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BRIEF COMMUNICATION

Effects of protein phosphatase inhibitors and calcium antagonists on self-incompatible reaction in buckwheat

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Abstract

Isolated pistils of dimorphic buckwheat (*Fagopyrum esculentum* Moench.) flowers were treated with phosphatase inhibitors (ocadaic acid and cantharidin) and with calcium antagonists (verapamil, La^{3+}, and A23187). They were subsequently cross- or self-pollinated, and the growth of pollen tubes was observed under the fluorescence microscope. All treatments suppressed inhibition of pollen tubes growth suggesting that protein phosphatases and calcium signaling may be involved in self-incompatibility signal transduction in buckwheat.

Additional key words: *Fagopyrum esculentum* Moench., ocadaic acid, cantharidin, verapamil, lanthanum chloride.

Self-incompatibility (SI) is an outbreeding mechanism, which enables plants to prevent self-fertilization by discriminating between the self- and non-self pollen grains. Known types of SI are gametophytic and sporophytic, and the latter can be homomorphic or heteromorphic. The heteromorphic sporophytic system is characterized by different positions of anthers and stigmas in dimorphic, or trimorphic flowers. Common buckwheat, belonging to this type, is a distylous species, with pin morph (long styles) and thrum morph (short styles). In this SI system the site of pollen tube arrest after illegitimate pollination may be on the stigma surface, within the stigma, or in the style. In contrast to the morphological phenomena, which are studied in details in certain species, very little is known about the biochemical mechanisms of SI reaction in heteromorphic systems (de Nettancourt 1997). In the present paper, we describe effects of protein phosphatase inhibitors and calcium antagonists on SI response in buckwheat. Protein phosporylation is part of signal transduction in animal and plant systems. Protein phosphatases type PP1, PP2A and PP2C have been found in plant cells. PP1 and PP2 are inhibited with ocadaic acid, that abolished sporophytic SI reaction in *Brassica* plants (Scutt *et al.* 1993). Ca^{2+} is not only essential for pollen tube growth (Trewavas and Malho 1998), but also acts as a second messenger in SI response, in the gametophytic system (Franklin-Tong 1999).

Buckwheat plants (*F. esculentum* cv. Darja) were grown in a greenhouse. Seeds gave rise to thrum plants, with short pistils, and pin plants, with long pistils. A day prior to the experiments, all opened flowers were removed. Freshly opened flowers were collected in the next morning and the pistils were isolated under sterile conditions, to minimize possible bacterial contamination. The pistils were pollinated with self or non-self pollen, by touching their surface with dehisced anthers. Buckwheat pistils consist of three closely adhering styles, and about 30 - 60 pollen grains were visible on each pistil upon pollination. About ten pistils of each pin and thrum plants were put in a Petri dish, containing the germination medium of Brewbacker and Kwack (1963). The medium consisted of 10 % agar, 15 % sucrose, and [mg dm^{-3}]: H_3BO_3 100, $Ca(NO_3)_2 . 4\ H_2O$ 300, $MgSO_4 . 7\ H_2O$ 200, and KNO_3 100. Inhibitors were added by wetting a piece of filter paper, which was put on agar surface below the pistils. In controls, the filter paper was wetted with distilled water. The calcium transport inhibitors were added in following concentrations: 1 mM verapamil, 0.1 mM La^{3+} (in the form of $LaCl_3$), and 50 μM A23187

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Abbreviations: CN - cantharidin; DMSO - dimethylsulphoxide; OA - ocadaic acid; SI - self-incompatibility.
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in 10 % dimethyl-sulphoxide (DMSO). Protein phosphatases inhibitors were added in following concentrations: 1 and 5 μM ocadaic acid (OA) in 10 % DMSO, 10, 15 or 20 mM cantharidin (CN). Because of high OA molecular mass, treated pistils were pollinated 2 h after incubation (Scutt *et al.* 1993), while all others were pollinated immediatelly after the treatment. The care was taken to prevent any contact of stigma surface with inhibitors. Treatments were repeated three times for every inhibitor concentration. After 24 h incubation, pollinated pistils were fixed overnight in ethanol:acetic acid (3:1, v:v), then washed in distilled water, macerated in 7 M NaOH for 24 h and washed again. Finally, they were stained with 0.1 % decolorized aniline blue in 0.1 % K_3PO_4, and mounted on a microscope slide in a drop of 50 % glycerol. The pollen tubes were then observed in UV light, using a *Leitz ARISTOPLAN* fluorescent microscope (12 V 100 W halogen lamp) and excitation block filter *I3-blue* (*Leica, Microsystems*, Heerbrugg, Switzerland).

