Involvement of Phytosulfokine in the Attenuation of Stress Response during the Transdifferentiation of Zinnia Mesophyll Cells into Tracheary Elements1[W][OA]

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Phytosulfokine (PSK) is a sulfated peptide hormone required for the proliferation and differentiation of plant cells. Here, we characterize the physiological roles of PSK in transdifferentiation of isolated mesophyll cells of zinnia (Zinnia elegans 'Canary Bird') into tracheary elements (TEs). Transcripts for a zinnia PSK precursor gene, ZePSK1, show two peaks of expression during TE differentiation; the first accumulation is transiently induced in response to wounding at 24 h of culture, and the second accumulation is induced in the final stage of TE differentiation and is dependent on endogenous brassinosteroids. Chlorate, a potent inhibitor of peptide sulfation, is successfully applied as an inhibitor of PSK action. Chlorate significantly suppresses TE differentiation. The chlorate-induced suppression of TE differentiation is overcome by exogenously applied PSK. In the presence of chlorate, expression of stress-related genes for proteinase inhibitors and a pathogenesis-related protein is enhanced and changed from a transient to a continuous pattern. On the contrary, administration of PSK significantly reduces the accumulation of transcripts for the stress-related genes. Even in the absence of auxin and cytokinin, addition of PSK suppresses stress-related gene expression. Microarray analysis reveals 66 genes down-regulated and 42 genes up-regulated in the presence of PSK. The large majority of down-regulated genes show significant similarity to various families of stress-related proteins, including chitinases, phenylpropanoid biosynthesis enzymes, 1-aminocyclopropane-1-carboxylic acid synthase, and receptor-like protein kinases. These results suggest the involvement of PSK in the attenuation of stress response and healing of wound-activated cells during the early stage of TE differentiation.

Intercellular communication is indispensable to coordinate the cellular behaviors of multicellular organisms during morphogenesis and responses to environmental stimuli. In animals, many peptide signals play important roles in cell-cell interactions. In contrast, plant cell-cell interactions have been thought to be mediated mainly by nonpeptide small organic compounds, such as abscisic acid, auxin, brassinosteroids, cytokinin, ethylene, gibberellic acid, jasmonate, and salicylic acid. Recently, several peptides and glycopeptides have been found as a new class of plant signaling molecules during the defense response (Pearce et al., 1991, 2001a, 2001b; Pearce and Ryan, 2003), cell proliferation and expansion (Matsubayashi and Sakagami, 1996; Amano et al., 2007), meristem formation (Fletcher et al., 1999; Fiers et al., 2005; Kondo et al., 2006; Kinoshita et al., 2007), stem cell fate (Ito et al., 2006), xylem differentiation (Motose et al., 2001b, 2004), stomatal development (Hara et al., 2007), and self-incompatibility (Schoepfer et al., 1999; Takayama et al., 2000). Some of these signaling peptides have been shown to interact with specific receptor-like protein kinases (RLKs) and activate intracellular signaling cascades (for review, see Matsubayashi, 2003; Fukuda et al., 2007).

Plant cells cultured at cell densities less than a critical value (typically, 1.0 × 10^6 cells mL^−1) cannot proliferate despite the administration of any classical phytohormones and defined nutrients. Addition of conditioned medium prepared from rapidly growing cell cultures stimulated the proliferation of cells cultured at low cell densities.
RESULTS

Expression Pattern of ZePSK1

We cloned a zinnia gene encoding a putative PSK precursor and analyzed its expression pattern in zinnia xylogenic culture. Isolation of zinnia cDNA for PSK was carried out by 3'-RACE followed by 5'-RACE. The resulting cDNA fragments were sequenced and determined to cover an open reading frame for 75 amino acid residues (Fig. 1A; accession no. AB089283). The gene represented by this cDNA is designated ZePSK1. ZePSK1 belongs to a PSK gene family including OsPSK and AtPSK (Fig. 1B), which were experimentally confirmed to encode PSK precursor (Yang et al., 1999, 2001; Matsubayashi et al., 2006). The deduced amino acid sequence contained an N-terminal signal sequence and the pentapeptide backbone of PSK (YIYTQ) near the C terminus of the open reading frame (Fig. 1A). The Asp residue, which was indispensable for Tyr O-sulfation of PSK (Hanai et al., 2000b), was conserved immediately N terminal to the PSK sequence. Two Arg residues (amino acids 56 and 59) and two Lys residues (amino acids 72 and 73) were found to border the PSK sequence, suggesting proteolytic processing of this precursor, as in the case of animal prohormone precursors. The signature motif Cx(4–9)[E/D/Q]xCx(2)RRx(3–4)AH[T/L/V]DYIYTQ derived from comparisons of 36 PSK genes (Lorbiecke and Sauter, 2002) was also conserved (Fig. 1B).

