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Purification of Phosphoproteins NrEP1 to 6, Markers for the Beginning of Pollen Embryogenesis in *Nicotiana rustica*, and Identification of Their N-terminal Sequences

Masaharu KYO[1]*, Shoji HATTORI[2], Naoki YAMAJI[1] and Hiroshi FUKUI[1]

[1]*Department of Life Sciences, Faculty of Agriculture, Kagawa University, Miki, Kagawa 761-0795, Japan*
[2] *Present address: Soda Aromatic Co., Ltd., Noda, Ciba 270-0233*
**Corresponding author* E-mail address: kyo@ag.kagawa-u.ac.jp

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Abstract

In pollen culture methods developed for *Nicotiana rustica* and *N. tabacum*, it is possible to induce embryogenic dedifferentiation, i.e., transformation process from immature pollen to embryogenic cells. As biochemical markers for the dedifferentiation of pollen attention has been focused on several phosphoproteins which characteristically appeared in the two-dimensional gel electrophoretogram of total protein from pollen fed with ^{32}Pi in both species. Here, the six phosphoproteins of *N. rustica*, NrEP1 to 6, were partially purified and their N-terminal amino acid sequences were identified. The sequences were similar to each other and also to those of the phosphoproteins (NtEPs) previously identified in *N. tabacum*. This suggests that a protein family possessing a common structure plays an important role in the induction process of pollen embryogenesis in *Nicotiana*.

Key words: *Nicotiana rustica*, N-terminal amino acid sequence, Phosphoproteins, Pollen embryogenesis.

Pollen embryogenesis is well known as an example of totipotency in plant cells but its induction mechanism is entirely unknown. This phenomenon is also a potential tool for producing doubled haploids useful in breeding work but the low productivity of haploids in pollen/anther culture makes the practical application difficult in general plant species. To provide insight the induction mechanism and to increase the productivity, many efforts have been reported as previously reviewed (Mohan Jain *et al.*, 1996; Raghavan, 1997). Considering the present situation of the studies on the induction mechanism (Reynolds, 1997; Pechan and Smykal, 2001), it is an important milestone to identify as many marker molecules for the phenomenon as possible.

We have been developing pollen culture methods in *Nicotiana* for studying the induction mechanism of pollen embryogenesis (Kyo and Harada, 1985, 1986). In the methods for *N. rustica* and *N. tabacum*, immature pollen at a specific developmental stage (mid-bicellular stage, Stage III) dedifferentiated to embryogenic cells in the media lacking carbon and nitrogen sources. Associated with the dedifferentiation, several phosphoproteins appeared on the two-dimensional gel electrophoretogram of total protein in both species (Kyo and Harada, 1990a, b). The subcellular localization of the phosphoproteins is unclear but they are probably bound to the membrane system (Kyo and Ohkawa, 1991). Recently, Kyo *et al.* (2000) developed a simple method for concentrating the phosphoproteins that enables one to clone a cDNA encoding one of the phosphoproteins in *N. tabacum*. In this study, we applied the method to purify the phosphoproteins of *N. rustica*.

Plants of *N. rustica* (seeds were supplied by Japan Tobacco Inc., Tokyo) were grown under a natural light condition in a room regulated at 20 ℃. Pollen population mainly consisting of Stage III pollen, which are characterized by possessing undeveloped amyloplasts and no or small central vacuole, was isolated from flower buds with a corolla length of 7 to 8 mm, prepared by two-step Percoll density gradient centrifugation and cultured in the basal medium (0.4 M mannitol with 20 mM KCl, 1 mM $MgSO_4$ and 1 mM $CaCl_2$) for inducing embryogenic dedifferentiation as described previously (Kyo and Harada, 1990).

Total protein obtained from Stage III pollen of *N.*

rustica fed with ^{32}Pi for three days under the condition for inducing embryogenic dedifferentiation, were developed by two-dimensional gel electrophoresis and visualized by autoradiography (**Fig. 1**) by the methods as described previously (Kyo and Harada, 1990). The major six phosphoproteins (1 to 6) shown in **Fig. 1** were reported to be associated with embryogenic dedifferentiation (Kyo and Harada, 1990b) and were referred to as *N. rustica* embryogenic pollen-abundant phosphoproteins 1 to 6 (NrEP1 to 6, Kyo *et al.*, 2000).

