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A SEARCH OF *BRASSICA* SI-INVOLVED ORTHOLOGS IN BUCKWHEAT LEADS TO NOVEL BUCKWHEAT SEQUENCE IDENTIFICATION: MLPK POSSIBLY INVOLVED IN SI RESPONSE

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Abstract - Self-incompatibility (SI) systems, gamethophytic (GSI) and sporophytic (SSI), prevent self-pollination in angiosperms. Buckwheat displays heteromorphic SSI, with pollination allowed only between different flower morphs - thrum and pin. The physiology of thrum and pin morph SI responses are entirely different, resembling homomorphic *Brassica* SSI and *Prunus* GSI responses, respectively. Considering angiosperm species may share ancestral SI genes, we examined the presence of *Brassica* and *Prunus* SI-involved gene orthologs in the buckwheat genome. We did not find evidence of *SRK*, *SLG* and *SP11 Brassica* or *S-RNase* and *SFB Prunus* orthologs in the buckwheat genome, but we found a *Brassica* MLPK ortholog. We report the partial nucleotide sequence of the buckwheat MLPK and discuss the possible implications of this finding.

Key words: Buckwheat, heteromorphic sporophytic self-incompatibility, MLPK

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INTRODUCTION

Self-incompatibility (SI) systems are widely distributed among angiosperms (in approximately 60% of all angiosperm species; Hiscock and Kues, 1999). They enable the discrimination between "self" and "non-self" pollen in flowering plants, forming intra- and interspecies reproductive barriers and preserving the genetic variability of species. Basic classification of SI systems to gametophytic (GSI) or sporophytic (SSI) is made according to pollen SI phenotype determination – in GSI systems the pollen self-incompatibility phenotype is determined by its own haploid genotype and in SSI systems it is determined by the diploid genotype of the mother plant.

Also, GSI displaying species are always homomorphic (one type of flower per species), while SSI plant species may be homomorphic or heteromorphic (more than one type of flower per species). Today, GSI systems and homomorphic SSI systems are far better studied than heteromorphic SSI, with research into the *Primula* (McCubbin, 2008) and *Fagopyrum* (Miljuš-Djukić et al., 1998; Matsui et al., 2004; Matsui et al., 2007).

Common buckwheat (*Fagopyrum esculentum* Moench.) is of interest as an important nutritive crop in Asia, Australia, the USA and Western Europe. Buckwheat displays a heteromorphic SSI system – it is a distylous plant with two flower types (one flower type per plant), pin (long styles, short stamens) and thrum (short styles, long stamens), equally distributed among the population. Legitimate pollination is possible only between different flower morphs. In the case of illegitimate pollination in thrum flower morph, self-pollen tubes are stopped at the junction between the stigma and the style, while in pin flower morph self-pollen tubes grow to 2/3 of the style length (Miljuš-Djukić et al., 1998) before termination.

Although the physiological data about the SI response upon incompatible pollination in buckwheat are abundant (Miljuš-Djukić et al., 1998; Matsui et al., 2004; Matsui et al., 2007), the data concerning the SI reaction at a molecular level

Table 1.

Primer name	5'→3' primer nucleotide sequence	Used in orientation / in combination with	Annealing temperature (°C)
SRKf1	TCITTYGAYTAYCCICANGAY	forward/SRKr1, SRKr2, SRKr3	61 -66 (with SRKr1) 64 -66 (with SRKr2) 66 -71 (with SRKr3)
SRKf2	GGIYTIYTITAYYTICAYCARGAY	forward/SRKr2, SRKr3	64 -68 (with SRKr2) 68 -71 (with SRKr3)
SRKr1	RTAIARIARICCYCKWGC	reverse/ SRKf1	61-66
SRKr2	CATICCRAARTCIGWDATYTTN	reverse/SRKf1, SRKf2	64 -66 (with SRKf1) 64 -68 (with SRKf2)
SRKr3	CATIGCRTAYTCIGGISWCATRTAN	reverse/SRKf1, SRKf2	66 -71 (with SRKf1) 68 -71 (with SRKf2)
SP11f1	TAACTAARATHCAYTAYYTNTG	forward/ SP11r	62 - 68
SP11f2	CACTIGAYGTWGGNGCN	forward/ SP11r	62 - 68
SP11r	TAIGAYTTIACYTTRCARCARTARC	reverse/SP11f1 and SP11f2	62 - 68
SLGf1	GGWGATGTYTTYGARYTNGG	forward/SLGr1 and SLGr2	65 - 67 (with SLGr1) 67 - 70 (with SLGr2)
SLGf2	AGITGGTATYTNGGWATHTGGTAY	forward/ SLGr2	68-70
SLGr1	RAAICCYTGDATRCARTTRCA	reverse/SLGf1	65 -67
SLGr2	GCAAAWGCIGTRCARTTRCARTC	reverse/SLGf1, SLGf2	67-70 (with SLGf1) 68-70 (with SLGf2)
FeMLPKf1	TWYAARGGDTGGATBGATG	forward/ FeMLPKr1	52.6
FeMLPKr1	GTRGAXACRTGRCTXTTVTCRCC	reverse/ FeMLPKf1	52.6