Reverse transcription (RT)-PCR analysis was carried out to detect ZePSK1 transcripts (Fig. 1, C–F), because transcripts for ZePSK1 could not be detected with RNA gel blot analysis. There are two peaks of accumulation of ZePSK1 transcripts in cells cultured in TE-inductive medium containing 0.1 μg L⁻¹ 1-naphthaleneacetic acid (NAA) and 0.2 μg L⁻¹ 6-benzyladenine (BA; D medium); the first peak is at 24 h and the second peak is at 72 h of culture (Fig. 1C). Effects of auxin and cytokinin on the expression of ZePSK1 were analyzed for cells cultured in medium with different combinations of phytohormones: hormone-free medium (C₀), medium containing only 0.1 μg L⁻¹ NAA (C₅₀), medium containing only 0.2 μg L⁻¹ BA (C₅₀), and medium containing 0.1 μg L⁻¹ NAA and 0.001 μg L⁻¹ BA (C₅₀). Cells cultured in C₅₀, C₅₀, and C₅₀ medium did not differentiate into TE s, and those in C₅₀ medium differentiated rarely into TE s. The transcripts for ZePSK1 accumulated even in these control cultures, although they did not accumulate significantly at 72 h in cells cultured in C₀, C₅₀, and C₅₀ media (Fig. 1, C and D).

Because the transient accumulation of the ZePSK1 transcripts at 24 h was not affected by phytohormones, the effect of wounding on the accumulation was examined. When zinnia first true leaves were cut into small pieces and incubated on the hormone-free TE-inductive medium containing 6-benzyladenine (BA; D medium), they did not accumulate significantly at 72 h in cells cultured in C₀, C₅₀, and C₅₀ media. The expression of ZePSK1 was induced at 12 and 24 h after wounding (Fig. 1E). This result suggests that the early increase in the ZePSK1 transcripts is due to wound stimuli.

Endogenous brassinosteroids are essential for TE transdifferentiation of zinnia mesophyll cells (Iwasaki and Shibaoka, 1991) and, in particular, for entry into the final stage of TE differentiation, which usually occurs between 48 and 72 h of culture (Yamamoto et al., 1997, 2001). To investigate whether brassinoste-
roids were required for the expression of ZePSK1, we analyzed the effect of uniconazole, which inhibits brassinosteroid biosynthesis, and brassinolide, a biologically active brassinosteroid, on the expression of ZePSK1 (Fig. 1F). Uniconazole is also known to inhibit gibberellin biosynthesis, but in zinnia xylemogenic culture, it is confirmed that brassinosteroids overcome uniconazole-induced suppression of TE differentiation while gibberellin does not (Iwasaki and Shibaoka, 1991). The accumulation of the ZePSK1 transcripts was suppressed at 60 and 72 h of culture by the administration of uniconazole at the start of culture, and this suppression was fully reversed by the exogenously supplied brassinolide (Fig. 1F). This result suggested that biosynthesis of brassinosteroids is required for the induction of ZePSK1 during the late stage of TE differentiation.

Chlorate as an Inhibitor of PSK

Inhibitors of PSK should be useful to analyze the function of PSK. However, specific inhibitors of PSK have not yet been described. Therefore, we applied chlorate, a potent inhibitor of peptide sulfation in animal cells (Baeuerle and Huttner, 1986), to suppress TyrO-sulfation of PSK precursor, which is indispensable for the expression of ZePSK1.