The pollen cultured in the medium for three days were collected by centrifugation and kept at -80 ℃ until use. Using the frozen pellets of approximately 5 x 10^6 pollen, NrEPs were purified following six steps as described previously (Kyo *et al.*, 2000) with minor modifications. An aliquot of sample prepared in each step (Fractions I to VI) was recovered to examine the two-dimensional gel electrophoretogram of the protein contained in the sample. **Fig. 2** shows the patterns of protein contained in the five fractions, developed by two-dimensional gel electrophoresis and visualized by silver staining as described previously (Kyo *et al.*, 2000). Since Fraction V contained no proteins detectable by silver staining, the data was not shown. The spots indicated by arrows (1 to 6) in **Fig. 2**, showed arrangement identical to that of NrEPs shown in **Fig. 1**, and suggested that NrEPs were highly concentrated in Fraction VI.

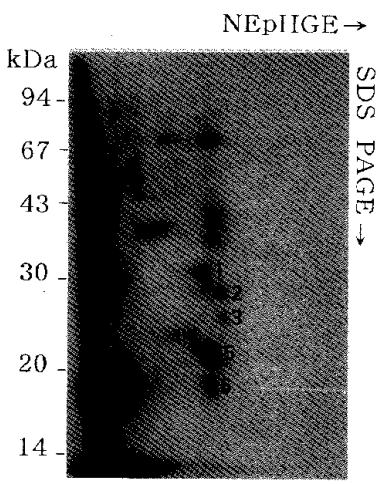

Fig. 1 Autoradiogram of two-dimensional gel electrophoretogram pattern of phosphoproteins of *Nicotiana rustica* pollen cultured for 3 days under the starvation condition. The first dimension was developed by nonequilibrium pH gradient gel electrophoresis (NEpHGE) and the second dimension SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The numbered spots (1 to 6) indicate NrEP 1 to 6, respectively.

The proteins contained in Fraction VI were developed by one- and two-dimensional gel electrophoresis, electrically transferred onto a PVDF membrane (Immobilon-P, Millipore, MA, USA) and visualized by CBB staining (**Fig. 3**). Although the intensity of staining was low, only six spots corresponding to NrEP 1 to 6 were detected (Panel C in **Fig. 3**). The lower detectability of proteins by CBB staining than by silver staining, revealed a clear pattern without signals of contaminated proteins.

The six spots on PVDF membrane for NrEP1 to 6, developed by two-dimensional gel electrophoresis, were carefully cut out and directly charged to a gas-phase peptide sequencer (model 477A, Applied Biosystems, Foster City, CA, USA; model 6625, Millipore) to analyze their N-terminal amino acid sequences, but significant signals for amino acids were not detected. We also obtained unsuccessful results using samples prepared from proteins developed by two-dimensional gel electrophoresis in our previous work (Kyo *et al.*, 2000), for an unknown reason. In the previous case, samples prepared from proteins developed by one-dimensional gel electrophoresis (SDS-PAGE) brought successful results.

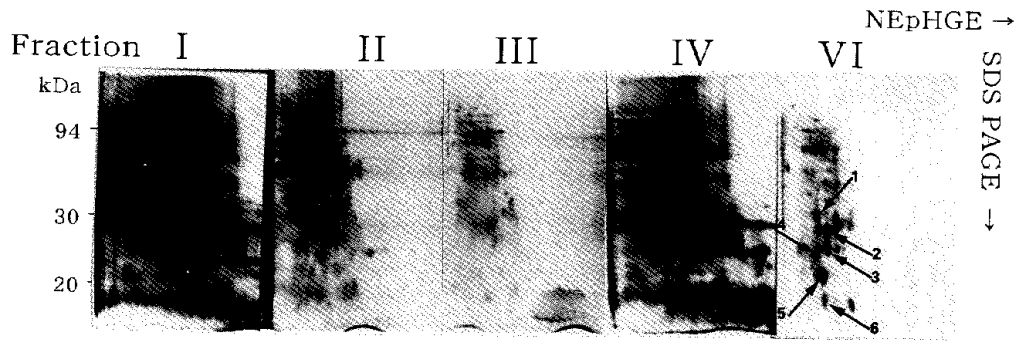

Fig. 2 Two-dimensional gel electrophoretogram of the protein in the fractions prepared in the purification procedure. The patterns were visualized by silver stain. Fraction VI corresponds to 6 × 10^5 pollen grains and others to 2 × 10^5 pollen grains. Numbered arrows (1 to 6) indicate the spots corresponding to NrEP1 to 6 shown in **Fig. 1**, respectively.

