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Regular Article

Evidence of *WUSCHEL* (*WOX2*) gene expression during induction of somatic embryogenesis from apical shoot buds of mature trees of *Pinus roxburghii*

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This study highlights for the first time the expression of transcription factor, *WOX2* (*WUSCHEL* homeobox 2) in the embryogenic tissue derived from the apical shoot buds of mature trees of 3 genotypes of *P. roxburghii*. Therefore, *PrWOX2* could be used as a potential molecular signature for the identification of embryogenic cultures during the early development of somatic embryos in conifers. On the other hand, the non-embryogenic tissue of shoot bud cultures which were initiated without cold treatment (control) failed to show the expression of *WOX2*. This clearly demonstrates the role of *WOX2* in the somatic embryogenesis pathway and might be directly related to the stress conditions.

Key words: Apical buds, Chir pine, meristematic tissue, pines, somatic embryogenesis,

Somatic embryogenesis is the development of embryo-like structures under *in vitro* conditions on tissues derived from somatic cells (Konar and Nataraja, 1965; Nataraja and Konar, 1970; Feher *et al*. 2003; Malabadi *et al*. 2011a, 2011b, 2011c, 2011d, 2011e, 2011f, 2011g). During cloning of mature conifers, isolated somatic cells (stem cells) under any external stress conditions of cold\heat or chemical are induced to form a somatic embryo. Embryogenic systems derived from vegetative shoot apices or secondary needles of mature pines have been well established in at least a few conifers (Litz *et al*. 1995; Malabadi and van Staden, 2003, 2005a, 2005b, 2005c, 2006; Malabadi, 2006; Malabadi and Nataraja, 2006a, 2006b; Aronen *et al*. 2007, 2008; Malabadi and Nataraja, 2007a, 2007c, 2007f, 2007g; Malabadi *et al*. 2008a, 2008b, 2008c; Park *et al*. 2009a; Malabadi and Teixeira da Silva, 2011). Furthermore, the tissue used, developmental phase of the source plant, stress source, stress chemical concentration, and duration of stress are important for induction of somatic embryos of mature conifers (Malabadi *et al*. 2004). In addition, these factors are very sensitive and affect each other. The fact indicates that each stress induces a common reaction, and the reaction is related to the plant somatic cell de-differentiation and re-differentiation to somatic embryogenesis (Ikeda-Iwai *et al*. 2003). Probably, each stress treatment induces expression of a factor that controls the start of somatic embryogenesis, through the common reaction (Ikeda-Iwai *et*

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al. 2003). Since most somatic cells are not naturally embryogenic, an induction phase is required for the cells to acquire embryogenic competence (Konar and Nataraja, 1965; Feher *et al*. 2003; Ikeda-Iwai *et al.* 2003; Namasivayam, 2007; Malabadi *et al.* 2009a). This is in contrast to the zygote in sexual reproduction which is intrinsically embryogenic. Stress conditions, for example temperature-related, hormonal or osmotic, can influence the fate of tissue cultured plants cells i.e. induce either apoptosis or a developmental switch, and this phenomenon could be utilized for initiation of somatic embryogenesis as well (Feher *et al*. 2003; Ikeda-Iwai *et al.* 2003; Namasivayam, 2007; Malabadi *et al.* 2009a). The molecular bases of this metabolic and developmental switch are poorly understood despite extensive research with different tissue culture systems.