are still very scarce. Considering the different SI responses in two buckwheat morphs upon self-pollination, it is to be expected that different genes and different mechanisms underlying the SI reactions in those morphs will be found.

In light of the fact that the SI response in buckwheat thrum and pin pistils physiologically resembles the SI response in *Brassica* and Solanaceae, respectively, that similar biochemical processes underlay different SI systems (Miljuš-Djukić

et al., 2003) and that phylogenetically distant flowering plant species may share ancestral genes, we decided to investigate if the SI responses in two buckwheat morphs involve similar SI components as those identified in *Brassica* and *Prunus*.

For the homomorphic SSI system in *Brassica* and *S*-RNase based GSI in *Prunus*, most of the SI-involved molecular components are well known. The SI reaction in *Brassica* includes *S*-locus receptor kinase (SRK), *S*-locus glycoprotein (SLG) and *S*-locus cysteine rich (SCR) protein (SP11 protein), with a myristoylated membrane bound kinase (MLPK) as SI signal transducer (Nasrallah et al., 1988; Stein et al., 1991; Goring et al., 1993; Nasrallah et al., 1994; Suzuki et al., 1999; Schopfer et al., 1999; Murase et al., 2004). In *Prunus* the SI response involves *S*-locus RNase (*S*-RNase) and *S*-locus F-box protein (SFB) (McClure et al., 1989; Lee et al., 1994; Ushijima et al., 2003). In contrast to the rapid SI response in *Brassica* with the immediate inhibition of self-pollen tube growth at the stigma surface, the *S*-RNase based GSI system allows self-pollen tube growth to 2/3 of the style's length before its termination.

In this paper we started with the identification of buckwheat SI-involved genes through a search for SI-involved orthologs. We investigated the presence of *SRK*, *SLG, SP11* and MLPK *Brassica* orthologs as well as *S-RNase* and *SFB Prunus* orthologs in the buckwheat genome, using PCR primers designed from conserved regions of *Brassica* and *Prunus* genes. Also, for additional *S*-RNase identification we separated the styles' protein extracts by IEF and stained IEF gel specifically for RNases. The implications of the results are discussed.

MATERIALS AND METHODS

Isolation of buckwheat genomic DNA

The fresh leaves of greenhouse-grown buckwheat (*Fagopyrum esculentum* Moench) were collected, frozen in liquid nitrogen, ground into a fine powder and used for genomic DNA isolation (DNeasy Plant Mini Kit, Qiagen).

PCR identification of target genes

Degenerate forward and reverse primers were designed according to published sequences of *SRK*, *SLG, SP11* and MLPK genes in the genus *Brassica* (*http://www.ncbi.nlm.nih.gov*) and adjusted according to buckwheat codone usage. All primer sequences and annealing temperatures used for the search of *Brassica* orthologs are given in Table 1. The PCR conditions were: 1. 94°C 2 min; 2. 94°C 1 min, Tann 1 min, 72°C 1 min; repeated 34 times; 3. 72°C 10 min. The study of *Prunus* orthologs was conducted using specific primers and PCR conditions as stated in Banović et al., (2009).