Therefore, the six bands for NrEP1 to 6 indicated by arrow heads on Panel B in **Fig. 3**, were also cut out and directly charged to peptide sequencers. In this case, at least fourteen amino acid residues could be identified in each sample (**Table 1**).

As previously reported, it was indicated that NtEPc possess a signal peptide sequence in the premature form (Kyo *et al.*, 2000). The N-terminal amino acids of NrEP1 to 6 (**Table 1**) were not methionine, probably because all these proteins possess signal peptide in their premature forms as NtEPc does. In fact NrEP1 to 6 were not solubilized until treated with a buffer containing a proper detergent in a step of the purification method. These suggest that NrEP1 to 6 are membrane-binding protein or included in some organelle. The N-terminal amino acid sequences of NrEP1, 2 and NtEPa were identical. The sequence of NrEP4 may be identical to NtEPb if the unclarified, eleventh amino acid is tryptophan. The sequence of NrEP3, 5 and 6 were not identical to each other nor to the sequences of NtEPs. However, seven (50%) of the 14 identified residues were common to all the sequences shown in **Table 1**, suggesting that they are categorized to a protein family distributed in *Nicotiana* species.

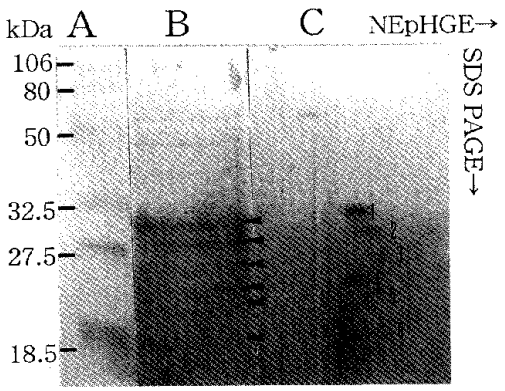

Fig. 3 Electrophoretograms of partially purified NrEPs (Fraction VI) transferred onto PVDF membrane. The patterns were visualized by CBB stain. A: Marker proteins. B: One-dimensional pattern. C: Two-dimensional pattern. Arrow heads in Panel B indicates the bands corresponding to the numbered spots (1 to 6) in panel C, which correspond to NrEP 1 to 6 in **Fig. 1**, respectively.

We examined whether the level of transcrips coding for a member of the protein family including NrEPs is associated with the dedifferentiation of *N. rustica* pollen. As cDNAs for NrEPs have not been obtained, we used cDNA for NtEPb1 (accession No. AB080969) isolated from *N. tabacum* cDNA library as a probe. According to a method described previously (Kyo *et al.*, 2000), total RNA samples prepared from *N. rustica* pollen undergoing maturation and dedifferentiation and anthers were reverse-transcribed, amplified using a specific primer set for NtEPb1 gene (5'-TCAAGTCGTGTATGTCTCCTCC, 5'-TGTTGGAAAAGAAATGGGGGCG), separated by gel-electrophoresis and blotted on nylon membrane. The DNA fragments homologous to NtEPb1 gene on the membrane were hybridized with digoxigenin-11-dUTP labeled cDNA for NtEPb1 and visualized by the digoxigenin detection system (Boehringer Mannheim). Therefore, the hybridization signals (**Fig. 4**) seemed to monitor the transcription level for one of NtEPb1 homologs in *N. rustica* though it is not clear which transcripts for NrEPs were detected.

Table 1 N-terminal amino acid sequences of NrEP1-6 and NtEP a-b.

Protein names	N-terminal amino acid sequences	References
Nicotiana rustica L.		This work
NrEP1	TEFTVGGDKGWVVP	
NrEP2	TEFTVGGDKGWVVP	
NrEP3	LEFQVGDNTGWVVP	
NrEP4	LEFQVGDTTGXAVP	
NrEP5	DEFAVGGDLGPAVP	
NrEP6	TEFAVGGDLGLAVP	
Nicotiana tabacum L.		Kyo *et al.* (2000)
NtEPa	TEFTVGGDKGWVVP	
NtEPb	LEFQVGDTTGWAVP	
NtEPc	TEFAVGGDKGWAVP	

Underlined types show common residues in all the sequences.
X: not identified.