During the past 2 decades, considerable efforts have been made to identify genes with altered expression pattern during somatic embryogenesis (Zuo *et al.* 2002; Arroyo-Herrera *et al.* 2008; Cairney and Pullman, 2007; Malabadi and Nataraja, 2007g; Malabadi *et al*. 2009b). Most of these genes however, are up regulated only in the developmental stages, suggesting that they do not play a direct role in the vegetative-to-embryogenic transition (Zuo *et al.* 2002; Malabadi and Nataraja, 2007g; Malabadi *et al*. 2009b). Cell differentiation depends on the proper and sequential expression of key genes required for somatic embryogenesis. Several aspects of control are required for this which includes: chromatin modifications, DNA methylation, correct amount of particular transcription factors, proper nuclear arrangement, etc (Malabadi and Nataraja, 2007g; Malabadi *et al*. 2009b). There are different groups or class of genes expressed during induction of somatic embryogenesis. For example, carrot somatic embryogenesis receptor kinase (*SERK*) gene, whose expression appears to mark vegetative-to-embryogenic transition; however its function remains unclear. Over expression of the *Plant Growth Activator 6 (PGA6)* gene promotes the formation of somatic embryos from various vegetative tissues, as well as from zygotic embryos independent of any external plant hormones (Malabadi *et al.* 2009b). These somatic embryos following a developmental process remarkably similar to that of zygotic embryogenesis were able to germinate and grow healthy, fertile plants, suggesting that *PGA6* is involved in the maintenance of embryonic cell identity. *PGA6* was found to be identical to *WUS*, a homeodomain protein previously characterized as a key regulator for the specification of meristem cell fate. During the last few years the homeobox transcription factor *WUSCHEL* (*WUS*) has been shown to cause dedifferentiation when expressed on somatic cells followed by a production of new stem cells that can lead to somatic embryogenesis or organogenesis. The homebox gene *WUSCHEL* (*WUS*) is required to specify stem cell identity (Baurle and Laux, 2005; Leibfried *et al.* 2005). WUSCHEL (*WUS)* was originally identified as a central regulator of shoot and floral meristems in *Arabidopsis*, where it was expressed in a small group of cells, and is required to maintain the overlying stem cells undifferentiated (Baurle and Laux, 2005; Leibfried *et al.* 2005). Among all the genes, *WUSCHEL* homeobox (*WOX2*) in *Picea abies* (Palovaara and Hakman, 2008; Palovaara *et al.* 2010) is one of the important putative marker gene expressed during the early development of somatic embryos. The transcription factor, *WOX2 (WUSCHEL* homeobox 2), which is one of the 15 *WOX* family proteins, play an important role in controlling many aspects of somatic embryogenesis in plants (Palovaara and Hakman, 2008; Palovaara *et al*. 2010). In the higher plants, some genes and transcription factors related to important mechanisms of embryogenesis are plant specific. Therefore, new findings such as discovery and

identification of new transcription factors that control the expression of embryogenesis-related genes and embryogenesis-specific phytohormone response be made in a near future. In our previous study, we were able to isolate cDNA clones of genes expressed in embryogenic and non-embryogenic tissue derived from the vegetative shoot apices of mature trees of *P. roxburghii* (Malabadi and Nataraja, 2007g). Furthermore, we have also constructed cDNA library in our previous study (Malabadi and Nataraja, 2007g). This study is the continuation of our previous work and highlights for the first time the confirmation of expressed gene particularly *WUSCHEL* (*WOX2*) (Palovaara and Hakman, 2008; Palovaara *et al.* 2010) in embryogenic tissue derived from the apical shoot buds of mature trees of *P. roxburghii*.

Materials and methods

Initiation of embryogenic tissue

Shoot apical domes from mature trees (14- years old) of *Pinus roxburghii* of 3 genotypes (PR11, PR105, and PR521) were collected from the Western Ghat Forests, India (14^0 5′ to 15^0 25′ N latitude and 74^0 45′ to 76^0 E longitude with an average rainfall of 80 cm.). They were cleansed with 1% Citramide (Sodium hypochlorite 3.5%) for 5 min and rinsed thoroughly with sterilized distilled water. These were surface decontaminated with 70% ethanol for 5 min followed by immersion in 0.5% $HgCl_2$ for 2 min and rinsed 4-times with sterile double distilled water. Transverse-thin sections of approximately 0.5-1.0 mm thick were cut using sharp sterilized blade or scalpel from shoot apical domes (upper part with 2 to 3 sections only) for the initiation of embryogenic tissue. These shoot apical dome sections were cultured individually on full strength (Inorganic salts) DCR (Gupta and Durzan, 1985) medium containing 0.2 g l^{-1} polyvinyl pyrollidine (PVP), 2.0 g l^{-1} Gellan gum (Sigma), 30 g l^{-1} maltose (Analar, Sigma) and 0.3 % activated charcoal (Sigma) without growth regulators. The cultures were raised in 25 mm X145 mm glass culture tubes (Borosil) containing 15 ml of medium. These cultures were incubated in dark at 4^0 C for 3 days. Thin apical dome sections after incubation in dark at 4^0 C for 3 days were subcultured on full strength DCR medium supplemented with 22.62 μM 2, 4-D, 26.85 μM NAA, and 8.87 μM BA (initiation medium) (Malabadi and Nataraja, 2006ab). The pH of the medium was adjusted to 5.8 with NaOH or HCl before Gellan gum was added. The media were then sterilized by autoclaving at 121^0 C and 1.05 kg/cm^2 for 15 min. L-glutamine and casein hydrosylate were filter sterilized and added to the media after it had cooled to below 50^0 C. All the cultures were maintained in the dark at 25 ± 2^0 C with 55-60% relative humidity.

