BIOLOGIA PLANTARUM 48 (2): 293-296, 2004

BRIEF COMMUNICATION

Detection of proteins possibly involved in self-incompatibility response in distylous buckwheat

J. MILJUŠ-ĐUKIĆ*[1], S. NINKOVIĆ**, S. RADOVIĆ***, V. MAKSIMOVIĆ*, J. BRKLJAČIĆ* and M. NEŠKOVIĆ**

*Institute of Molecular Genetics and Genetic Engineering, Vojvode Stepe, 444a, 11000 Belgrade, Serbia and Montenegro**
*Institute for Biological Research "S. Stanković", 29. Novembra 142, 11060 Belgrade, Serbia and Montenegro***
*Faculty of Biological Sciences, Studentski trg 3/II, 11000 Belgrade, Serbia and Montenegro****

Abstract

Buckwheat (*Fagopyrum esculentum* Moench) is a heterostylous plant displaying heteromorphic sporophytic self-incompatibility (SI). In order to detect proteins involved in SI, pistils from both long and short styles were isolated and then selfed or cross-pollinated. One-dimensional gel electrophoresis revealed that short pistils 2 h after selfing contained an unique 50 kDa protein. In the two-dimensional electrophoresis two distinct groups of proteins possibly involved in SI response were detected in the short, and one in the long pistils.

Additional key words: *Fagopyrum esculentum*, heteromorphic self-incompatibility.

Self-incompatibility (SI) is an inherited outbreeding mechanism, which enables plants to prevent self-fertilization by discriminating between the self- and non-self pollen grains. It is widely distributed among plant families; it has been estimated to occur in more than half of all angiosperm species. Two types of SI are known: gametophytic and sporophytic, and the latter can be homomorphic or heteromorphic (De Nettancourt 1997). Self-incompatibility is the main barrier for breeders who want to produce lines with selected traits, what makes studying on SI very warranted.

Buckwheat (*Fagopyrum esculentum* Moench) is a heterostylous plant displaying sporophytic heteromorphic type of self-incompatibility. It is a distylous species, with long styles (pin morph) and short styles (thrum morph). The legitimate pollination is possible only between these two types. In heteromorphic sporophytic SI the site of pollen tube arrest after illegitimate pollination may be on the stigma surface, within the stigma, or in the style (Dulberger 1992). In buckwheat, the self-pollen tubes grow till the 2/3 style length in long styles, while in short styles they stop growing at the junction between the stylar and the stigmatic tissue (Schoch-Bodmer 1934).

In contrast to the morphological phenomena, data about the molecular mechanisms responsible for SI are still very scarce (De Nettancourt 1997). Recent study on *Averrhoa carambola* (Wong 1994a,b) demonstrated the presence of several proteins specific for stamen and style of both short and long morphs. Athanasiou and Shore (1997) reported identification of short style specific proteins in distylous *Turnera*, but their function is still unknown.

In this study we have isolated buckwheat proteins after cross and self-pollination, and separated them by sodium dodecyl sulfate-polyacrylamide gel electro-

Received 24 March 2003, *accepted* 15 January 2004.
Abbreviations: IEF - isoelectric focusing, SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SI - self-incompatibility.
Acknowledgement: This work was supported by the Ministry of Science, Technology and Development of Serbia, grant No. 1451.
[1] Corresponding author; fax: (+381) 011 3975 808, e-mail: heljda@sezampro.yu

phoresis (SDS-PAGE) and isoelectric focusing (IEF), in an attempt to relate differences in morphological aspects of SI reaction with differences in protein profiles between the two styles.

