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Chemical properties of pollen tube growth promoters extracted from transmitting tissue in Pione grape (*Vitis vinifera* x *V. labrusca*) pistils

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Summary

The contribution of extracellular matrix (ECM) in pollen tube transmitting tissue (TT) to pollen tube growth in grape pistils was examined. At bloom the style and ovary septum tissues were excised from blooming Pione (*Vitis vinifera* × *V. labrusca*, tetraploid) pistils using a micro capillary. The TT-ECM was extracted by an apoplast extraction method using 1 M NaCl and 50 mM $MgCl_2$ solutions as extraction solvents. NaCl extracts, except for those with M.W. < 5,000, showed a promotive activity on *in vitro* pollen germination. By gel filtration of the crude extract two active fractions were recovered; the molecular masses were approximately 40 kDa and < 13 kDa, respectively. The UV spectrum of the higher molecular active fraction, lacking maximum absorption at 280 nm and 260 nm, suggested that the putative pollen tube growth promoter (PGP) might not be nucleic acids or proteins. Carbohydrate component analysis of the 40 kDa PGP using GLC revealed that almost only D-glucose was the constituent. Methylation analysis showed that the PGP consists of a 1→3 linked glucose unit, a 1→4 linked glucose unit, and a 1→6 linked glucose unit at a molar ratio of 5.6:2.0:1.0. From these results, we postulate that this glucose polymer is one of the PGPs in Pione pistils.

K e y w o r d s : Pollen tube growth promoter (PGP), transmitting tissue (TT), extracellular matrix (ECM), pistil, Pione grape.

Introduction

In most higher plants, pollen grains germinate on the stigma and the tubes elongate into the style and ovary through a transmitting tissue (TT) developed around the central axis of pistils. Transmitting tissue extracellular matrix (TT-ECM), found among TT cells in *Nicotiana* plants, has been reported to contain polysaccharides and glycoproteins (Atkinson *et al.* 1994). Pollen tube growth in the style is initially supported by the mobilization of storage material in pollen grains and subsequently by components of TT-ECM. The role of TT-ECM on pollen tube growth has been reported for several plants such as peaches and kiwifruit (Herrero and Arbeloa 1989; Gonzalez *et al.* 1996).

In grape, we have reported the developmental difference of the TT among cultivars and shoot vigor that correspond with the number of pollen tubes penetrating the TT in an ovary (Okamoto *et al.* 2001, 2002). However, physiological contributions of TT-ECM on pollen germination and/or pollen tube growth have not been investigated. Therefore, we tried to find out bioactive compound(s) for pollen tube growth in grape TT-EMC. In this report, we describe partial purification and preliminary characterization of a bioactive compound prepared from TT-EMC of cv. Pione pistils.

Material and Methods

P l a n t m a t e r i a l a n d a p o p l a s t e x t r a c t i o n o f T T - E C M : Pistils were collected from inflorescences of mature vines (cv. Pione, *Vitis vinifera* × *V. Labrusca*, tetraploid) at full bloom (March and April 2003). The vines were cultivated in heated commercial plastic houses located in Okayama, Japan. After cutting off a lower part of ovaries, the pistils were soaked in 0.05 M phosphate buffer (pH 5.5). A micro capillary (I.D. 150 µm) was inserted from the style top into a pistil to excise the central part including the TT. One hundred micro capillaries holding an excised tissue for each were decompressed in distilled water and then centrifuged at 3,000 rpm for 10 min to obtain H_2O extracts. Next, they were decompressed in 1 M NaCl solution, and centrifuged as mentioned above to obtain NaCl extracts. Finally, using 50 mM $MgCl_2$ solution, the $MgCl_2$ extract was obtained. Two ml of the NaCl and $MgCl_2$ extracts were poured individually into a membrane filter (MWCO 100) and dialyzed against 5 l of distilled water for 2 h at 4 °C, 3 times. Each extract was divided into 4 molecular weight ranges, < 5,000, 5,000-10,000, 10,000-30,000, > 30,000, using a Vivaspin concentrator (Vivascience, Germany). Each fraction of the 4 extracts was stored at -20 °C until analyzed.