All the cultures were maintained in the dark at 25 ± 2^0 C for four weeks. Embryogenic tissue was initiated according to our previous existing protocols (Malabadi *et al*., 2004; Malabadi and Nataraja, 2006ab; Malabadi and van Staden, 2006). For the initiation of non-embryogenic tissue, thin sections of shoot apical dome without cold-pretreatment subcultured on the initiation medium (Malabadi and Nataraja, 2006ab) were served as the control. They were also maintained in dark for four weeks.

Maintenance of embryogenic tissue

The embryogenic tissue showing proembryonal masses was again subcultured onto maintenance medium. The full-strength DCR(Gupta and Durzan, 1985) basal medium containing 60 g l^{-1} maltose, 2 g l^{-1} Gellan gum supplemented with 2.26 μM 2,4-D, 2.68 μM NAA and 0.88 μM BA (maintenance medium) was used for this purpose in accordance with our existing previous protocols (Malabadi and Nataraja, 2006ab). On the maintenance medium, embryonal suspensor masses were cultured for 30 days with two subcultures. The presence of embryonal masses was

determined by morphological and microscopic observations. The non-embryogenic cultures (control) were also subcultured on the maintenance medium and maintained in dark condition.

For the following experiments of *WUSCHEL (WOX2)* (Palovaara and Hakman, 2008) gene expression studies, two types of plant material, 1) embryogenic tissue initiated by cold-pre treatment, and 2) control (non-embryogenic tissue) induced without cold pre-treatment have been used for the isolation of total RNA. For total RNA isolation, one gram fresh wt of embryogenic tissue and control (non-embryogenic tissue) were then placed in a cryostorage vial partially immersed in liquid nitrogen. Ten vials of plant tissues (embryogenic tissue and non-embryogenic tissue-control) were collected. Frozen tissues were stored at -70^{o}C until further analyses were performed.

RNA preparation and cDNA synthesis

Frozen samples of embryogenic tissue and control (non-embryogenic tissue) derived from the apical shoot buds of *Pinus roxburghii* of 3 genotypes (PR11, PR105, and PR521) were ground in a mortar and pestle with liquid nitrogen. Total RNA was isolated according to the modified method of Chang *et al*. (1993). To remove residual genomic DNA, 25 µg of RNA was treated with TURBO-DNase™ (Ambion, Austin, TX, USA). cDNA was generated from 1 µg of DNase-treated RNA using the Superscript II RT system (Invitrogen, CA, USA) according to the manufacturer's protocol (Park *et al.* 2009). Each reaction was run in duplicate, generating two independent cDNA samples for each RNA sample isolated from 3 genotypes (PR11, PR105, and PR521) of *P. roxburghii* as reported in our previous study (Malabadi and Nataraja, 2007g).

Cloning of *P. roxburghii WOX2*

For the isolation of the *WOX2* gene, following gene specific primers were designed based on homologous sequences publically available, including Pa*WOX2* (*P. abies WOX2*, Acc. AM286747) and Pt*WOX2* (*P. taeda WOX2*, Acc. DR693345; Cairney *et al.* 2006; Park *et al*. 2009). The gene specific primers were custom synthesized and used for the PCR amplification of the PR *WOX2* gene using cDNA as the template. Gene alignment was done using Bio Edit programme. Following PCR amplification from *P. roxburghii* cDNAs, the resulting amplicons were cloned into the TOPO-TA cloning vector (Invitrogen) (Park *et al.* 2009). The gene *WOX2* was confirmed by the DNA sequence analysis. (Forward: 5'-ATG GCC GAG GGT CAA TCC ACC ATG A-3'); (Reverse 3' CTT GCC AGG ATG CTG AGG GAT A-5')