Figure 1. Structure and expression of ZePSK1. A, Nucleotide and deduced amino acid sequences of ZePSK1 cDNA. The deduced amino acid sequence with single-letter abbreviations is shown below the nucleotide sequence of ZePSK1. The potential N-terminal signal sequence is underlined, and the PSK sequence is underscored with double lines. The Asp residue near the PSK sequence is printed in boldface, and the dibasic pairs (putative processing sites) are printed in italic. The nucleotide sequence reported in this paper has been submitted to the DNA Data Bank of Japan under accession number AB089283. B, Amino acid sequence comparison of PSK precursors. Single-letter abbreviations for amino acid residues are used. Gaps are shown as dashes. The sequence motif is according to Lorbiecke and Sauter (2002). C to F, Expression patterns of ZePSK1. C, Zinnia mesophyll cells were cultured in D medium or CN medium and collected every 12 h. Total RNAs were isolated and subjected to RT-PCR of ZePSK1 and 18S rRNA. D, Effects of auxin and cytokinin on the expression of ZePSK1. Zinnia mesophyll cells were cultured in C0, C B, C N, C P, and D media for 24, 48, and 72 h for RT-PCR analysis of ZePSK1 and 18S rRNA. E, Effect of wounding on the expression of ZePSK1. First true leaves were cut into small pieces and cultured for 24 h on hormone-free medium. Total RNA was isolated from leaf pieces cultured for 12 and 24 h and subjected to RT-PCR of ZePSK1 and 18S rRNA. F, Effects of uniconazole and brassinolide on the expression of ZePSK1. Isolated mesophyll cells were cultured for 48, 60, and 72 h in D medium without uniconazole or brassinolide (D), D medium with 5 μM uniconazole (U), or D medium with 5 μM uniconazole and 10 nM brassinolide (UB). Total RNA was isolated for the expression analysis of ZePSK1 and 18S rRNA by RT-PCR.
able for the biological activities of PSK and the binding of PSK to PSK receptor (Matsubayashi et al., 1996, 1997, 2002; Matsubayashi and Sakagami, 1999, 2000). Chlorate added at concentrations of more than 2 mM suppressed TE differentiation completely (Fig. 2, B and D) and cell division strongly (Fig. 2E). Administration of PSK at concentrations above $1.0 \times 10^{-8}$ m restored the chlorate-dependent inhibition of TE differentiation (Fig. 2, C and F) but not cell division (Fig. 2G). These results suggested that chlorate would be useful for analyzing the roles of PSK in TE differentiation, apart from its roles in cell division.

To investigate the effect of chlorate on the Tyr sulfation of PSK, PSK was purified from cell suspensions cultured with or without chlorate and was subjected to immunodot-blot assay using an anti-sulfotyrosine monoclonal antibody called PSG2 (Hoffhines et al., 2006). PSG2 binding was significantly decreased in the PSK fraction derived from culture treated with chlorate (Fig. 3), suggesting that chlorate inhibits Tyr sulfation of PSK in zinnia xylogetic culture.

Stage-Dependent PSK Functions

The process of TE transdifferentiation of zinnia mesophyll cells is divided into three stages (Fukuda, 1997): stage 1 (first 24 h), during which mesophyll cells dedifferentiate to become pluricompetent cells; stage 2 (next 24 h), during which dedifferentiated cells restrict their potency of differentiation to become the precursors of TEs via procambial cells; and stage 3 (final 24–48 h), during which TE precursors form secondary cell walls and execute programmed cell death. Based on this categorization, we investigated stage-dependent PSK functions.

First, we characterized changes in the responsiveness of cells to PSK with chlorate. Chlorate was added at the start of culture and PSK was added at various times thereafter (Fig. 4A). PSK, when added to the culture within 24 h of culture, most effectively reversed the chlorate-induced inhibition of TE differentiation, and thereafter, the reversal effect of PSK decreased with time. Similarly, we examined the time requirement for PSK in low-density cell culture ($5.0 \times 10^3$ cells mL$^{-1}$; Fig. 4B). PSK was most effective at inducing TE differentiation when PSK was added at the start of culture or at 12 h of culture. TE differentiation was severely suppressed when mesophyll cells were cultured for 36 h without exogenously supplied PSK. These results imply that PSK is required for TE differentiation before 24 h of culture.