The PCR mixture contained (Taq DNA polymerase kit, Qiagen); 1X PCR buffer; 2 uL solution Q; 1mM MgCl2; 0.2 mM dNTPs; 0.25 uM of each primer; 0.5 U Taq; 100 ng DNK.

The PCR product of the targetted MLPK gene was purified (PCR Purification Kit, Qiagen), ligated with deoxyadenine and cloned into the pGEM-T Easy Vector System (Promega). Plasmids with inserts of an appropriate size were selected by PCR amplification using MLPK specific primers and by restriction digestion with EcoRI and KpnI (New England BioLabs, UK).

The nucleotide sequence was obtained using an ABI3730XL DNA Analyzer (Applied Biosystems) (commercially done by Macrogen company).

Isoelectric focusing of stylar protein extracts and staining for RNase activity

Whole protein stylar extracts were prepared, separated by isoelectric focusing (IEF) and the IEF gel was stained for ribonuclease activity as described by Bošković and Tobutt (1996). The conditions for electrophoresis were as in Banović et al., (2009).

Computer-assisted analysis

The obtained partial MLPK nucleotide sequence published in this paper was deposited at the NCBI

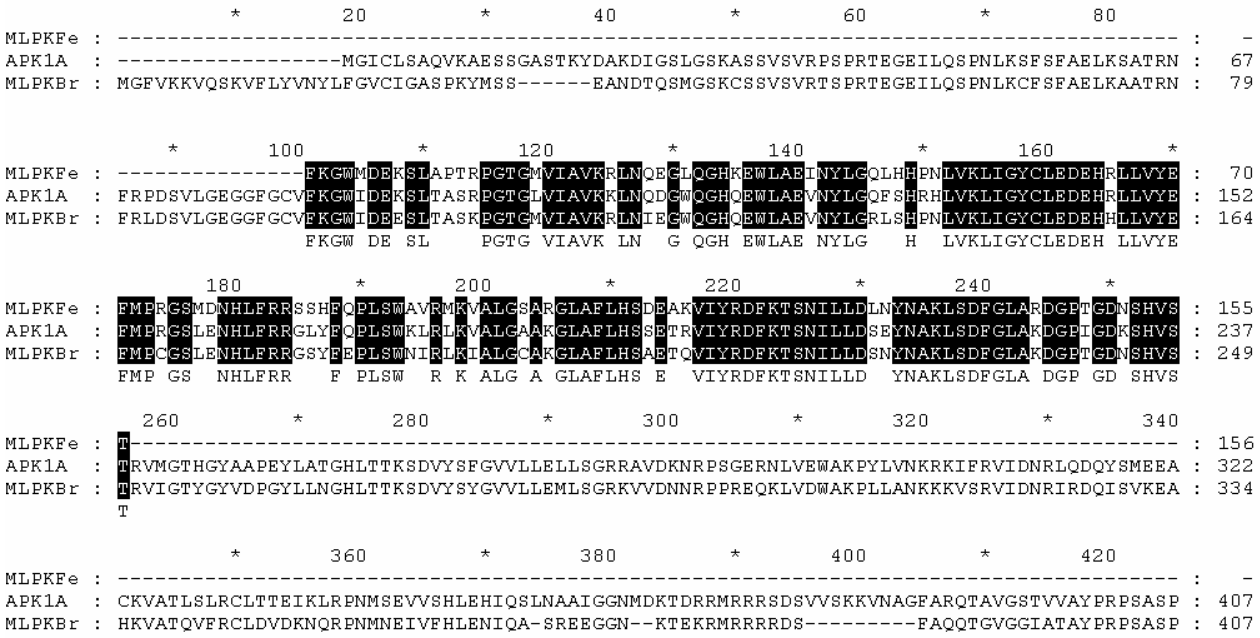

Figure 1.

data base under accession number FJ858190. The deduced amino acid sequence was compared to other protein sequences in the NCBI data base using the BLASTP search program (www.ncbi.nlm.nih.gov Web server).