Maturation of somatic embryos

For maturation, embryogenic tissue clumps of each of the 3 genotypes (PR11, PR105, and PR521) were partially desiccated. One gram fresh weight of tissue of each embryogenic line were transferred to sterile empty Petri dishes (60 mm diam.) containing two sterile Whatman filter paper disks (50 mm) (Schleicher and Schuell, qualitative circles). The Petri dishes were sealed with Parafilm and kept at 25 ± 2°C in the dark for 24 hr to obtain the desired extent of desiccation. After desiccation, the partially desiccated tissue of each embryogenic line was transferred to maturation medium to induce cotyledonary embryo development. The full strength DCR (Gupta and Durzan, 1985) medium with 60 g l^{-1} maltose, 37.84 µM abscisic acid (ABA) and 5 g l^{-1} Gellan gum (maturation medium) was tested for this purpose. All the cultures were placed in the dark at 25 ± 2°C and these maintained for 8 to 12 weeks (Malabadi, 2006b; Malabadi and Nataraja 2006a, 2006b).

Germination and plantlet recovery

After 6 to 8 weeks of maturation in the presence of ABA and higher concentrations of

maltose (60 g l^{-1} maltose) the cotyledonary somatic embryos were recovered from the cultures for germination. Before germination, the cotyledonary somatic embryos of the all embryogenic lines were cold pre-treated at 2°C and kept in dark for 5 days. The germination medium (GM) used was half DCR (Gupta and Durzan, 1985) medium with 2 g l^{-1} Gellan gum (Malabadi and Nataraja 2006a, 2006b). In the first week of germination, cultures were kept in darkness, and then transferred to diffuse light in the second week, and thereafter to a 16-hr photoperiod under a light intensity of 50 µmol m^{-2} s^{-1} for hardening. Somatic embryos were considered germinated as soon as radical elongation occurred and conversion to plantlets was based on the presence of epicotyls. After 4 to 6 weeks on germination medium, plantlets were transferred to vermiculite in a controlled growth room. All the experiments were repeated over 3 different and consecutive years. Data were arcsine transformed before being analyzed for significance using ANOVA (analysis of variance, $p<0.05$) or evaluated for independence using a Chi-square test. Further, the differences were contrasted using a Duncan's multiple range test ($a=0.05$) following ANOVA. All statistical analysis was performed using SPSS statistical software package version 13.0.1.1, Microsoft Windows.

Results and discussion

In our present study, we were able differentiate both embryogenic and non-embryogenic tissue on the basis of gene expression studies. We have established a reproducible protocol for the induction of somatic embryogenesis using apical shoot buds of mature trees of many recalcitrant pines (Malabadi and van Staden, 2003, 2005a, 2005b, 2005c, 2006; Malabadi, 2006; Malabadi and Nataraja, 2006a, 2006b; Aronen *et al.* 2007, 2008; Malabadi and Nataraja, 2007a, 2007c, 2007f, 2007g; Malabadi *et al.* 2008a, 2008b, 2008c; Park *et al.* 2009a; Malabadi and Teixeira da Silva, 2011). This is one of the major breakthroughs in the forest biotechnology. Embryogenic tissue derived from apical shoot buds could be easily separated and identified on the basis of microscopic observation. This is the first hand information for the confirmation of induction of somatic embryogenesis from shoot buds of mature trees of recalcitrant pines. The second most important feature for the identification of somatic embryogenesis is the nature, and type of the cells which was well documented and demonstrated in our previous papers (Malabadi and van Staden, 2003, 2005a, 2005b, 2005c, 2006; Malabadi, 2006; Malabadi and Nataraja, 2006a, 2006b; Aronen *et al.* 2007, 2008; Malabadi and Nataraja, 2007a, 2007c, 2007f, 2007g; Malabadi *et al.* 2008a, 2008b, 2008c; Park *et al.* 2009a; Malabadi and Teixeira da Silva, 2011). In case of pines, the transverse thin layer of apical shoot buds showed outermost epidermis layer, then internal layer of cortex region, followed by thin cambial region and central pith or medullar region respectively. The active dividing and totipotent cells (stem cells) are positioned only at the cambial layer of the apical meristematic tissue in conifers, so that their growth and division leads to a continuous flow of progeny cells. These cambial layer cells (stem cells) under stress conditions such as cold/heat or chemical treatment undergo differentiation and leading to the embryogenic pathway in conifers. But activation of the cambial layer cells is one of the important steps for the successful induction of somatic embryogenesis in conifers. This has been achieved and successful in many recalcitrant conifers (Malabadi and van Staden, 2003, 2005a, 2005b, 2005c, 2006; Malabadi, 2006; Malabadi and Nataraja, 2006a, 2006b; Aronen *et al.* 2007, 2008; Malabadi and Nataraja, 2007a, 2007c, 2007f, 2007g; Malabadi *et al.* 2008a, 2008b, 2008c; Park *et al.* 2009a; Malabadi and Teixeira da Silva, 2011). These cells (stem cells) are very small in size