In the isolated control pistils of the thrum morph, self-pollen tubes were arrested at the junction between the stigmatic tissue and the style (Fig. 1*A*). CN in all applied concentrations counteracted the growth inhibition and the self-pollen tubes elongated down the style, like in the compatible control (Table 1). The effect of OA (Fig. 1*B*) was similar to that of CN, *i.e.*, at both concentrations (1 and 5 μM) the SI reaction was overcome and self-pollen tubes elongated down the style (Table 1). The SI reaction was also suppressed when Ca^{2+} antagonists were applied (Fig. 1*C*). There was no difference between the response to Ca^{2+} entry blockers, verapamil and La^{3+}, and the ionophore A23187 (Table 1).

In the isolated control pistils of the pin morph, self-pollen tubes were arrested at the 2/3 of the style length (Fig. 1*D*). At the highest CN concentration (20 mM), the self-pollen tubes were elongated further than the 2/3 of the style length, like in compatible control (Fig. 1*E*), while at lower concentrations, not all pollen tubes responded. Ocadaic acid at both concentrations (1 and 5 μM) also produced the breakdown of SI reaction (Table 1). The treatment with the three Ca^{2+} antagonists abolished the SI reaction and the pollen tubes reached the bottom of the style, as seen at compatible crosses (Table 1, Fig. 1*F*).

It can be concluded, therefore, that both protein phosphatases inhibitors and the three calcium antagonists overcame the self-incompatible reaction in pin and thrum morphs alike. It is noteworthy that they did not affect the growth of pollen tubes at compatible crosses (Table 1).

It is well known that in animal and plant cells the phosphorylation state of proteins is dependent on the activity of both kinases and phosphatases. Membrane-bound receptor-kinase is one of the main constitutents of SI reaction in sporophytic systems (Dickinson 1999), so it can be expected that protein phosphatases are part of it too. Recent data gave evidence that in *Brassica* plants, the treatment of isolated pistils with OA and other phosphatase inhibitors abolished SI reaction (Rundle *et al.* 1993, Scutt *et al.* 1993). Since CN and OA are extremely specific, it can be assumed that their effects on SI reaction in buckwheat may be that of inhibiting PP1 and/or PP2A (Smith and Walker 1996). Obermeyer *et al.* (1998) reported that OA inhibited the lily (*Lilium longiflorum*) pollen tube growth *in vitro*. However, as OA did not affect the pollen tube growth on isolated pistils in buckwheat, similarly as in *Brassica* (Scutt *et al.* 1993) we can assume that it specifically affected the SI components on pistils only.

Franklin-Tong (1999) reported that the action of S proteins on self-pollen in the gametophytic system (*Papaver rhoeas*), is associated with the increase in Ca^{2+} concentration leading to inhibition of self-pollen tube growth. Recently, the same authors (Franklin-Tong *et al.* 2002) found that extracellular calcium influx is necessary for SI response, which is blocked by verapamil. Wehling *et al.* (1994) also stated that in another gametophytic system (*Secale cereale*) Ca^{2+} antagonists blocked the SI reaction. However, in a sporophytic system (*Brassica*

Table 1. Effects of phosphatase inhibitors and calcium antagonists on the elongation of pollen tubes in the isolated pistils. Each pistil was pollinated with 30 - 60 pollen grains. Pollen tubes were counted in 3 × 10 pistils, in three independent treatments. The numbers represent average number ± SE of pollen tubes per pistil. (-) pollen tubes arrested at the junction between the stigma and the style; (+) pollen tubes arrested at the 2/3 of the style length; (++) pollen tubes elongated down the style.

Treatment	[μM]	Pin/pin	Pin/thr	Thr/thr	Thr/pin
Control		41.6 ± 1.5 (+)	46.5 ± 2.1 (++)	0 (-)	39.2 ± 1.6 (++)
Ocadaic acid	1	50.1 ± 2.5 (++)	50.9 ± 3.2 (++)	48.9 ± 2.1 (++)	40.9 ± 2.4 (++)
	5	54.7 ± 2.8 (++)	43.3 ± 2.3 (++)	46.1 ± 2.4 (++)	42.4 ± 2.5 (++)
Cantharidin	10000	39.8 ± 3.5 (+)	41.5 ± 3.2 (++)	42.5 ± 3.5 (++)	40.2 ± 3.4 (++)
	15000	37.2 ± 3.4 (+)	36.5 ± 3.2 (++)	47.6 ± 5.6 (++)	45.8 ± 4.5 (++)
	20000	40.6 ± 2.5 (++)	38.4 ± 3.4 (++)	30.9 ± 3.5 (++)	33.7 ± 3.2 (++)
Verapamil	1000	39.4 ± 3.2 (++)	39.7 ± 2.4 (++)	33.9 ± 2.5 (++)	41.0 ± 3.4 (++)
La^{3+}	100	51.6 ± 4.0 (++)	35.9 ± 3.1 (++)	31.4 ± 3.4 (++)	42.8 ± 2.5 (++)
A23187	50	48.1 ± 3.3 (++)	36.0 ± 3.3 (++)	30.0 ± 2.8 (++)	40.6 ± 3.3 (++)