Buckwheat plants *(Fagopyrum esculentum* Moench cv. Darja) from field-collected seeds were grown in a greenhouse. Seeds gave rise to plants with short pistils and plants with long pistils. The day prior to experiments, all opened flowers were removed. Freshly opened flowers were collected the next morning and pistils were isolated under sterile conditions. Pistils were then selfed or cross-pollinated and incubated in a Petri dish, containing the germination medium (Brewbacker and Kwack 1963). Pistils (30) were homogenized in 285 mM Tris pH 6.8, 2 % sodium dodecylsulfate (SDS), 20 % glycerol, 4 % β-mercaptoethanol. The homogenate was then centrifuged for 15 min at 13 000 *g*, and supernatant was used for protein analysis. Protein concentration was determined by the method of Lowry *et al.* (1951). Two-dimensional electrophoresis (2D PAGE) was performed with IEF in the first dimension and SDS-PAGE in the second. IEF was performed in a 4 % acrylamide gel containing 0.4 % ampholines, pH range 3 - 10 (O'Farell 1975). Protein samples (0.04 cm^3 containing approx. 40 µg protein), were loaded on the tube gels, and IEF was carried out at 700 V for 12 h. Separation in the second dimension was realized on a discontinuous system (Laemmli 1970) using vertical slab gel of 12 % polyacrylamide. Molecular mass marker (*Sigma-Aldrich Fine Chemicals*, St. Louis, USA) was included in the electrophoresis, which was carried out under the constant current of 20 mA. Proteins were visualized by silver staining (Merill 1984). Protein separation by one-dimensional electrophoresis was the same as described for the second dimensional gel electrophoresis of 2D PAGE (Laemmli 1970). However, after extraction, the supernatant was added to a buffer containing 2 % SDS, 2 % β-mercaptoethanol, with 0.1 % bromophenol blue as indicator. The mixture was dipped into boiling water for 5 min.

With the aim to identify proteins associated with SI reaction, protein profiles of self and cross-pollinated short and long pistils 2 and 4 h after pollination were compared. After one-dimensional separation and silver staining many common proteins were visible in a range from 15 to 100 kDa, while the 50 kDa protein was detected only in short pistils 2 h after self-pollination (Fig. 1, *lane* 5). This protein was not found in the same pistils 4 h after self-pollination (not shown), what could be a consequence of its degradation, in the situation where irreversible inhibition of the self-pollen tubes occurred. The assumption that 50 kDa protein is specifically induced by SI reaction remains to be confirmed. Analysis of long self-pollinated pistils did not reveal any differences compared to compatible polinations. It was reported previously that in heteromorphic *Linum grandiflorum,* SDS-PAGE was not suitable to separate morphs specific proteins (Ghosh and Shivana 1980).

2D PAGE of protein extracts from self and cross-pollinated long and short pistils, 2 h after pollination revealed a unique group of polypeptides - 25 - 35 kDa, pI 4.7 - 5.5 among long pistil proteins after illegitimate pollination (Fig. 2*A*). Proteins common for both self and cross-pollinated (Fig. 2*B*) pistils were in a range of

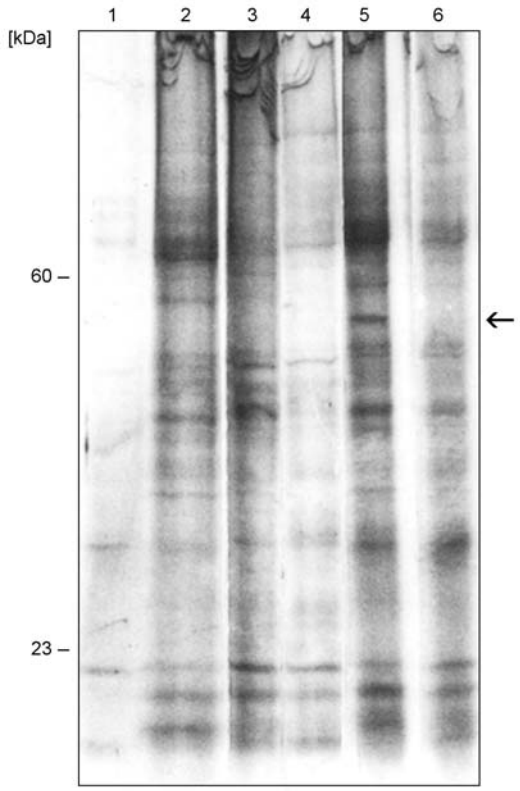

Fig. 1. SDS PAGE profiles of protein extracts from short and long styles 2 h after pollination. *Lines 1* and *4* - unpollinated long and short pistils. *Lines 2* and *5* - selfed long and short pistils. *Lanes 3* and *6* - cross-pollinated long and short pistils. *Arrow* indicates the 50 kDa protein in *lane 5*.