and slow in growth at the initial stages of the development. The embryogenic cells were small, richly cytoplasmic and actively dividing with a prominent nucleus. These cells were generally present in small, compact aggregates, rich in starch grains and showed competence for embryogenesis. On the other hand the rest of the layers (epidermis, cortex region and central pith or medulla) have induced nonembryogenic tissue in conifers. Microscopic observation of non-embryogenic tissue confirmed loosely arranged, elongated thin parachymatous cells. The non-embryogenic tissue is composed of large cells, vacuolated and often elongated with sparse cytoplasm and few starch grains. These cells can survive up to 6 months with multiple subcultures, ultimately leading to death due to the exudation of high phenolic compounds and other factors. Therefore, there is a clear distinction between embryogenic and non-embryogenic tissue on the basis of nature, and type of the cells without any molecular marker. A well experienced Scientist in plant tissue culture can recognize and identify the embryogenic cells very easily without the help of molecular marker. Therefore, inducing somatic embryo-genesis using apical shoot buds of mature conifers is an important step in the commercial forestry. Therefore, somatic embryogenesis is still a very challenging process and considered as a fundamental science but not a technique. On the other hand plant tissue culture is an important *in vitro* technique.

For further characterization of the embryogenic tissue, we have developed a potential molecular marker on the basis of the expression of one of the important transcription factor, *WOX2* (*WUSCHEL* homeobox 2) in the embryogenic tissue of *P. roxburghii.* Transcription factor, *WOX2* (*WUSCHEL* homeobox 2) was strongly expressed in the embryogenic tissue but not in non-embryogenic tissue (control). In our present study, embryogenic tissue was developed under cold stress conditions. On the other hand non-embryogenic tissue was induced without cold treatment. Expression of *WOX2* (*WUSCHEL* homeobox 2) in the embryogenic tissue of 3 genotypes (PR11, PR105, and PR521) of *P. roxburghii* clearly confirmed its involvement in the somatic embryogenic pathway and might be directly related to stress conditions. Therefore, stress conditions always induce somatic embryogenesis in many plant species which also directly related to the expression of genes. This is the first evidence of expression of *WOX2* in *P. roxburghii* and could be used as marker for the identification of embryogenic tissue in pines. In gymnosperms, no *WUS* homolog was found (Palovaara and Hakman, 2008) and as such it is believed that the *WOX* and *WUS* genes have not diverged in some species (Kiselev *et al.* 2009; Park *et al.* 2009). An interrogation of the *WOX* family members in gymnosperms obtained from the public databases identified that *PrWOX2* has 80% similarity to *P. abies PaWOX2* and is almost identical to other pine species sequence such as *PsWOX2* of *Pinus sylvestris* (99% homology) and *PtWOX2* of *P. taeda* (100% homology) (Park *et al*. 2009). These findings are consistent with previous findings (Malik *et al.* 2007, Palovaara and Hakman, 2008, Palovaara *et al.* 2010) which suggested that *WOX2* may be a potential marker to predict the embryogenic potential of spruce and *Brassica* cultivars (Park *et al*. 2009). Therefore, on the basis of this wonderful study by our laboratory further strengthens the concept of cloning of mature trees of pines. *PrWOX2* could be used as a potential genetic marker for the identification of the embryogenic cultures, which is just an added advantage for developing SE protocols for recalcitrant pines.

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