiselev et al. 2008; Dubrovina et al. 2009). Sequenced fragments (homeobox) of *PgWUS* genes were deposited into GenBank (GenBank accession numbers indicated in parentheses): *PgWUSI-1* (EU293843), *PgWUSI-2* (EU293844), *PgWUSI-3* (EU293845), *PgWUSI-7* (EU939528), *PgWUSII-6* (EU939529), *PgWUSII-3-6* (EU939530), *PgWUSII-5* (EU939527), and *PgWUSII-8* (EU939531).

Results

Using PCR with degenerate primers for cDNAs of 2c3, 2c3-DD, 2c3-ES, and 2c3-LS cultures, we obtained amplicons of *WUS* genes of expected sizes; these products were then cloned and sequenced. Using degenerate primers and ginseng cDNA as template, we sequenced the homeobox domains of several *WUS* genes (eight genes). All of the obtained amino acid sequences of homeobox domains were very similar to those of known plant *WUS* genes (Figs. 3, 4). The sequences of *PgWUS* genes that were obtained after the alignment by the program BioEdit were divided into two *PgWUS* subfamilies (Figs. 3, 4), named *PgWUSI* and *PgWUSII*. Eight *WUS* genes were divided into two subfamilies of four closely related genes (Figs. 3, 4). We divided the genes into separate genes subfamilies based on differences of more than one amino acid. Genes in the subfamily were differentiated by DNA sequence, but the amino acid sequences of the homeobox domains are identical. The DNA sequences of these genes, which were obtained in three independent experiments, have been deposited into Genbank.

The expression of *PgWUS* genes in ginseng cell cultures was measured by the frequency analysis of RT–PCR products generated using degenerate primers (Fig. 2b). The expression of *PgWUS* genes in the control GV and 2c2 cell cultures was insignificant (Fig. 2b). In the 2c3 cell culture and in the different developmental aggregates of the 2c3 culture (2c3-DD, 2c3-ES, 2c3-LS), *PgWUS* genes were

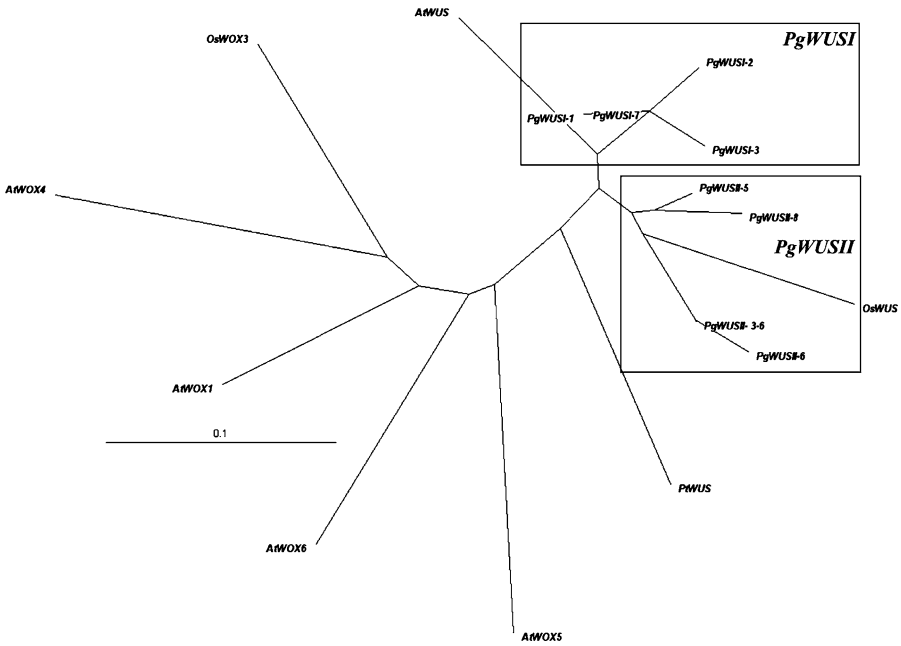

Fig. 3 Phylogenetic tree built from amino acid sequences of the homeobox domain of the *WUS* gene, which is expressed in ginseng 2c3 cell culture, and *WUS/WOX* genes expressed in *Arabidopsis*, *Oryza*, and *Petunia*

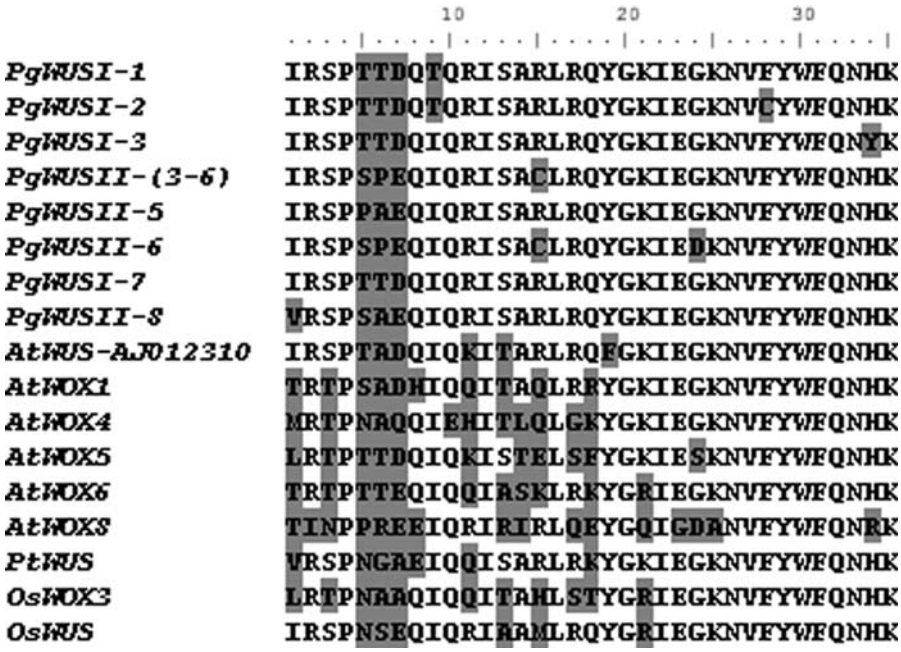

Fig. 4 Comparison of the amino acid sequences of the homeobox of PgWUSs with WUSs and WOXs from other plant species. *Gray* letters indicate differences in amino acid sequence

significantly more expressed than in control cells, correlated with *rolC* expression (Fig. 2b). We calculated the expression of certain *PgWUS* genes using total *WUS* expression data and qualitative composition of the ginseng *PgWUS*.

