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TREATMENT OF ISOLATED PISTILS WITH PROTEASE INHIBITORS OVERCOMES THE SELF-INCOMPATIBILITY RESPONSE IN BUCKWHEAT

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Abstract – Isolated pistils of distylous buckwheat (*Fagopyrum esculentum* Moench) were treated with protease inhibitors (PMSF, pepstatin A, and antipain). Pistils were cross- or self- pollinated, and growth of pollen tubes was observed under a fluorescence microscope. Treatments with all inhibitors suppressed inhibition of self-pollen tube growth, suggesting that activity of proteases is involved in rejection of self-pollen during the SI response.

Key words: Buckwheat, heteromorphic self-incompatibility, protease inhibitors

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INTRODUCTION

Self-incompatibility (SI) is an outbreeding mechanism which enables plants to discriminate between self and non-self pollen grains. It is broadly distributed among the flowering plants: more than half of all flowering plant species display some of the SI types. There are two main well-defined types of SI, gametophytic and sporophytic, and the latter can be homomorphic or heteromorphic (for recent reviews, see D i x i t and N a s r a l l a h, 2001; H i s c o c k and Mc I n n i s, 2003; and T a k a y a m a and I s o g a i, 2005). While SI is considered advantageous from the evolutionary point of view, it nevertheless represents a drawback for plant breeders, since it prevents the creation and selection of homozygous lines.

The heteromorphic SI system is characterized by different positions of stigmas and anthers in dimorphic or trimorphic flowers (de N e t t a n c o u r t, 1997). It is rare and occurs in 24 families of flowering plants. Among plants of this type, common buckwheat (*Fagopyrum esculentum* Moench) is of the greatest economical importance. It posseses two flower morphs: pin (with long pistil and short anthers) and thrum (with short pistil and long anthers), which are equally distributed among the population. Legitimate pollination is possible only between these two types. In contrast to homomorphic systems, very little is known about the molecular mechanism of the SI response in heteromorphic systems (de N e t t a n c o u r t, 1997) operating in buckwheat.

Our previous papers on SI in buckwheat (M i lj u š – Dj u k i c et al., 1998, 2003) showed that processes such as protein synthesis and glycosylation, together with protein phosphorylation, can be part of the SI response, such processes being common in plants which display one of the two SI systems (sporophytic in Brassicaceae, gametophytic in Solanaceae). On the other hand, the SI response in Papaveraceae belongs to quite a different system, where Ca^{2+} serves as an important SI response mediator (F r a n k l i n – T o n g et al., 2002).

In the present paper, we describe effects of protease inhibitors on the self-incompatibility response in order to further "dissect" the SI response in buckwheat and analyze the possible role of proteases in this complex process. It could be predicted that proteases are involved in programmed cell death (PCD). Processes of PCD processes are very important for sexual plant reproduction, even when hidden from view (W u and C h e u n g, 2000). Cell death occurs in both pollen and the pistil. For example, in plants with a solid style, the transmitting tis-

sue must pass through degeneration in order to let pollen tubes grow (W a n g et al., 1996).

Isolated buckwheat pistils, cross and self-pollinated, were treated with three different protease inhibitors: the serine protease inhibitor PMSF, the aspartic protease inhibitor pepstatin A, and the cysteine protease inhibitor antipain.

MATERIAL AND METHODS

Plant material

Buckwheat plants (*Fagopyrum esculentum* Moench, cv. Darja) were grown in a greenhouse. Field-collected seeds gave rise to thrum plants (with short pistils) and pin plants (with pistils about three times longer). One day prior to experiments, all opened flowers were removed. Freshly opened flowers were collected the next morning and pistils were isolated under sterile conditions to minimize possible bacterial contamination. About 10 pistils of each morph were put in a Petri dish containing the germination medium (B r e w b a c k e r and K w a c k, 1963). The medium consisted of 10% agar, 15% sucrose, and (in mg L^{-1}): 100 H_3BO_3, 300 $Ca(NO_3)_2.4\ H_2O$, 200 $MgSO_4.7H_2O$, and 100 KNO_3. The pistils were pollinated with self and non-self pollen by touching their surfaces with dehisced anthers. Buckwheat pistils consist of three closely adhering styles, and about 10-20 pollen grains were visible on each stigma upon pollination.