Second, we investigated the effects of PSK and chlorate on the accumulation of mRNAs for various stage marker genes (Yamamoto et al., 1997; Demura et al., 2002). Transcripts for marker genes of stage 1, ZePR (for Zinnia elegans pathogenesis-related protein), ZePI1 (for Zinnia elegans proteinase inhibitor1), and ZePI2, accumulated transiently between 12 and 36 h of culture (Fig. 5). In the presence of chlorate, ZePR, ZePI1, and ZePI2 continuously accumulated at high level. On the contrary, administration of PSK suppressed the accumulation of the ZePR, ZePI1, and ZePI2 transcripts. Accumulation of the ZePAL3 (for Zinnia elegans phenylalanine ammonia-lyase3) mRNA peaked at 24 and 72 h of culture (Fig. 5). Chlorate enhanced the accumulation of the ZePAL3 transcripts, which was similar to that of the ZePR, ZePI1, and ZePI2 transcripts. Addition of PSK suppressed the first peak of accumulation of the ZePAL3 transcripts but not the second peak. These results indicate that stress-related gene expression is suppressed preferentially in the presence of PSK. This suppression occurred in the absence of auxin and cytokinin (Fig. 6A), suggesting that auxin and cytokinin are not required for the suppression of stress response by PSK. The suppressive effect was a little weaker in cultures with only auxin or only cytokinin than in culture with both phytohormones or in hormone-free culture (Fig. 6A). Besides the suppressive effect of PSK on stress response, PSK also promoted TE differentiation in the presence of auxin and cytokinin (Fig. 6B), suggesting that PSK stimulates TE differentiation via the suppression of stress response.
Chlorate treatment delayed the appearance of transcripts for stage 2 marker genes for 24 h, including transcripts for TED3 (for TE differentiation) and TED4 (Fig. 5). However, it did not significantly affect peak transcript levels for these two genes. The chlorate-induced delay was reversed by exogenously supplied PSK. In contrast, chlorate almost completely suppressed the accumulation of the ZCP4 mRNA, a stage 3 marker, and PSK restored the chlorate-induced suppression.

Microarray Analysis

To study PSK function in detail, large-scale expression analysis was performed with zinnia microarrays consisting of approximately 9,000 genes (Demura et al., 2002). The cDNA populations for hybridizations to the microarray were prepared from freshly isolated zinnia mesophyll cells (indicated as 0-h cells) and cells cultured for 24 h in four different culture conditions: D medium without PSK or chlorate (indicated as mock), with \(1.0 \times 10^{-7} \text{ M PSK} \) (indicated as PSK), with \(2 \text{ mM chlorate} \) (indicated as \(\text{KClO}_3\)), or with \(2 \text{ mM PSK} \) (indicated as \(\text{KClO}_3 + \text{PSK}\)). Gene expression profiles were compared between mock-treated and PSK-treated cells and between chlorate-treated and chlorate + PSK-treated cells, and genes were identified as being differentially expressed if the signal values deviated either positively or negatively by 2-fold or more in both sets in two or three independent microarray experiments (Fig. 7).

As a result, transcript levels of 66 genes and 42 genes were down-regulated and up-regulated in the presence of PSK, respectively (Fig. 7; Supplemental Tables S1 and S2). Expression patterns of these genes were compared with the previous comprehensive microarray data on changes in transcript accumulation during TE differentiation (Demura et al., 2002). Of the 66 genes down-regulated by PSK, 46 genes (46 of 66 = 69.7%) were clustered into group C, which mainly included genes induced at stage 1 with a peak at 24 h of culture (Fig. 7; Supplemental Table S1). Functionally, 21 (21 of 66 = 31.8%) of the 66 genes were classified as genes encoding stress-related proteins, including four putative chitinases (Z1809, Z2220, Z2326, and Z7553), seven enzymes involved in the biosynthesis of phenylpropanoids (Z293, Z2345, ZePAL6, Z5823, Z2452, Z8712, and Z9331), a putative laccase involved in the polymerization of lignin monomers (Z8815), three redox-related enzymes (Z5164, Z7351, and ZeGSAT), 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (Z1862), and defense-response proteins (Z1987, Z5204, ZePR, ZePI1, and ZePI2; Supplemental Table S1).

