Phytosulfokine Stimulates Somatic Embryogenesis in *Cryptomeria japonica*

Tomohiro Igasaki 1,4, Noriko Akashi 1, Tokuko Ujino-Ihara 2, Yoshikatsu Matsubayashi 3, Youji Sakagami 3 and Kenji Shinohara 1

1 Department of Molecular and Cell Biology, Forestry and Forest Products Research Institute (FFPRI), Tsukuba, 305-8687 Japan
2 Department of Forest Genetics, Forestry and Forest Products Research Institute (FFPRI), Tsukuba, 305-8687 Japan
3 Graduate School of Bio-Agricultural Sciences, Nagoya University, Nagoya, 464-8601 Japan

Phytosulfokine (PSK), which has been identified as a plant growth factor, had a dramatic stimulatory effect on the formation of somatic embryos of sugi (*Cryptomeria japonica*) in the presence of polyethylene glycol. The resultant somatic embryos germinated with synchronous sprouting of cotyledons, hypocotyls and roots, and most of the seedlings grew normally. A cDNA clone for the precursor to the PSK peptide of *C. japonica* was identified in an expressed sequence tags database. Our results support the existence of a PSK signaling pathway in *C. japonica*.

**Keywords**: *Cryptomeria japonica* — Phytosulfokine — Regeneration — Somatic embryogenesis — Sugi — Zygotic embryo.

Abbreviations: EST, expressed sequence tags; PEG, polyethylene glycol; PSK, phytosulfokine.

Sugi, *Cryptomeria japonica* D. Don (Taxodiaceae), is one of the most commercially important conifers in Japan. However, sugi pollinosis is one of the most serious allergic diseases in Japan. We are interested in the genetic engineering of transgenic *C. japonica* that produces allergen-free pollen grains. Recently, we established a simple and reliable procedure for introducing DNA into mature zygotic embryos of three species of Japanese conifer, including *C. japonica* (Mohri et al. 2000) and a system for the reproducible regeneration of plants via somatic embryogenesis (Igasaki et al. 2003). However, appropriate techniques are now necessary to enhance the efficiency of regeneration of plants.

Phytosulfokine (PSK), a small sulfated peptide, acts as an extracellular ligand involved in the initial step of cellular dedifferentiation, proliferation and re-differentiation. PSK has been found in both monocotyledonous and dicotyledonous plants, for example, *Asparagus officinalis*, *Oryza sativa*, *Daucus carota* and *Arabidopsis thaliana* (Matsubayashi and Sakagami 1996, Matsubayashi et al. 1996, Matsubayashi et al. 1997, Yang et al. 1999, Yang et al. 2000, Hanai et al. 2000, Yang et al. 2001). PSK has also been shown to stimulate somatic embryogenesis in carrot (Kobayashi et al. 1999). Therefore, we examined the effects of PSK on somatic embryogenesis in *C. japonica*. In the present study, we found that the addition of synthesized PSK to both the medium used for proliferation and that used for embryogenesis has a dramatic stimulatory effect on the formation of somatic embryos. We also found evidence for the expression of the gene for the precursor to PSK in *C. japonica*.

We used ten lines of embryogenic cells whose ability to produce somatic embryos had been confirmed (Table 1). In many cases, embryogenic cells that had been maintained in standard liquid medium lost the capacity to proliferate and to regenerate and they often turned brown during repeated subculture. However, the addition of PSK to the medium maintained both the capacity to proliferate and regenerate and freshness (bright yellow color) of embryogenic cells (Fig. 1A, B). This observation suggests that PSK might play an important role in the maintenance of the capacity for cell division and the juvenility of embryogenic cells.

Polyethylene glycol (PEG) has a stimulatory effect on the formation of somatic embryos of *Picea glauca, Chamae-

**Table 1** Effects of PSK on the frequency of formation of embryos, in the presence and absence of PEG, in ten lines of embryogenic cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Number of embryos per Petri dish</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>+PEG</td>
</tr>
<tr>
<td>L-1</td>
<td>26.3±4.7</td>
</tr>
<tr>
<td>L-2</td>
<td>2.3±0.3</td>
</tr>
<tr>
<td>L-3</td>
<td>4.2±1.9</td>
</tr>
<tr>
<td>L-4</td>
<td>32.3±6.4</td>
</tr>
<tr>
<td>L-5</td>
<td>8.3±3.9</td>
</tr>
<tr>
<td>L-6</td>
<td>11.3±2.0</td>
</tr>
<tr>
<td>L-7</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>L-8</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>L-9</td>
<td>6.7±1.4</td>
</tr>
<tr>
<td>L-10</td>
<td>5.5±1.9</td>
</tr>
</tbody>
</table>

Values are means ± SE of results (n = 3–6). See text for full details.

4 Corresponding author: E-mail: iga@ffpri.affrc.go.jp; Fax, +81-298-73-0507.
cyparis pisifera and C. japonica (Attree et al. 1995, Maruyama et al. 2002, Igasaki et al. 2003). We examined the effects of PSK on the development of somatic embryos of C. japonica in the presence and absence of PEG (Fig. 2A–D, Table 1). The addition of either PSK or PEG could increase the efficiency of formation of somatic embryos. Moreover, the addition of both PSK and PEG had a dramatic stimulatory effect on the formation of somatic embryos (Fig. 2D). Furthermore, while cell line L-5 never produced somatic embryos in the absence of PSK, it formed the embryos in the presence of PSK (Table 1). It seems likely that embryogenic cells of C. japonica produce a smaller amount of active PSK as compared with those of P. grauca and...
Somatic embryogenesis in *C. japonica*

