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Effect of Phytosulfokine-α on *Agrobacterium*-Mediated Transformation in Rice

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Abstract: Phytosulfokine-α (PSK-α), a biologically active peptide acting as a growth factor, plays a key role in cellular differentiation and proliferation. To test if PSK-α has some influence on *agrobacterium*-mediated transformation in rice, PSK-α at a series of concentrations was added into co-culture medium respectively. The results showed that PSK-α indeed affected the recovery of resistant calli and the transformation frequency of rice varieties Taipei 309 and Lijiangxintuanheigu. PSK-α at the concentration of 10 nmol/L could increase induction of resistant callus and efficiency of transformation, with a 11% and 4.9% top increase, respectively than the control. However, PSK-α at 200 nmol/L could inhibit the induction of the resistant calli. Further more, the effect of PSK-α on *agrobacterium*-mediated transformation is related with the concentration of 2, 4-D in selection medium. Higher induction rate of resistant calli was obtained from tissues treated with PSK-α plus 2 mg/L 2, 4-D.

Key words: phytosulfokine; rice; *Agrobacterium*-mediated transformation

Phytosulfokine (PSK) is a new peptide plant hormone, which was isolated in the conditioned medium of cultures derived from both monocotyledonous and dicotyledonous plants, such as *Asparagus officinalis* mesophyll [1], rice [2], zinnia [3], and carrot [4]. PSK has two types of structure: PSK-α and PSK-β. The former is a sulfated pentapeptide [H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH], the latter is a sulfated terapeptide [H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-OH]. Both are heat-stable, susceptible to pronase digestion, and resistant to glycosidase treatment [1]. The corresponding synthetic PSK has the same function as that of the natural PSK, e.g. stimulating tracheary element differentiation of isolated mesophyll cells of *Zinnia elegans* [3], promoting the chlorophyll formation in etiolated cytoledons of cucumber [5], as well as the growth and chlorophyll formation of *Arabidopsis* seedling under high nigh-time temperature conditions [6]. PSK also can induce the formation of adventitious roots on cucumber hypocotyls [7], promote the formation of adventitious root and bud in *Antirrihium* callus [8], and simulate somatic embryogenesis in carrot [9]. In the present study, we investigated the effect of PSK-α on *Agrobacterium*-mediated transformation in rice and determined the optimal concentration for promoting the transformation efficiency.

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MATERIALS AND METHODS

Plant material and callus preparation

The japonica rice varieties Taipei 309 (T309) and Lijiangxintuanheigu (LTH) were used to prepare calli. Dehusked mature seeds and immature embryos were disinfected with 75% ethanol for 2 min, and then placed in $HgCl_2$ solution (0.1%) for 15-20 min. After rinsed 4-5 times with sterile distilled water, immature embryos were dissected with scalpel then incubated on induction medium (Table 1). Calli initiation was carried out at 25℃ in darkness for 2-3 weeks. The proliferating calli were transferred to fresh induction medium for subculture, and selected for transformation.

Bacterial strain and plasmid

Agrobacterium tumefacientions strain LBA4404 and four plasmids were used in the experiment. Plasmid 1 is pCAMBIA1301 containing blast resistance gene *pi-d*(t)*2* with a part of self-promoter; Plasmid 2 is pCAMBIA1301 carrying resistant blast gene of *pi-d*(t)*2* with self-promoter; Plasmid 3 is

pZH01 containing the cDNA of T309 corresponding with gene *pi-d*(t)*2* under the control of the CaMV(cauliflower mosaic virus) 35s promoter; Plasmid 4 is pZH01 containing the cDNA of *pi-d*(t)*2* gene and 35s. The four plasmids carry selective marker gene of *hpt* that confers resistance to the hygromycin.

Culture medium

The media used in this research were listed in Table 1.