Seven cDNAs down-regulated by PSK exhibited high degrees of sequence similarity to putative RLKs, which were classified to three different subfamilies: LRR-RLKs (Z1175, Z7149, Z8544, and Z8757), wall-associated kinase (Z6194), and S-locus RLK (Z2332). Of
these genes, Z8544 closely resembled rice LRR-RLK Xa21, involved in disease resistance to Xanthomonas oryzae pv oryzae (Song et al., 1995). Z8757 showed a high degree of homology with the carrot PSK receptor kinase DcPSKR (Matsubayashi et al., 2002). The zinnia microarray contained three cDNAs (Z4541, Z8757, and Z9214) with significant similarities to the PSK receptor kinase, of which Z8757 was most preferentially expressed in zinnia xylogenic culture and potently down-regulated by exogenously supplied PSK. These data suggest that suppression of RLKs is involved in the down-regulation of stress response and negative feedback regulation of PSK signaling.

Genes down-regulated by PSK also included genes involved in various cytological processes: transcription (ZeH88), transport (Z2631, Z3438, Z4657, Z6170, Z6277, and Z7460), protein destination (Z1947, Z2804, Z2715, Z5307, and Z9251), cell wall metabolism and function (Z4367, Z6105, and Z8790), and lipid metabolism (Z1733, Z3304, and Z3892).

Of the 42 genes up-regulated in the presence of PSK, 31 genes (31 of 42 = 73.8%) belonged to group A categorized by Demura et al. (2002; Fig. 7; Supplemental Table S2), of which mRNA levels were high in freshly isolated cells (0-h cells) and decreased rapidly after 24 h of culture. Of these 31 genes, 20 genes encoded proteins related to various chloroplast (plastid) functions, including photosynthesis, chlorophyll synthesis, carbon fixation, and chloroplast protein synthesis. This result suggests that PSK maintains chloroplast activity, which is in agreement with previous reports showing the promotion of chlorophyll content by PSK (Yamakawa et al., 1998, 1999). PSK also promoted the accumulation of transcripts for genes encoding nitrite transporters (Z144 and Z7443), acid phosphatases (Z8691 and Z8692), receptor-like protein kinase (Z4539), signal recognition particle subunit (Z6904), and glycosyl hydrolase (Z6245).

**DISCUSSION**

**Chlorate as an Inhibitor of PSK**

We chose chlorate as an inhibitor of PSK because Tyr O-sulfation of PSK is essential for its biological activity.
(Matsubayashi et al., 1996) and chlorate has been widely used as a potent inhibitor of peptide sulfation in animal cells (Baeuerle and Hutner, 1986; Beinfeld, 1994; Maiti et al., 1998; Farzan et al., 1999). In this study, we show that chlorate completely inhibited TE differentiation of zinnia mesophyll cells (Fig. 2) and suppressed Tyr sulfation of PSK (Fig. 3). The chlorate-induced inhibition of TE differentiation was recovered by exogenously supplied PSK (Fig. 2). This reversal effect is specific for PSK, since other phytohormones had no effect on the suppression of TE differentiation by chlorate (Supplemental Table S3). These results suggest that chlorate inhibits Tyr sulfation of PSK, resulting in the suppression of TE differentiation. Chlorate provides an advantage to investigate the roles of PSK. It is difficult to prepare enough RNA for analysis from cultures at low cell densities, which have been used for highly sensitive bioassay for PSK (Matsubayashi and Sakagami, 1996; Matsubayashi et al., 1999). Because Henmi et al. (2001) reported the requirement of glutathione, which was biosynthesized from Cys, for TE differentiation of zinnia mesophyll cells, there is a possibility that chlorate inhibits the biosynthesis of Cys and glutathione, resulting in the inhibition of TE differentiation. Cys, a reduced form of glutathione, or glutathione disulfide, however, had no reversal effect on the chlorate-induced suppression of TE differentiation (Supplemental Table S3), suggesting that chlorate-induced inhibition of TE differentiation did not result from the inhibition of Cys and glutathione biosynthesis.