Expression of *PgWUS* in the GV cell culture

The analysis of the PCR products showed that the GV cell culture did not express *PgWUS* genes. Sometimes a small amount of PCR product appeared in the analyzed region, but further cloning and sequencing of these products indicated that they were not homologous to the *WUS* genes or any other known plant genes. Therefore, these products were likely a result of a nonspecific annealing of the degenerate primers.

Expression of *PgWUS* genes in 2c3-DD

PgWUS subfamilies had low expression levels in the dedifferentiated friable callus. The gene *PgWUSI-7* was the only gene whose expression approached the level of 0.04 r.u. (Fig. 1f).

Expression of *PgWUS* in 2c3-ES

There were consistent differences between 2c3 cell cultures and 2c3-DD cell aggregates in the expression of the *PgWUSII* subfamily in somatic embryos during early developmental stages (Fig. 1f, g). In 2c3-ES cell aggregates, the expression level of *PgWUSII-8* increased reliably to 0.06 r.u. The expression of two new genes *PgWUSII-6* and *PgWUSII-5* was also detected. The expression of all other genes was not substantially different from that in 2c3-DD.

Expression of *PgWUS* in 2c3-LS

The expression pattern of *PgWUS* in somatic embryos at the later stage of development was different in 2c3-LS than in the cultures noted above. The *PgWUS* genes that were predominantly expressed were *PgWUSI-7* and *PgWUSII-5*; the expression of these genes was between three and eight times higher in these cultures than in 2c3-ES (Fig. 1h). The expression of *PgWUSII-8*, however, was lower in 2c3-LS (Fig. 1h).

Discussion

The gene families are known to have arisen from multiple gene duplications. The appearance of a "superfluous" gene copy in the genome opens up the freedom for evolutionary experimentation. One example of this phenomenon that has been studied in detail is the biosynthesis pathway for secondary metabolites in plants (Des Marais and Rausher 2008). A similar process probably occurs with the case of the *WUS* genes. It remains unknown why several plant genes that are homologous to the *WUS* genes have been identified, and yet no *WOX* gene has been found. Degenerate primers are able to amplify related genes. In *Arabidopsis* and *Petunia*, only one *WUS* gene is known to exist. The *WUS* genes that we found in ginseng are probably analogous to the *Arabidopsis* and *Petunia WOX* genes. We suppose that because ginseng is an evolutionary ancient species, its *WOX* and *WUS* genes have not diverged.

The diversity of the *PgWUS* genes and their different expression levels during the different stages of ginseng somatic embryo development suggest that these genes have distinct functions. We suggest that the expression of the *PgWUSI* subfamily plays a role in *P. ginseng* cell culture embryogenesis. It was previously shown that the *WUS* genes are not expressed in GV cell culture, and somatic embryogenesis could not be activated in this line (Gorpenchenko et al. 2006). In cultures of 2c3-DD cells, however, the *PgWUSI* subfamily is expressed. 2c3-DD cells have a tendency to form somatic embryos. In this aggregate, somatic embryos may spontaneously appear during cultivation. The reason that there is a spontaneous signal for the activation of somatic embryo development is unknown. We cannot yet predict when or in what part of the callus will this process begin; we know only that local change in temperature, salt concentration, or phytohormones can stimulate this process. For example, if the 2c3 cell culture is incubated for more than half a year on medium lacking hormones, the number of somatic embryos will increase. Also, cultures of 2cR2 cells (callus with the high level of *rolC* gene expression) will grow embryo-like structures (data not published) when cultured in the same conditions. It is possible that there is a link between *WOX* gene expression and the hormone compound in the medium.

It is informative to compare *PgWUS* gene expression in 2c3-ES and 2c3-LS. During the passage from early to late stages of embryo development, there is an increase in *PgWUSI-7* and *PgWUSII-5* expression, while *PgWUSII-8* expression is decreased. This pattern is similar to that of the *WOX* genes during somatic embryo development, which was described earlier (Zuo et al. 2002).

Thus, our research demonstrates the correlation between *rolC*, *PgWUS* genes expression, and the initiation of the *P. ginseng* somatic embryogenesis; however detailed understanding of the PgWUS functions will be achieved by using such methods as Agrobacterial transformation with *PgWUS* genes or RNAi in the future.

Research on the biochemical function of the Rol proteins has been reviewed by several authors (Costantino et al. 1994; Nilsson and Olsson 1997) but the mechanism by which *rolC* causes the expression of the *WUS* genes is still unknown. Early research has shown that the expression of the *rolC* gene in the *P. ginseng* cells changes the expression of different *CDPK* genes and induces the generation of new *CDPK* transcripts with modified sequences in the catalytic Ser/Thr kinase subdomains (Kiselev et al. 2008). Perhaps *rolC* affects multiple biochemical processes in the transformed cells by changing the expression of various *CDPK* genes and generating new *CDPK* transcripts. Further study is needed to confirm this hypothesis.

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