***Agrobacterium* culture and callus transformation**

Agrobacterium cultures were grown about 48 h in YEP liquid medium (5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, pH 7.0) containing 25 mg/L rifampicin, 50 mg/L kanamycin at 28℃ in darkness. When the OD_{595} of the cell density was 0.5, the cultures were harvested by centrifuging at 4000 r/min for 6 min, followed by resuspending in the equal volumes of the AAM liquid medium and cultured for 2 h under slow shaking.

The calli described above were inoculated in bacterium suspension for 15 min, then blotted on sterile filter paper and incubated on co-culture medium at 21℃ for 2-3 days. After co-cultivation, the calli were washed with sterile distilled water until the supernatant was clear, and suspended in sterile water supplemented with 500 mg/L cefotaxine at slowly continuous shaking for 2-3 h. The calli were blotted dry by blowing on a sterile filter and placed on the selection medium 1 or 2 (as described in Table 1). The first cycle of selection was under low selective pressure for 3 weeks, and then transferred the calli to fresh selection medium with high selective pressure for 2-3 weeks. After the second cycle of selection, the calli were transferred to regeneration medium and incubated in the dark for a week, and then the calli were cultured under photoperiod of 12 h illuminations. The regenerated plants with 2-5 cm in height were transferred to the rooting medium. When the plantlets reached about 10 cm in height, the tube lids were pulled and added some water into the tubes. After four days of culture, the plants were transplanted to field.

Molecular analysis of transformants

The primers were designed according to the *hpt* gene sequence. The forward primer was 5'-GACGG TGTCGTCCATCACAGTTT-3' and the reverse primer was 5'-ACTCACCGCGACGTCTGTCGA GAA-3'. The 500 bp band was amplified. To test the interest gene of *pi-d*(t)*2* inserting into the rice genome, the primer pairs with a 1.1 kb amplifying product was designed. Because a homologous gene of interest gene exists in rice genome, the only difference was that the interest gene had a *Mlu* I enzyme digestion site. So the enzyme digestion analysis to the PCR product was needed. The enzyme digestion reaction was performed overnight at 37℃ in a total volume of 20 μL containing 10 μL PCR product, 4 μL 10×buffer, 3 U *Mlu* I, and ddH_2O.

RESULTS

Effects of different PSK-α concentrations on transformation

Calli of LTH were transformed with plasmid 1 and plasmid 2. Calli of T309 were transformed with plasmid 3. Meanwhile, calli were treated on the co-culture medium supplemented with 1, 10, 100 nmol/L of PSK-α, respectively. The controls were without PSK-α. The treated calli were selected on

Table 1. Medium used for rice transformation experiment.

Medium	Culture medium constituents
Callus induction medium (NB2)	NB + 2, 4-dichlorophenoxyacetic acid (2, 4-D) 2 mg/L
AAM liquid medium	AAM + casamino acid 0.5 g/L + glucose 36 g/L + sucrose 68.5 g/L+ acetosyinringone 100 μmol/L
Co-cultivation medium	NB2 + acetosyinringone 100 μmol /L + PSK (1-200 nmol/L)
Selection medium 1	NB + 2, 4-D 1 mg/L + hygromycin 30-50 mg/L + cefotaxine 500 mg/L
Selection medium 2	NB + 2, 4-D 2 mg/L + hygromycin 30-50 mg/L + cefotaxine 500 mg/L
Regeneration medium	MS + 6-benzylaminopurine 2 mg/L + naphthaleneaceticacid 0.2 mg/L + hygromycin 20-50 mg/L
Rooting medium	1/2 MS + NAA 0.2 mg/L + hygromycin (10-30 mg/L)

Table 2. Effect of PSK-α concentration on frequency of transformation mediated by *Agrobacterium*.