Chlorate is an analog of nitrate and reduced to toxic chlorite by nitrate reductase (Aberg, 1947; Solomonson and Vennesland, 1972; Nakagawa and Yamashita, 1986). Chlorate has been used as an herbicide and applied to isolate mutants that are defective in nitrate reduction (Oostindi and Feenstra, 1973; Braaksma and Feenstra, 1982; Willkinson and Crawford, 1991; LaBrie et al., 1992; Tsay et al., 1993; Lin and Cheng, 1997). Therefore, the toxic chlorite produced from chlorate by

Figure 7. Summary of microarray analysis. A and B, Expression patterns of genes down-regulated (A) or up-regulated (B) by PSK. Freshly isolated mesophyll cells were cultured for 24 h in D medium without PSK or KClO₃ (Mock), with 200 nM PSK (PSK), with 2 mM KClO₃ (KClO₃), or with 200 nM PSK and 2 mM KClO₃ (PSK + KClO₃). Poly (A⁺) RNA was prepared from 0-h cells and cells cultured in mock, PSK, KClO₃, and PSK + KClO₃ for microarray analysis. C and D, Clustering of genes down-regulated (C) or up-regulated (D) by PSK according to the classification described in E. E, Schematic expression patterns of six different groups of genes during TE differentiation as described by Demura et al. (2002).
nitrate reductase might inhibit TE differentiation. However, a toxic effect of chlorate seems unlikely for the following reasons: (1) zinnia culture medium contains nitrate at a concentration of 20 mM, which is enough for nitrate to function as a potent competitive inhibitor of chlorate reduction (Solomonson and Vennesland, 1972; Nakagawa and Yamashita, 1986); (2) chlorate did not affect the viability of zinnia cultured cells in the presence of nitrate at concentrations of more than 20 mM (Supplemental Fig. S1); and (3) chlorate added after 12 h of culture had no effect on TE differentiation (Supplemental Fig. S2). The third observation implies that a sufficient amount of 5'-adenylylsulfate may be synthesized by ATP-sulfurylase by 12 h of culture.

Roles of PSK in TE Differentiation

It has been shown that endogenous PSK is required for transdifferentiation of zinnia mesophyll cells into TEs (Matsubayashi et al., 1999). In this study, we investigated the functions of PSK in TE differentiation and obtained the following results. (1) ZePSK1 transcripts accumulated in response to wounding at 24 h of culture, after which wound-induced genes were rapidly down-regulated (Figs. 1 and 5). (2) The inhibition of active PSK biosynthesis by chlorate enhanced the expression of stress-related genes, which was significantly suppressed by exogenously supplied PSK (Fig. 5). (3) Microarray analysis identified various kinds of stress-response genes down-regulated in the presence of PSK (Supplemental Table S1). (4) The stress-response genes were down-regulated by the addition of PSK in the absence of auxin and cytokinin (Fig. 6). These results strongly suggest the involvement of PSK in the attenuation of stress response occurring in the early stage of TE transdifferentiation. Although it remains to be identified which signaling pathway(s) of stress response is down-regulated, our microarray data imply that genes for phenylpropanoid biosynthesis enzymes, chitinases, RLKs, and ACC synthase probably represent a stress-response pathway downstream of PSK. The suppression of PAL genes and an ACC synthase gene could result in down-regulation of the biosynthesis of salicylic acid and ethylene, two potent signals mediating stress response.

The effects of PSK and chlorate on stage-specific marker genes led to the conclusion that PSK is required both for the entry into stage 2 and for the transition from stage 2 to stage 3. Chlorate induced continuous stress-response gene expression, delayed stage 2 marker expression, and suppressed stage 3 marker expression (Fig. 5). These data suggest that chlorate-treated cells are delayed in entering stage 2 and finally stopped before the transition from stage 2 to stage 3 with stress response continuously activated. On the contrary, addition of PSK suppressed stress-response genes, overcame chlorate-induced delay of stage 2 marker expression, and restored the suppression of stage 3 marker gene (Fig. 5). These results suggest that PSK is essential for the progression of stage 2 and the entry into stage 3. The continuous stress response might be incompatible with the entry into the final step of TE differentiation.

It is noteworthy that the second induction of ZePSK1 was dependent on the biosynthesis of brassinosteroids, an essential factor for the transition from stage 2 to stage 3 (Iwasaki and Shibaoka, 1991; Yamamoto et al., 1997, 2001). Brassinosteroid-dependent induction of ZePSK1 evokes the possibility that endogenous brassinosteroids induce the biosynthesis of PSK, which acts cooperatively with brassinosteroids during the transition from stage 2 to stage 3. Interestingly, expression of three zinnia HD-Zip III homeobox genes was induced by brassinosteroids during TE differentiation (Ohashi-Ito et al., 2002). These transcriptional factors might function with brassinosteroids and PSK during the entry into the final stage of TE differentiation.