Experiments with inhibitors

Inhibitors were added by wetting a piece of filter paper, which was put on the agar surface under the pistils. In the controls, this filter paper was wetted with distilled water. Care was taken to prevent any contact of stigma surface with inhibitors. Incubation lasted 24 h. The serine protease inhibitor PMSF (phenylmethylsulfonylfluoride), aspartic protease inhibitor pepstatin A, and cysteine protease inhibitor antipain were added in the following concentrations: 1 and 10 mM PMSF; and 1 and 10 μM pepstatin or antipain. All experiments were repeated three times, with 10 pistils in each sample.

Microscopy

After incubation, the pistils were fixed overnight in ethanol:acetic acid (3:1, v:v), then washed in distilled water, macerated in 7 N NaOH for 24 h, and washed again. Finally, they were stained with 0.1% decolorized aniline blue in 0.1 N K_3PO_4 and mounted on a microscope slide in a drop of 50% glycerol. Pollen tubes were then observed in UV light using a Leitz ARISTOPLAN fluorescence microscope (12 V 100 W halogen lamp) and an excitation block filter (I3-blue, Leica).

RESULTS AND DISCUSSION

We examined the effects of different protease inhibitors to establish possible protease involvement in the SI response of buckwheat.

In isolated control pistils of the thrum morph, self-pollen tubes were arrested at the junction between stigmatic tissue and the style (Fig. 1E). Treatment of isolated pistils with PMSF resulted in overcoming of the SI response at both applied concentrations (Fig. 1F), and self-pollen tubes elongated down the style as in the compatible control. The effect of pepstatin A (Fig. 1G) was similar to that of PMSF, i.e., at both concentrations (1 and 10 μM), the SI response was abolished and self-pollen tubes elongated down the style. The same results were obtained after treatment of isolated pistils with both applied antipain concentrations (Fig. 1H).

In isolated control pistils of the pin morph, self-pollen tubes were arrested at two thirds of the style's length (Fig. 1A). At both concentrations (1 and 10 mM), PMSF produced a breakdown of the SI response, and self-pollen tubes elongated beyond two thirds of the style's length, as in the compatible control (Fig. 1B). Treatment with pepstatin A and antipain abolished the SI response and self-pollen tubes reached the bottom of the style, as in compatible crosses (Figs. 1C and 1D).

In experiments with protease inhibitors in buckwheat, the SI response was overcome and self-pollen tubes elongated down the style with all three inhibitors, while compatible crosses were not affected (Table 1). This finding indicates that proteases could be important players in the SI system operating in buckwheat. The SI response is complex: besides proteins at the stigma surface directly involved in self-pollen discrimination (thrum morph), there must also be others involved in processes leading to interruption of pollen tube elongation (pin morph).

It could be expected that proteases induced PCD during the SI response to prevent self-pollen tube elongation in buckwheat. There are recent data indicating a role for proteases in programmed cell death occuring in the