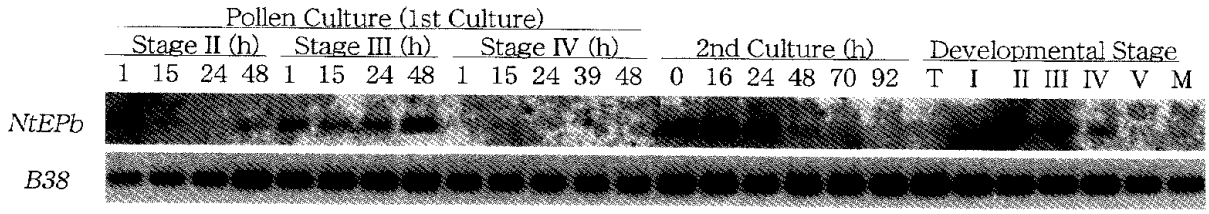

Fig. 4 Expression analysis of *NtEPb* homologs in *N. rustica* during pollen culture and anther/pollen development. The PCR products amplified from cDNA prepared from total RNA isolated from each samples were detected by digoxigenin-11-dUTP labeled probes. *B38* is a cDNA clone coding for DnaJ homolog (accession No. AB032545, primers: 5'-GAGAAGGTTGTGCAGGAGAAGA, 5'-GGCCTTTGGTA-CATTGGCATTC) and was used as an internal standard in RT-PCR. Sizes of the amplified products for *NtEPb* and *B38* were proper judging from the sizes predicted from the positions of their primer sets in the registered sequences.

The immature *N. rustica* pollen at the early-bicellular (Stage II), mid-bicellular (Stage III) and late-bicellular (Stage IV) stages was prepared from flower buds with corolla length of 5-6, 7-8 and 9-10 mm, respectively, by the method described previously (Kyo and Harada, 1990b). When cultured in the basal medium for inducing the dedifferentiation as the 1st culture, the hybridization signal was remarkably detected only in Stage III pollen at 48 h (**Fig. 4**). For inducing embryogenic cell division, Stage III pollen was transferred to 0.4 M mannitol with 20 mM KCl, 5 mM glutamine, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1mM KH_2PO_4-KH_2PO_4, 1 mM sucrose (pH 6.8) at 72 h after the beginning of the 1st culture, i.e., time 0 in the 2nd culture. The hybridization signal was maximized at 16 h, then it was decreased and undetectable after 48 h. As previously reported (Kyo and Harada, 1985, 1990b), *N. rustica* pollen embryo was originated from Stage III pollen and the first pollen cell division starts asynchronously after 24 h in the 2nd culture. Therefore, the stage specificity and the temporality in the gene expression manner were consistent with the appearance of NrEPs.

The anthers containing tetrad (T), unicellular (I), Stage II, III, IV V and mature (M) pollen were isolated from flower buds with calyx or corolla length of 3-3.5 (calyx), 3-4, 5.5-6, 7.5, 9, 13-14 and 21 mm, respectively. The signal was detectable in anthers containing pollen at Stages II, III and IV but their intensity was much lower than those observed at 48 h in the 1st culture of Stage III pollen or in the 2nd culture (**Fig. 4**). Though the physiological role of those gene expression at low level in certain developmental stages of pollen maturation is unclear, we speculate that it may related to the stage-specific feasibility for pollen dedifferentiation and embryogenesis.

As described above, expression of the genes for the protein family including NrEPs showed pollen stage-specific and temporal manner being coincident with the induction of the dedifferentiation of pollen. As the appearance of the phosphoproteins, NrEPs (Kyo and Harada, 1990b), their gene expression also seemed to be a reliable marker for the pollen dedifferentiation in *N. rustica*. NtEPs showed moderate similarity with some copper binding glycoproteins (Kyo *et al.*, 2000), whose function is not clear in spite of many studies on their structure related to the redox potential (for example, Dennison and Lawler, 2001). Therefore, the function and physiological role of NrEPs in the pollen dedifferentiation are also remained to be examined.

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