PSK not only activates the proliferation of cultured cells (Matsubayashi and Sakagami, 1996) but also stimulates TE differentiation (Matsubayashi et al., 1999), somatic embryogenesis (Kobayashi et al., 1999; Hanai et al., 2000a; Igasaki et al., 2003), and pollen germination (Chen et al., 2000). To explain the multiple functions of PSK, Matsubayashi (2003) proposed a hypothesis that PSK confers cellular competence to respond to signals such as auxin and cytokinin, which ultimately determine cellular behavior. Here, we report that one of the downstream effects of PSK action is the attenuation of stress response. The PSK-mediated suppression of stress response might be a prerequisite for the acquisition of competence for redifferentiation and responsiveness to signals such as auxin and cytokinin. As an alternative hypothesis, PSK might increase general physiological and metabolic activity, resulting in the faster down-regulation of stress response and multifunctional effects of PSK. Detailed analysis of the Arabidopsis PSK receptor AtPSKR1 indicated that PSK is required for cellular longevity and potential for growth (Matsubayashi et al., 2006). Phenotypic analysis of the triple mutant of AtPSKR1, AtPSKR2, and Atgs172300, which encodes a receptor of another Tyr-sulfated peptide called PSY1, showed that PSK and PSY1 were involved in the promotion of cellular proliferation and expansion, tissue repair after wounding, and inhibition of premature senescence (Amano et al., 2007). Since the triple mutant of PSK/PSY1 receptors did not exhibit any significant defect in xylem development, PSK and PSY1 might not be essential for xylem differentiation in planta. Instead, PSK may participate in tissue repair and xylem regeneration after wounding. Our study suggests that PSK might promote the formation of a bypass of regenerated xylem through the suppression of stress response during vascular regeneration after wounding. Further analysis of PSK/PSY1 signaling will shed new light on the flexible ability of plant cells for growth and differentiation.
MATERIALS AND METHODS

Cell Suspension Culture

Seeds of zinnia (Zinnia elegans 'Canary Bird') were purchased from Takii Shubyo. Zinnia seedlings were grown on vermiculite at 25°C under a daily 14-h light period. The first true leaves of 14-d-old seedlings were used as the source material for the isolation of mesophyll cells. Suspension culture of zinnia mesophyll cells was performed according to the procedure of Sugiyama and Fukuda (1995). The culture medium was a slightly modified version of that described by Fukuda and Komamine (1980). The culture medium for the induction of TE differentiation (D medium) contained 0.1 mg L⁻¹ NAA and 0.2 mg L⁻¹ BA. For control nondifferentiation cultures, C₀ medium that was free of NAA and BA, C₀ medium containing 0.2 mg L⁻¹ BA, C₅ medium containing 0.1 mg L⁻¹ NAA, and C₅ medium containing 0.1 mg L⁻¹ NAA and 0.001 mg L⁻¹ BA was used instead of the D medium. Isolated mesophyll cells were cultured at a density of 5.0 × 10⁶ cells mL⁻¹ unless stated otherwise.

TEs, divided cells, and dead cells, which are morphologically distinguishable, were counted with a light microscope. The frequencies of TE differentiation and cell division were calculated as the proportions of TEs and divided cells to the number of living cells, respectively. Cell viability was calculated as the proportions of living cells and TEs to total cell number.

Culture of Leaf Pieces

Surface-sterilized first true leaves of 14-d-old seedlings were cut into small pieces with a razor blade after removing the midribs. Leaf pieces were transferred onto hormone-free C₀ medium gelled with 0.25% gellan gum in plastic dishes and cultured in the dark at 27°C.

PSK

PSK and unsulfated PSK were synthesized according to Matsubayashi et al. (1996).

Cloning of ZePSK1

Zinnia mesophyll cells were cultured in D medium and harvested after 24, 36, 48, and 60 h in culture. From a mixed sample of these cells, poly(A) RNA was prepared by use of the FastTrack mRNA Isolation Kit version 3.5 (Invitrogen). For 3’RACE, first-strand cDNA was reverse transcribed from poly(A) RNA with dT₁⁷-LL-BamA primer (5’-GATTAGGATCCACTAATA-TCATTITTTTTCCTTTTTTCTTTT-3’). A degenerate primer, PSK-F2a (5’-GAYTAYATHTAYACIAC-3’), was designed for the amino acid sequence of the PSK domain. cDNA fragments were amplified from first-strand cDNA by PCR with primers PSK-F2a and LL-BamA. After agarose gel electrophoresis, DNA of 0.39 kb. This PCR product was cloned into pGEM-T-Easy vector (Promega).