Cultivar	Plasmid	Concentration of PSK (nmol/L)	No .of tested calli (A) [a]	No .of resistant calli (B)	Rate of resistant calli (B/A,%)[b]	No.of positive plants (C)	Frequency of transformation (C/A, %)
LTH (Immature embryos)	Plasmid 1 (Treatment 1)	1	163	59	36.2±0.1 bB	2	1.2
		10	191	81	42.9±0.7 aA	6	3.1
		100	186	64	35.4±0.5 bB	4	2.2
		0	188	67	35.6±0.7 bB	2	1.1
	Plasmid 2 (Treatment 2)	1	205	89	43.4±0.1 cB	9	4.4
		10	182	91	50.1±0.8 aA	11	6.0
		100	202	91	45.1±0.2 bB	3	1.5
		0	181	72	40.1±0.4 dC	2	1.1
T309 (Mature embryos)	Plasmid 3 (Treatment 3)	1	147	31	20.1±0.8 cC	13	8.8
		10	144	44	31.1±0.6 aA	15	10.4
		100	148	40	27.0±0.7 bB	14	9.5
		0	154	29	20.0±0.4 cC	10	6.5

[a, b] The three treatments were repeated twice, and each treatment consisted of about 20 calli with 4 replication. After the first cycle of selection was finished, no. of resistant calli were recorded. Mean analysis was conducted by DPS software. Values with different lowercase and uppercase letters in the same treatment indicate significant differences at 0.05 and 0.01 levels, respectively, according to LSD test.

Table 3. Effect of 2, 4-D in selected medium and PSK in co-culture medium concentration combinations on rate of resistant calli of rice variety T309.

Plasmid	2,4-D (mg/L)	PSK-α concentration (nmol/L)	No. of resistant calli / No. of tested calli	Rate of resistant calli (%)[a]
Plasmid 3	1	70	32/182	18.0 b
		200	0/172	0.0 d
		0	24/159	15.1 c
	2	70	51/182	28.1 a
		200	3/194	1.5 d
		0	37/189	19.5 b
Plasmid 4	1	70	30/176	17.1 a
		200	0/189	0.0 d
		0	28/201	13.9 b
	2	70	34/206	17.3 a
		200	3/197	1.5 c
		0	28/198	14.1 b

[a] Each treatment was considered as an independent experiment which has two factors and repeats. In the same plasmids, different small letter mean the significant difference at 0.05 level (LSD).

selection medium 2.

In the Table 2, the results of treatment 1 indicated that 1 nmol/L PSK in co-culture medium had no obvious effect on the induction rate of resistant calli. Increasing the PSK concentration to 10 nmol/L, resulted in a significantly increased induction rate of resistant calli. However, when further increasing the concentration of PSK, we could not see noticeable positive effect there. In the treatment 2, the three concentrations of PSK obviously enhanced the induction rate of resistant calli, and the rate peaked at 50.1%, above 10 percent point than that of the control. In the treatment 3, the resistant calli frequency was also increased from 20.0 %(the control) to 31.1 % in the presence of 10 nmol/L PSK.

The effect of plasmid on transformation was also investigated in the research. Different combinations of bacterium strains and plasmids have great effects on transformation efficiency. The result of statistical analysis showed that LBA4404 (plasmid 2) is much more efficient than LBA4404 (plasmid 1).

The resistant calli differentiated and the transgenic plants were obtained. The result indicated that PSK enhanced the frequency of transformation, especially at the concentration of 10 nmol/L in the co-culture medium (Table 2). The highest increase in the frequency of transformation was 2.0 percent point in plasmid 1, 4.9 percent point in plasmid 2, and 3.9 percent point in plasmid 3, respectively, suggesting that the function of PSK is stable.

Effect of 2, 4-D at various concentrations on PSK-α treatment

PSK responded to externally added auxin and cytokine and triggered cell proliferation at normal concentrations in collaboration with plant hormones[10]. To investigate the relationship between PSK-α and hormones in transformation, we tried two concentrations of 2, 4-D in selection medium after treatment with PSK-α. As presented in Table 3, selection medium with 2 mg/L 2, 4-D were more suitable for callus growth than that with 1 mg/L 2, 4-D.