From *C. pisifera*, from which one can easily induce somatic embryogenesis by the addition of only PEG, we observed and obtained embryos at the early to the mature stages after about 4 weeks (Fig. 2E, F). The time required for generation of somatic embryos was unaffected by PSK (data not shown). The optimal concentration of PSK for the formation of somatic embryos was 32 nM. At 1 nM, PSK already had an obvious effect, but at levels above 32 nM PSK was no more effective than it was at 32 nM. Our results are almost consistent with previous findings of *D. carota* by Kobayashi et al. (1999), who found that 100 nM PSK was most effective for somatic embryogenesis in *D. carota*, but did not test at 32 nM. Approximately 80% of the induced somatic embryos germinated, with synchronous sprouting of cotyledons, hypocotyls and roots, and the germinated seedlings grew normally (Fig. 2G, H). Thus, PSK clearly had a positive effect on the development of somatic embryos, and our results suggest that the PSK signaling pathway, previously identified by Matsubayashi et al. (2002) in angiosperms is also operative in *C. japonica*.

To investigate the possible presence and expression of a gene for the precursor to PSK in *C. japonica*, we surveyed a database of *C. japonica* expressed sequence tags (EST) using the amino acid sequence of PSK (YIYTQ). We found one EST clone, CC4124 (accession nos. BP176104 and AB105536) that encoded PSK within a putative open reading frame (ORF). The ORF that we identified is 306 bp long and encodes 102 amino acids (Fig. 3A). Application of the rules proposed by von Heijne (1986) allowed us to predict that the ORF encodes an amino-terminal hydrophobic signal sequence of 28 amino acids. The predicted polypeptide includes the sequence YIYTQ at amino acid positions 93 through 97 and a conserved Asp residue at position 92 (Fig. 3A, B). These three features are conserved in other precursors to PSK in angiosperms (Yang et al. 1999, Yang et al. 2000). Thus, a gene for the precursor to PSK is present and expressed in *C. japonica*, supporting the hypothesis that a PSK signaling pathway exists in this conifer.

In the present study, we found evidence that suggests that a PSK signaling pathway is present in a gymnosperm, as it is in angiosperms. Moreover, PSK had positive effects both on the proliferation and maintenance of embryogenic cells and on the formation of somatic embryos of *C. japonica* (Fig. 1, 2, Table 1). Furthermore, the gene for a precursor to PSK was found in the genome of *C. japonica* (Fig. 3). Our findings
allowed us to establish a simple and reliable procedure for somatic embryogenesis and the regeneration of *C. japonica*. In our system, embryogenic cells can be induced from various genotypes of *C. japonica* (Igasaki et al. 2003), and somatic embryos can be easily produced in all seasons of the year by the addition of PSK. Our system also allowed us to repeat the induction of somatic embryos via embryogenic cells from newly induced somatic embryos. Such a system for the reproducible regeneration of plants from embryogenic callus is essential for the genetic transformation of *C. japonica*.

In previous studies, we established a simple and reliable procedure for the regeneration of transgenic Japanese broad-leaved trees (Mohri et al. 1996, Mohri et al. 1997, Mohri et al. 1999, Igasaki et al. 2000). However, to our knowledge, no studies of the transformation of Japanese coniferous species have been reported. Recently, we established an effective procedure for the introduction of DNA into mature zygotic embryos of *C. japonica* (Igasaki et al. 2001) from *C. japonica*. Therefore, in the near future, the present system for the regeneration of *C. japonica* should allow the genetic engineering of transgenic *C. japonica* with allergen-free pollen grains.

Ten different lines of embryogenic cells (Igasaki et al. 2003) were used in this study. All cells were cultured in a medium that contained MSG basal salts and vitamins (Becwar et al. 1988), 2003) were used in this study. All cells were cultured in a medium that contained MSG basal salts and vitamins (Becwar et al. 1988), 0.01% (w/v) myo-inositol, 0.15% (w/v) L-glutamine, 3% (w/v) sucrose, 3.2 μM N6-benzyladenine and 32 nM PSK (LMSGP medium; 10 ml per 50-ml flask), on a rotary shaker operated at 110 rpm, in darkness, at 25°C. Cells were subcultured at 2-week intervals in the same medium.

After proliferation, embryogenic cells were collected on a cell strainer with 100-μm pores (Falcon 2360; Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.) and rinsed twice with a solution (LEMM medium) that contained EM basal salts, vitamins, inositol and amino acids (Smith 1996), 5% (w/v) PEG 4,000 (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 3% (w/v) maltose and 100 μM ABA. Approximately 1 × 10^7 to 2 × 10^7 cells in 1 ml of LEMM medium were plated on individual filter-paper disks (Advantec no. 2, 70 mm diameter; Toyo Roshi Kaisha, Ltd, Tokyo, Japan) in Petri dishes (90 mm i.d. × 20 mm) that contained LEMM medium supplemented with 32 mM PSK, 0.2% (w/v) activated charcoal and 0.3% (w/v) gellan gum (SEMM medium; 50 ml per Petri dish). Petri dishes were sealed with Parafilm® "M" (American National Can™, Chicago, IL, U.S.A.) and incubated in darkness at 24°C/16°C (day/night; 12 h/12 h) for 4–8 weeks.

Somatic embryos were collected from the SEMM medium and transferred to Smith's germination medium (Smith 1996) that has been supplemented with 0.2% (w/v) activated charcoal and 10 μM GA3. Cultures were kept in darkness at 24°C/16°C (day/night; 12 h/12 h). After germination, plantlets were transferred to the same medium without GA3 and maintained at 25°C under cool white fluorescent light (30 μmol m⁻² s⁻¹, 16-h photoperiod) for regeneration of plantlets.

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**References**


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