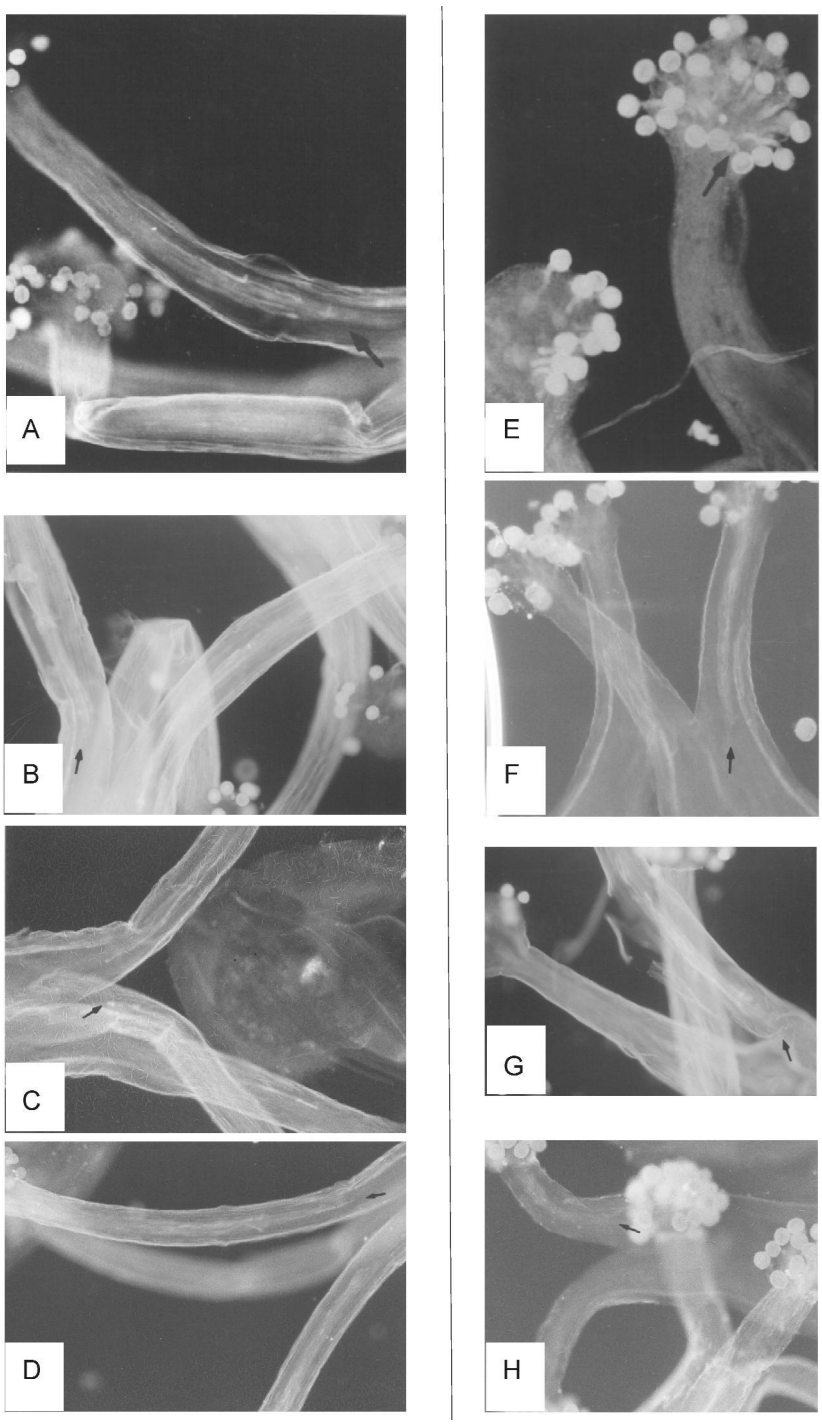

Fig. 1. Examples of overcoming of the incompatibility response in pin (A, B, C, D) and thrum (E, F, G, H) pistils under treatment with different protease inhibitors. A and E-controls without treatments; treatments with: B – 10 mM PMSF; C – 1 μM pepstatin A; D – 10 μM antipain; F – 1 mM PMSF; G – 10 μM pepstatin A; H – 10 μM antipain. Arrows indicate the site of pollen tube arrest.

Table 1. Effects of protease inhibitors on elongation of pollen tubes in isolated pistils. Each pistil was pollinated with 30-60 pollen grains. Pollen tubes were observed under a fluorescence microscope in 3x10 pistils subjected to three independent treatments. (-) pollen tubes arrested at the junction between the stigma and the style; (+) pollen tubes arrested at two thirds of the style's length; (++) pollen tubes elongated down the style.

Treatment	[μM]	*Pin/pin*	*Pin/thrum*	*Thrum/thrum*	*Thrum/pin*
control	/	+	++	-	++
PMSF	1000	++	++	++	++
	10000	++	++	++	++
Pepstatin A	1	++	++	++	++
	10	++	++	++	++
Antipain	1	++	++	++	++
	10	++	++	++	++

presence of incompatible pollen tubes during the SI response in other plants. Aspartic proteases were found in reproductive tissues, including pistils, of many plants, although their exact roles are not clear (W u and C h e u n g, 2000). This was shown for cardosin, an aspartic proteinase from cardoon that is involved in adhesion-mediated proteolytic mechanisms in pollen recognition and growth (F a r o et al., 1999). The question which remains to be answered is what mechanism activates proteases in the buckwheat two morphs, where the site of self-pollen tube arrest differs.