25 - 70 kDa, pI 5.5 - 6.5. In selfed short pistils two groups of proteins could be associated with SI reaction (Fig. 2*C*). The one was a group of proteins from 25 - 55 kDa, pI 5.2, and another was a poorly separated group in a range from 35 - 65 kDa, pI 5.5. Besides, a group of proteins 25 - 35 kDa was also present after legitimate pollination (Fig. 2*D*), but it was less abundant. Quantitative differences in abundance among proteins (25 - 35 kDa, pI 5.5) noticed between selfed and cross-pollinated pistils could not be ignored, suggesting their involvement in SI reaction. Proteins in a range of 25 - 70 kDa, pI 5.5 - 6.5 were common for both selfed and cross-pollinated pistils.

It is clear that the proteins responsible for SI response in the short and in the long styles are not identical, neither in their abundance, nor in mass and pI values, which is in

agreement with morphological data obtained from fluorescence micrographs (Miljuš-Đukić *et al.* 2003). We can assume that some of them have functions in adhesion to stigma surface, or to the inhibition of pollen tube elongation. Athanasiou and Shore (1997) have found proteins specific for short style of distylous *Turnera* plants, which can be involved in SI reaction, but function is unknown. They suggested different SI mechanisms for plants with short and long styles. According to our results, this could be suggested for buckwheat, too. The complexity of heteromorphic SI in buckwheat could be elucidated by functional and structural characterization of proteins considered to be involved in SI.