A s s a y f o r p o l l e n t u b e g r o w t h : The *in vitro* pollen germination ratio was used as indicator of pollen tube growth because of wide variations of tube length after incubation (Okamoto *et al.* 1989 b). Pollen grains were collected from mature Muscat of Alexandria (*V. vinifera* L.) vines cultivated in commercial, heated greenhouses in Okayama in 2003. After drying in a desiccator, they were stored at -20 °C until used. Certain volumes of the TT-ECM extracts were lyophilized, then dissolved in a liquid culture media consisting of 50 µl of 0.05 M phosphate buffer solution (pH 5.5), 10 µl of mineral solution (100 mg H_3BO_3, 300 mg $CaCl_2$, 200 mg $MgSO_4$, 50 mg KNO_3 per l), 25 µl of 40 % sucrose solution, and 15 µl of distilled water. After spotting 12.5 µl of

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the medium on a glass plate, pollen grains were gently scattered onto the drops using a small brush. The population of pollen grains was adjusted between 200-400 per mm^2 under a binocular. The glass plate was immediately turned upside-down, set on the U-shaped slide glass holders, and sealed in a plastic box with high humidity. Pollen grains were incubated at 25 °C for 5 h, then germination ratios were evaluated under a light microscope.

Gel filtration of TT-ECM: NaCl extracts from 100 pistils were dialyzed by MWCO 3,500 membrane filter to remove small molecule extracts and salts. They were freeze-dried and then re-dissolved in 25 µl of 0.025 M phosphate buffer (pH 5.5) containing 0.2 M NaCl. The sample was injected into a Jasco 880-PU HPLC analyser with a TSKgel G3000SWXL column (I. D. 7.8 mm x 30 cm) previously equilibrated with the same buffer. The column was developed in the same buffer at a flow rate of 0.5 $ml \cdot min^{-1}$. The eluate was monitored by the absorption of 220 nm and 1 ml each fraction was collected by a fraction collector. Bovine serum albumin (BSA, 67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease (14 kDa) were used as molecular markers. The UV spectrum of the partially purified active compound was measured with a spectrophotometer (Shimadzu UV-2000).

Sugar component analysis: The neutral sugars were detected by the phenol-H_2SO_4 method. The sugar component of the neutral sugar positive fraction was analyzed by GLC as TMS-derivatives after methanolysis by the method of MEGA *et al.* (1982). The samples were dissolved in 100 µl of 1.4 M anhydrous methanol hydrochloric acid, and heated at 100 °C for 2 h. After removal of the methanol hydrochloric acid under a stream of N_2, the methanolysates were acetylated by addition of 200 µl of 10 % pyridine methanol and 10 µl of acetic acid anhydride for 30 min at room temperature. After acetylation, the methanolysates were trimethyl-silylated by addition with 100 µl of trimethylsilyl reagent (Tri-SIL). The reaction mixture was evaporated to dryness under a stream of N_2 and the resulting trimethylsilyl derivatives were dissolved in 100 µl of n-hexane. An aliquot of 1 µl was injected to a GC-14A (Shimadzu) gas chromatograph equipped with a capillary column (DB-1, 30 m x 0.25 mm I.D.) and N_2 as carrier gas. A flame ionization detector was used for detection of TMS-derivatives. The injector temperature was 250 °C and the detector temperature was 280 °C. After injection, oven temperature was kept at 110 °C for 2 min, then programmed at a rate of 4 $°C \cdot min^{-1}$ to a final temperature of 250 °C.