In poppy, a caspase inhibitor appears to reduce the level of DNA fragmentation (a marker for PCD) that occurs in pollen tubes elongating in the presence of incompatible S-proteins (T h o m a s and F r a n k l i n – T o n g, 2004). The authors showed that Ca^{2+} acts as a second messenger in the SI response, and that increase of intracellular Ca^{2+} concentration can be linked with cytochrome c leakage, the classical marker for PCD. To find out if the situation in buckwheat is similar, the phenomenon of PCD during the SI response in buckwheat should be futher examined in the light of our previous results with Ca^{2+} antagonists (M i lj u š – Dj u k i ć et al., 2003).

Abbreviations used: SI) self-incompatibility; PCD) programmed cell death, PMSF) phenylmethylsulfonylfluoride.

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REFERENCES

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

De Nettancourt, D. (1997). Incompatibility in Angiosperms. – *Sex. Plant Reprod.* **10**: 185-199.

Dixit, R. and *Nasrallah, J.B.* (2001). Recognizing self in the self-incompatibility response. – *Plant Physiology*. **125**: 105-108.

Faro, C., Ramalho-Santos, M., Vieira, M., Mendes, A., Simões, I., Andrade, R., Verissimo, P., Lin, X., Tang, J., and *Pires, E.* (1999). Cloning and characterization of cDNA encoding cardosin A, an RGD-containing plant aspartic proteinase. – *J. Biol. Chem.* **274**: 28724-28729.

Franklin-Tong, V.E., Holdawey-Clarke, T.L., Straatman, R., Kunkel, J.G., and *Hepler, P.K.* (2002). Involvement of extracellular calcium influx in the self-incompatibility response of *Papaver rhoeas*. – *Plant J.* **29:** 333-345.

Hiscock, S.J., and *McInnis, S.M.* (2003). Pollen recognition and rejection during the sporophytic self-incompatibility response: *Brassica* and beyond. – *Trends in Plant Sci.* **8**(12):606-612.

Miljuš-Đukić, J., Ninković, S., Maksimović, V., Radović, S., Brkljačić, J., and *Nešković, M.* (1998). Effects of protein metabolism inhibitors on SI reaction in buckwheat (*Fagopyrum esculentum* Moench). – In: *Campbell, C.,* and *Przybylski, R. (ed.): Proceedings of the VII International Symposium on Buckwheat*. Vol. V. Pp. 9-18. International Buckweat Research Association, Winnipeg.

Miljuš-Đukić, J., Ninković, S., and *Nešković, M.* (2003). Effects of protein phosphatase inhibitors and calcium antagonists on self-incompatible reaction in buckwheat. – *Biologia Plantarum*. **46** (3): 475-478.

Takayama, S. and *Isogai, A.* (2005). Self-incompatibility in plants. – *Annu. Rev. Plant Biol.* **56:** 467-489.

Thomas, S.G., and. *Franklin-Tong, V.E.* (2004). Self-incompatibility triggers programmed cell death in *Papaver* pollen. - *Nature* **42:** 305-309.

Wang, H., Wu, H.-M., and *Cheung, A.Y.* (1996). Pollination induces mRNA poly(A) tail-shortening and cell deterioration in flower transmitting tissue. - *Plant J.* **9:** 715-727.

Wu, H., and *Cheung, A.Y.* (2000). Programmed cell death in plant reproduction. – *Plant Mol. Biol.* **44:** 276-281.

ТРЕТИРАЊЕ ИЗОЛОВАНИХ ТУЧКОВА ИНХИБИТОРИМА ПРОТЕАЗА ДОВОДИ ДО ПРЕВЛАДАВАЊА АУТО-ИНКОМПАТИБИЛНЕ РЕАКЦИЈЕ КОД ХЕЉДЕ

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Хељда (*Fagopyrum esculentum* Moench) је биљка код које постоји хетероморфни спорофитни систем инкомпатибилности. У популацији постоје биљке са пин типом цвета (дугачак тучак) и трам типом (кратак тучак). Легитимно опрашивање је могуће само између ова два типа. Изоловани тучкови су третирани инхибиторима протеаза (PMSF, пепстатином А и антипаином), а затим опрашени сопственим (инкомпатибилним) или поленом са супротног морфа (компатибилним). Раст поленових цевчица је праћен под флуоресцентним микроскопом. Резултати су показали да третмани доводе до превладавања ауто-инкомпатиблине реакције код хељде и раста сопствених поленових цевчица као код компатибилне контроле. То упућује на закључак да протеазе имају важну улогу у ауто-инкомпатибилној реакцији код хељде.