Immunoblotting

Conditioned medium (200 mL) prepared from zinnia cell suspension cultures was buffered (Tris-HCl at a final concentration of 20 mM at pH 8.0) and applied to a DEAE Sephadex column (GE Healthcare) equilibrated with 20 mM Tris-HCl (pH 8.0). The column was washed with 3.0 mL of equilibration buffer and with 3.0 mL of buffer containing 0.5 mM KCl and then eluted with 1.0 mL of buffer containing 2.0 mM KCl. The eluate was desalted by dialysis (Spectra/Pol molecular weight cutoff, 1,000), lyophilized, and dissolved in 100 μL of water. This solution was diluted 5- or 25-fold, and a 2-μL aliquot of each diluted solution was dot blotted on a polyvinylidene difluoride membrane (Hybond-P; GE Healthcare). The immunodecoration of sulfoyrosine was performed with an anti-sulfoyrosine monoclonal antibody, PSG2, as described by Hoffines et al. (2006). The anti-sulfoyrosine monoclonal antibody PSG2 or control IgG4 monoclonal antibody was used as a primary antibody at 50 ng mL⁻¹, and horseradish peroxidase-conjugated anti-human IgG was used as the secondary antibody. The antibody binding was detected using ECL Plus (GE Healthcare).

Microarray Analysis

Zinnia cDNA microarray analysis was performed according to Demura et al. (2002). On zinnia microarray slides, PCR-amplified cDNAs were spotted twice on each slide for the reliability of microarray analysis. Poly(A) RNA was isolated from freshly isolated mesophyll cells (0-h cells) and cells cultured for 24 h in four different culture conditions: D medium without PSK or chlorate (D), PSK with 2.0 × 10⁻⁷ M PSK (PSK), with 2 m M chlorate and 1.0 × 10⁻⁷ M PSK (KClO₃ + PSK), using the FastTrack RNA Isolation Kit (Invitrogen). A Cy5-labeled cDNA population was synthesized from 2 μg of poly(A) RNA using SuperScript II (Invitrogen). The hybridization to the microarray and scanning were performed according to Endo et al. (2002). Microarray experiments were done in three independent RNA samples derived from three independent cultures. Gene expression profiles were compared between mock-treated and PSK-treated cells and between chlorate-treated and chlorate + PSK-treated cells, and genes were identified as being differentially expressed if the signal values deviated either positively or negatively by 2-fold or more in both comparisons (mock versus PSK and chlorate versus chlorate + PSK) in two or three independent microarray experiments.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AB089928 (ZePSK1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effects of KNO₃ on TE differentiation, cell division, and viability in the presence of KCl, KClO₃, and PSK.

Supplemental Figure S2. Changes in the responsiveness of cells to KClO₃ during xylogenic culture.

Supplemental Table S1. Summary of genes down-regulated by PSK.

Supplemental Table S2. Summary of genes up-regulated by PSK.

Supplemental Table S3. Effects of Cys, glutathione, and phytohormones on the inhibition by chlorate of TE differentiation.

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RT-PCR

Total RNA was extracted as described previously (Ozeki et al., 1990). After treatment with RNase-free DNase I (Invitrogen), first-strand cDNA was synthesized with SuperScript II (Invitrogen) according to the manufacturer’s instructions and used as a template for PCR using primers PSK-F0 (5’-ACA-


CTCAACACCACACTT-3’) and PSK-R4 for 20 cycles. PCR products were separated by agarose electrophoresis, transferred to a positively charged nylon membrane (Roche), and hybridized with a digoxigenin-labeled antisense RNA probe. Hybridization signals were visualized by the immunological method with anti-digoxigenin-AP Fab fragments (Roche) according to the manufacturer’s instructions. As a control, 18S rRNA fragment was amplified with 20 cycles using primers 5’-TAGTAGGCAACTATCTACATACTCCGGA-3’ and 5’-ATTCTAGACCTAATACGCTGAACAAACC-3’.

Roles of Phytosulfokine in Tracheary Element Differentiation

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