Methylation analysis: The branching structure of the putative biological active polysaccharide was identified by a methylation analysis. The lyophilized polysaccharide fraction obtained by gel-filtration was methylated by 100 µl of NaOH-saturated dimethyl sulfoxide and 40 µl of methyliodide. After adding 1 ml of 2 % acetic acid and 1 ml chloroform, the methylated polysaccharide, recovered in the chloroform layer, was dried by flushing with N_2. The samples were hydrolyzed using 2 N trifluoroacetic acid at 100 °C for 2 h. After removal of the reagent, the hydrolysate was reduced by addition of 100 µl $NaBO_4$ at room temperature overnight. The reduction was stopped by addition of acetic acid, and then 50 µl of methanol were added and evaporated to dryness to remove boric acid. The chasing of the boric acid with methanol was repeated 5 times. After drying, the samples were acetylated using 100 µl of acetic anhydride at 120 °C for 3 h. One-hundred µl of toluene were added under N_2, and the alditol acetates were extracted after adding 200 µl of dichloromethane. The sample was dried by flushing with N_2 gas and dissolved in 200 µl of *n*-hexane. The resulting partially methylated alditol acetates were analyzed by GC-MS system (Shimadzu GC-MS QP-5000, capillary column, DB-1). After injection, oven temperature was kept at 40 °C for 3 min, then increased to 180 °C at a rate of 20 $°C \cdot min^{-1}$ and to final temperature of 250 °C at a rate of 1.5 $°C \cdot min^{-1}$.

Results and Discussion

Apoplast extraction and detection of pollen tube growth promoter (PGP): The pollen germination test for each extract, prepared by the apoplast extraction from TT-ECM of Pione pistils, revealed that NaCl extracts had strong promotive activities as shown in the Table. In water and $MgCl_2$ extractions, inhibitory activities were detected depending on the molecular weight range (data not shown). OKAMOTO *et al.* (1995) have already reported the existence of pollen tube growth inhibitors in methanolic extracts from homogenized whole pistils of various grape cultivars. In our present study, we at first excised TT moiety from sampled pistils using a micro capillary to prevent a contamination of other tissues. For effective extraction of TT-ECM, mineral solutions were used to release ion-bound compounds on TT cell wall.

Table

Effect of apoplast NaCl extracts from the TT in cv. Pione pistils on *in vitro* pollen germination[1)]

Molecular weight (range)	No. of pistils extracted	Pollen germination[2)] (%)
< 5,000	100	0.0**
	50	0.0**
	25	0.0**
5,000-10,000	100	49.7 ± 3.3*
	50	70.9 ± 4.3**
	25	73.4 ± 2.9**
10,000-30,000	100	0.0**
	50	68.5 ± 3.8**
	25	75.8 ± 1.8**
> 30,000	100	51.9 ± 3.8*
	50	59.1 ± 4.0**
	25	74.3 ± 4.0**
Blank		40.4 ± 1.5

[1)] Extracts were dried in vacuo and added to 100 µl of pollen germination media.

[2)] Mean ± SE. Means were compared with blank test (t-test). * $p < 0.05$; ** $p < 0.01$.

Japanese tetraploid grapes such as Pione and Kyoho usually have poor berry setting (Okamoto *et al.* 2002). Nevertheless, our finding indicated that the tetraploid grape pistils bear both pollen tube growth inhibitors (PGIs) and promoters (PGPs), suggesting that an expression balance of PGIs and PGPs may regulate pollen tube growth. The filtrate containing small molecules (M.W. < 5,000) showed inhibitory activity for pollen germination, which may be due to a contamination of extracting salts. Therefore, in later analyses, extracts were dialyzed exhaustively by another membrane filter (MWCO 3,500) to remove small molecules.

Purification of PGP: To purify the putative pollen tube growth factor(s), the total extract prepared from TT was applied to gel-filtration using a TSKgel G3000SWXL column. As shown in the Figure, 10 fractions (A to J) were collected and each fraction was assayed for pollen tube growth activity after dialysis using the MWCO 3,500 membrane. Fraction C, D and G showed apparent PGP activities compared to blank control. Since fraction D contained a sharp peak, we proceeded to biochemical analyses using this fraction. The UV spectrum of fraction D showed no maximum absorption at 260 nm and 280 nm, which suggested that it contained no nucleic acid polymers or proteinous compounds. On the other hand, the phenol-sulfuric acid reaction gave a positive result and the GLC analysis after methanolysis showed that the fraction consisted of D-glucose as constituent. Thus, we assumed that the PGP in fraction D would be a polysaccharide(s) with the molecular mass about 40 kDa consisting of glucose as a main constituent.