The highest rate of resistant calli produced in the medium contained 2 mg/L 2, 4-D, but this was obtained with a 70 nmol/L PSK treatment in co-cultivation. Once the PSK concentration reached 200 nmol/L in the co-culture medium, although on selection medium with the suitable concentration of 2, 4-D supplemented, PSK had no any positive affection on the callus growth. The results showed that PSK could promote the growth of the resistant calli in an available concentration range, and some other plant hormones can accelerate the effect of PSK.

It was worth paying attention to the efficiency of PSK and 2,4-D in combination of the plasmids. In this study, the effect of plasmid 3 is higher than that of plasmid 4. Therefore the efficiency of PSK was related not only to the concentration of 2, 4-D, but also to the structure of plasmid.

Effect of PSK on the callus growth and the tendency

In this experiment, the calli treated with PSK showed good growth state, such as fresh yellow color, good quality, and active division. What's more, when PSK at the concentration of 10 nmol/L was used in the co-culture medium, the mortality of calli hardly reached zero and more embryos were produced after the second selection. At the same time, the earlier occurrence of regeneration of plants and the better growth of regenerated plants were observed. This suggested that PSK had strongly affected the development of rice embryos.

According to the results of the treatment of T309 with different concentrations of PSK-α, we deduced the trend about the function of PSK-α in *Agrobacterium*-mediated transformation (Fig. 1). With the increase of PSK-α concentration in the co-culture medium, PSK-α showed positive function on the frequency of resistant calli, even at a low concentration of 1 nmol/L. As the PSK concentration increased, the effect of PSK-α increased to reach the maximum, and then began to decrease, indicating that selecting a suitable concentration of PSK-α is a key step to obtain high rate of resistant calli.

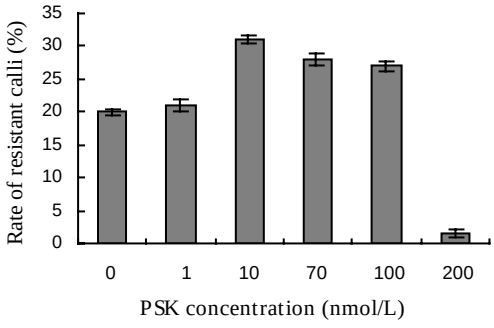

Fig. 1. Effect of different PSK-α concentrations on the rate of resistant calli of a rice variety T309.

Molecular analysis of the transgenic plants

PCR performed with *hpt* primers showed a 500 bp band in the transgenic plants, which proved that the *hpt* gene had been integrated into the rice genome (Fig. 2).

The result of enzyme digestion analysis with *Mlu* I showed that the positive plants had 2-3 bands and the negative only had one band (Fig. 3). The result indicated the interest gene also integrated into rice genome.

DISCUSSION

PSK is a small sulfated peptide secreted by cells. Many studies showed that PSK stimulated cell proliferation strongly at low concentration. It was

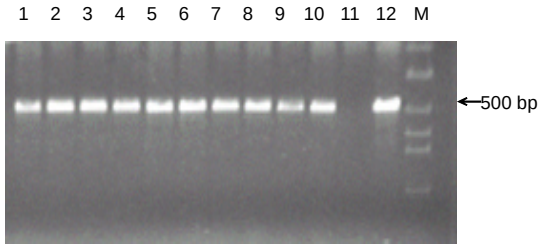

Fig. 2. PCR assay of *hpt* gene in transgenic plants.

Lanes 1-10, T_0 transformants; Lane 11, Non-transformed plant; Lane 12, Positive control; Lane M, Marker of DL2000.

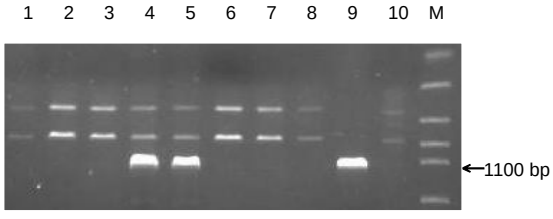

Fig. 3. Enzyme digestion assay of PCR products from interest gene.