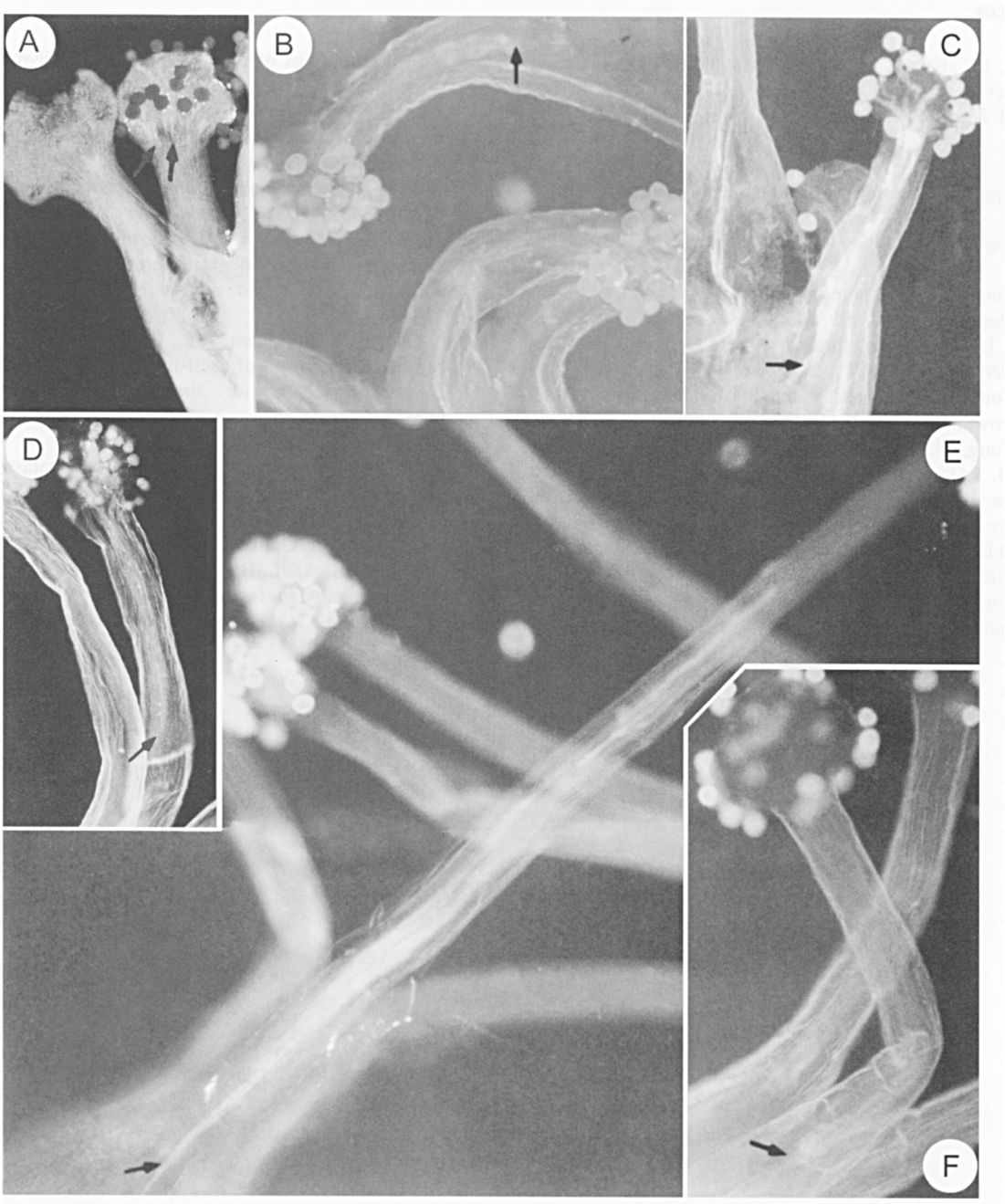

Fig. 1. Representative examples on the overcoming the incompatible reaction in thrum (*A*, *B*, *C*) and pin (*D*, *E*, *F*) pistils: *A* and *D* - controls, *B* - 5 μM ocadaic acid, *C* - 1 mM verapamil, *E* - 20 mM cantharidin, *F* - 50 μM A23187. *Arows* indicate the site of pollen tube arrest.

napus) Ca^{2+} fluxes do not seem to be specifically involved in SI response (Dearnaley *et al.* 1997). Our results demonstrate that the SI response in buckwheat is abolished with the three Ca^{2+} antagonists that may interfere with transient Ca^{2+} fluxes. If an early effect of Ca^{2+} ions is to promote callose formation, it is clear than why they affect the initiation of pollen tube growth only at incompatible crosses.

Our previous findings with actinomycin, cycloheximide, and tunicamycin (Miljuš-Đukić *et al.* 1988) showed that protein synthesis and glycosylation are part of SI response in buckwheat. Together with results presented here, showing that phosphorylation and Ca^{2+}-mediated signal transduction are also involved, it may be concluded that the basic elements of SI reaction are shared among all types of self-incompatible plants.

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