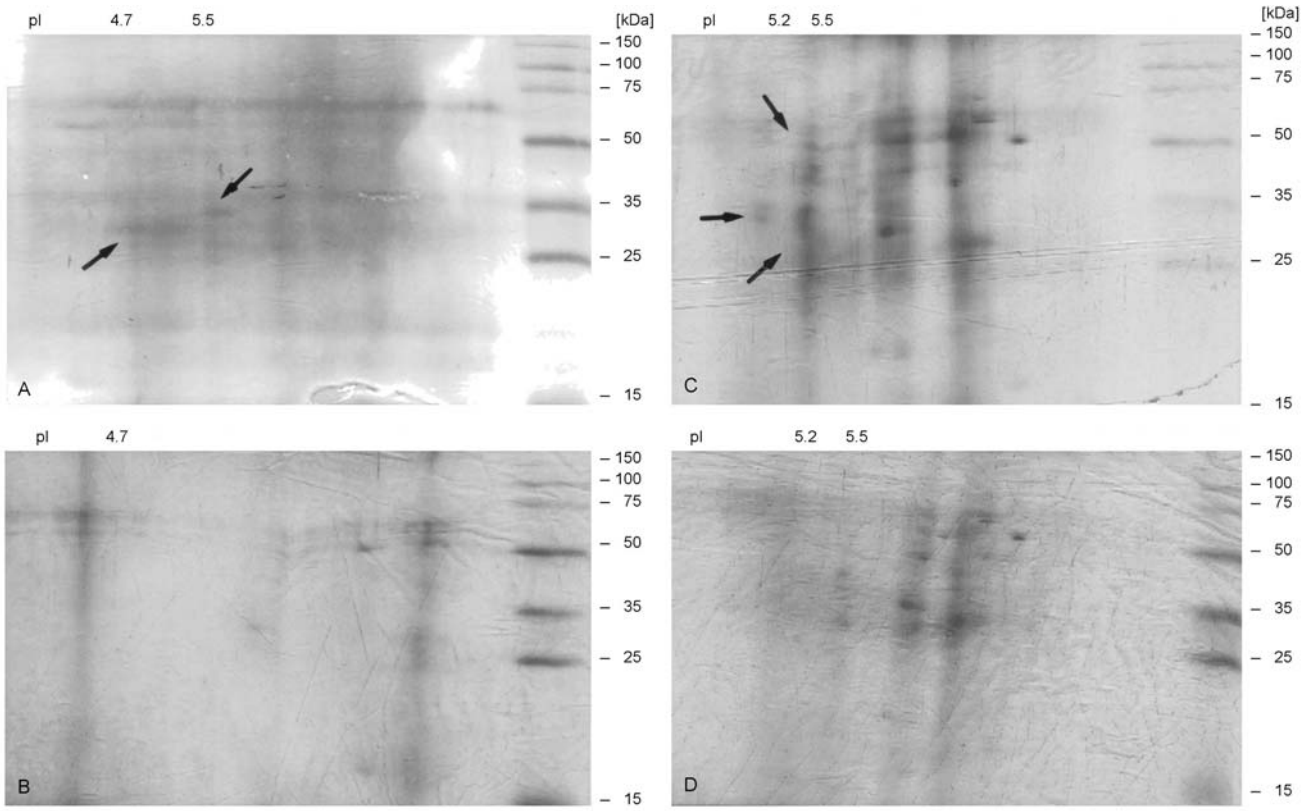

Fig. 2. 2D PAGE profiles of protein extracts from long (*A*, *B*) and short (*C*, *D*) pistils 2 h after illegitimate (*A*, *C*) and legitimate pollination (*B*, *D*). *Arrows* indicate groups of proteins from 25 - 35 kDa, pI 4.7 - 5.5 (*A*), 25 - 35 kDa, pI 5.2, and 35 - 65 kDa, pI 5.5 (*C*).

References

Athanasiou, A., Shore, J.S.: Morph-specific proteins in pollen and style of distylous *Turnera* (*Turneraceae*). - Genetics **146**: 669-679, 1997.

Brewbaker, J.L., Kwack, B.H.: The essential role of calcium ion in pollen germination and pollen tube growth. - Amer. J. Bot. **50**: 859-865, 1963.

De Nettancourt, D.: Incompatibility in angiosperms. - Sex. Plant Reprod. **10**: 185-199, 1997.

Dulberger, R.: Floral polymorphism and their functional significance in the heterostylous syndrom. - In: Barrette, S.C.H. (ed.): Evolution and Function of Heterostyly. Pp. 41-84. Springer-Verlag, Berlin - Heidelberg - New York 1992.

Ghosh, S., Shivanna, K.R.: Pollen-pistil interaction in *Linum grandiflorum*. - Planta **149**: 257-261, 1980.

Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **367**: 560-563, 1970.

Lowry, O.M., Rosenbrough, N.J., Farrar, I., Randall, R.J.: Protein measurement with folin phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

Merill, C.R., Goldman, D., Van Kauren, M.L.: Gel protein stains: Silver stain. - Methods Enzymol. **104**: 441-447, 1984.

Miljuš-Đukić, J., Ninković, S., Nešković, M.: Effects of protein phosphatase inhibitors and calcium antagonists on self-incompatible reaction in buckwheat. - Biol. Plant. **46**: 475-478, 2003.

O'Farell, P.H.: High resolution two-dimensional electrophoresis of proteins. - J. biol. Chem. **250**: 4007-4021, 1975.

Schoch-Bodmer, H.: Zum Heterostylieproblem: Griffelbeschaffenheit und Pollenschlauchwachstum bei *Fagopyrum esculentum*. - Planta **22**: 149-152, 1934.

Wong, K.C., Watanabe, M., Hinata, K.: Protein profiles in pin and thrum floral organs of distylous *Averrhoa carambola* L. - Sex. Plant Reprod. **7**: 107-115, 1994a.

Wong, K.C., Watanabe, M., Hinata, K.: Fluorescence and scanning electron microscopic study on self-incompatibility in distylous *Averrhoa carambola* L. - Sex. Plant Reprod. **7**: 116-121, 1994b.