Lanes 1-8, *Pi-d* (t)*2* positive plants; Lane 9, Negative plant; Lane 10, Positive control; Lane M, Marker of DL2000.

reported the PSK function in the *Agrobacterium*-mediated transformation of rice in the study. We concluded that PSK stimulates the resistant calli growth at a suitable concentration range. Matusubayashi et al [3] reported that the low concentration of PSK-α at 1 nmol/L initiated the division of asparagus, which corresponded with our results. In the study, a set of concentrations of PSK-α were used, but this is not enough. The concentrations of PSK-α should be enriched so as to find the best suitable concentration producing the highest frequency of transformation.

In the effective range of PSK concentration, the effect of PSK was related with the exogenous hormones. In the study on asparagus cells, the result showed the mesophyll cells immediately were arrested in G_0/G_1 after isolation and the cell cycle proceeded, only when all the three factors, the NAA, 6-BA and PSK existed in the medium. The results showed that the production and expression of biological activation of PSK-α is closely correlated with the signal transduction pathway mediated by auxin and cytokinin[10]. Although we only studied primarily interaction between PSK and 2,4-D, the close relationship between them was found. Whether the high efficient combination for PSK with other hormones will enhance the frequency of transformation or not remains to be further studied.

It is clear that PSK has obvious function in the plant growth. PSK-α identified in conditioned medium from rice and maize cell culture, apparently promoted cell growth by interacting with specific binding sites distributed upon plasma membranes [2]. Transforming rice cells with sense and antisense the *O. sativa PSK* gene driven by the actin promoter results in sense transformants with cell division rates two times faster than those of wild-type cells, and antisense transformants that divided much more slowly than the wild type [11]. The addition of PSK to selective media significantly improves genetic transformation efficiency of carrot by promoting the proliferation of surviving transgenic cells [12]. In this study, we made it clear that an amount of PSK-α supplemented in co-culture medium stimulates callus growth of rice, at the same time, it also stimulates cell proliferation and maintains the calli in an excellent embryonic status. Subsequently, the rate of resistant calli and the frequency of transformation are enhanced. The stimulation may be caused by promotion of cell division. In general, the relative growth rate of plant cell in vitro is considerably affected by initial cell density, but a lot of low mitotic activities in surviving transgenic cells produced under antibiotic selection, and the growth of low-density transgenic cells was inhibited. PSK-α just can compensate for cell growth suppression in low density cultures at a concentration as low as 1 nmol/L. However, why a certain higher concentration of PSK-α have inhibition to the growth of the resistant calli remain unclear. Yet to date, *Oryza sativa* PSK (OsPSK) cDNA encoding a PSK-α precursor has been isolated, and the specific high affinity binding sites for PSK-α have been found in rice plasma membrane [2,13]. Further studies revealed the 120- and 160-kD proteins from rice were PSK-α receptors that mediated the biological activities of PSK-α [14]. In fact, the 120-kD membrane protein purified from carrot microsmal fractions is a leucine-rich repeats receptor kinase acting as ligand-receptor pair with PSK-α to induce proliferation of plant cells [15].

The system of transformation mediated by *Agrobacterium* is affected by many factors, and the crucial factors include the genotype of plants, the type and status of tissues inoculated by the *Agrobacterium* strains and the kind of vector system, conditions of tissues culture and the compositions of medium and so on. Researchers had adopted several creative strategies to improve the efficiency [16, 17]. In the present experiment, the higher percentage of hygromycin resistant calli with better growth status as well as fast differentiation capability were obtained by appending a suitable concentration of PSK to the co-culture medium. This work establishes groundwork for the creation of high efficient and stable transformation system. The system should be applied to the rice varieties in which transformation has previously been unsuccessful due to low mitotic activity of surviving transgenic cells. Besides this, the following aspects should be considered: 1) selection of suitable explants such as immature embryos, 2) making use of high efficiency combinations of bacterium strains and vectors such as super-virulent

strains of *A. tumefaciens* and super- binary vector with high efficient promoter, 3) selective reagent, 4) adding suitable concentration of PSK to the co-cluture medium or the selection medium.

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