To clarify the branching structure of the polysaccharide having the putative promoter activity, we carried out a methylation analysis. The results showed that the 40 kDa substances have glucose moieties substituted at C-3 (11.32 min), C-4 (11.49 min) and C-5 (11.80 min) at a molar ratio of 5.6:2.0:1.0, indicating that this polysaccharide fraction consists of 1→3, 1→4, and 1→6 branching structures. However, it is noteworthy that, in addition to these substituted glucoses, high amounts of C1-substituted glucose and fructose were detected suggesting occurrence of glucose and ketose (fructose) monomers or sucrose in fraction D. On the gel filtration, fraction D was eluted at the elution position corresponding to about 40 kDa protein as shown in the Figure. Furthermore, before sugar composition and methylation analysis, the fraction was exhaustively dialyzed against distilled water. Therefore, the C1-substituted sugars must be constituents of the pollen tube growth factor, PGP, and could be linked to the main sugar chains by non-covalent bonds such as hydrogen bonds or ionic bonds via calcium or other ions. Ishii *et al.* (1999) reported that pectic polysaccharides in plant cell wall are cross-linked by a borate diester. There is a possibility that complex structures are formed between sucrose and glucose polymer or among glucose polymer with boron. The cross linkages *via* borate diesters might be involved in the assembling of mono-saccharides or di-saccharides to the 40 kDa PGP.

Conclusions

It can be concluded that the TT-ECM in Pione pistils contain a PGP of which the molecular mass is about 40 kDa. The PGP is neither nucleic acid nor protein but a kind of carbohydrate composed of D-glucose. We have already reported that the Pione pistils contain high levels of pollen tube growth inhibitors (PGI), such as quercetin-glucosides (Okamoto *et al.* 1995). Since the PGIs are rapidly diffusible into agar blocks from intact pistils, the PGIs may be localized in vascular systems (Okamoto *et al.* 1989 a). The PGP, analyzed in our current work, must be attached to the TT surface to play a promotive role for pollen tube growth, although the negative effect of PGIs seems to be more dominant.

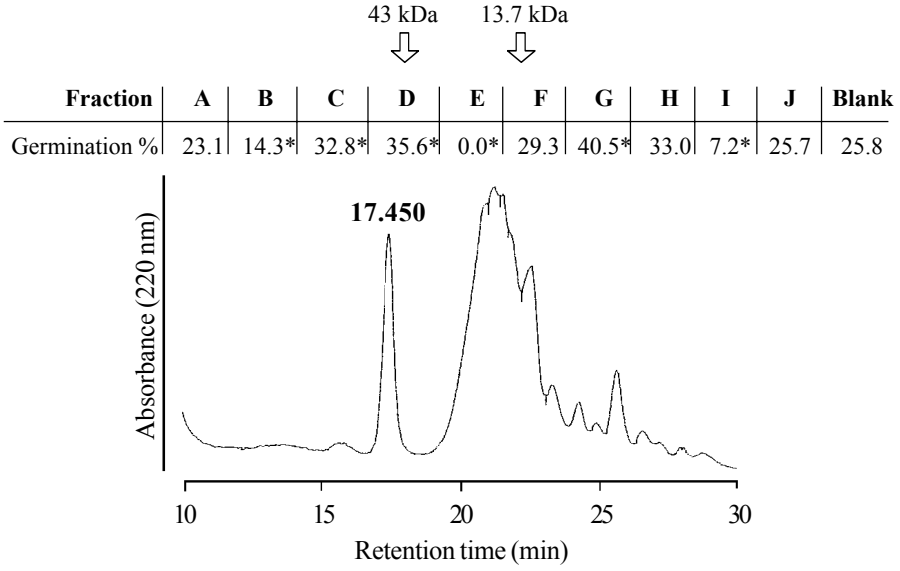

Figure: Gel-filtration (TSK-GEL G3000SW) profile of apoplast NaCl extracts from the TT in cv. Pione pistils. Peaks of ovalbumin (43 kDa) and ribonuclease A (13.7 kDa) are indicated by arrows. Each 2 min-portion of extracts A-J was used for germination tests. Asterisks indicate significantly different values compared to blank test (t-test, * $p < 0.01$).

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