RESULTS AND DISCUSSION

A search for *Brassica* and *Prunus* SI-involved orthologs in buckwheat genome gave further results. We did not find evidence for *SRK*, *SLG* or *SP11 Brassica* orthologs in the buckwheat genome - all PCR amplifications using gene specific primers (Table 1) were without amplification product. However, PCR amplification using MLPK specific primers (Table 1) gave a specific amplification product.

We obtained partial a *MLPK* nucleotide sequence that is 728 nucleotides long, contains 4 exons and corresponds to the kinase region of MLPK. It shows a high similarity at the amino acid level to the protein kinases of other plant species ranging from 81% (*Trifolium pratense*) to 89% (*Populus trichocarpa*).

MLPKs being orthologs and sharing a general function in the mediation of signaling processes does not exclude the possibility that those signaling processes may be involved in a spectrum of different roles, only one of which is SI response. With respect to the similar manifestation of the SI response in the thrum pistils of buckwheat and the SI response in *Brassica*, there is a reasonable possibility that buckwheat MLPK may be involved in SI response as well.

Further, buckwheat's partial MLPK sequence showed a 80% similarity to the MLPKf2 of *Brassica rapa* (AB121973) and a 80% similarity to the APK1A of *Arabidopsis thaliana* (AT1G07570) at the amino acid level (Figure 1). According to Murase et al., (2004) molecular mechanisms that produce two alternative MLPK (or APK1) transcripts and regulate their expression patterns are conserved in the genera of *Brassica* and *Arabidopsis*. It is probable that these mechanisms are conserved in other plant species sharing SI-involved MLPK orthologs. Therefore, in the forthcoming period we are going to obtain a full

MLPK sequence and if present in isoforms, to investigate their expression pattern. The next step will be to deduce the possible involvement of MLPK in the SI cascade underlying incompatible pollinations in the thrum flower morph of buckwheat.

Regarding the *Prunus* orthologs *S-RNase* and *SFB*, we find no evidence of their presence in the buckwheat genome. PCR amplifications using gene specific primers gave no amplification product. Also, IEF protein separation of the buckwheat style protein extracts specifically stained for RNases revealed no presence of basic *S*-RNases in either of the unpollinated buckwheat styles or in the self and non-self pollinated styles of both morphs. Therefore, in the buckwheat pin morph flower that shares a similar SI response physiology with *Prunus*, the SI response is not based on *S*-RNases and SFB. It remains to uncover the molecules that are SI-involved in the pin morph as well as the thrum, through *S*-locus mapping and 2D-PAGE style protein extracts' separation and identification.

Collecting buckwheat heteromorphic SSI molecular data will contribute to the data depository of components included in plant SI systems which is necessary to elucidate the evolution and preservation of SI systems in angiosperms. Moreover, as buckwheat is an important nutrition crop, it will provide knowledge to eventually gain genetic control over crosses in buckwheat and obtain lines and hybrids with desirable nutritional and/or physiological characteristics.

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НОВА ГЕНСКА СЕКВЕНЦА ИДЕНТИФИКОВАНА КОД ХЕЉДЕ: MLPK СА МОГУЋОМ УЛОГОМ У SSI ОДГОВОРУ

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Код биљака цветница постоје генетички одређени системи селф-инкомпатибилности (SI), који спречавају самоопрашивање и укрштање у сродству одржавајући генетичку разноврсност врста. SI се јавља у два облика, као гаметофитна и спорофитна SI, које се разликују у начину одређивања SI фенотипа полена - код GSI је SI фенотип полена одређен поленовим сопственим хаплоидним геномом, док је код SSI одређен диполидним генотипом мајке биљке.

SSI се јавља као хомоморфна (један тип цвета у биљака једне врсте) и хетероморфна (два или три типа цвета у биљака једне врсте). Хетероморфна SSI је у поређењу са хомоморфном SSI и GSI изузетно мало проучена и за сада је упознавање на молекуларном нивоу тек започело.

Код хељде је присутна дистилна хетероморфна SSI, о којој је сакупљено доста података на физиолошком нивоу, али о којој за сада нема молекуларних података. На основу физиолошке сличности SI одговора биљака родова *Brassica* и *Prunus* са трам и пин морфом хељде, респективно, затим на основу тога што постоје докази да слични биохемијски механизми леже у основи различитих SI одговора и на основу тога што и еволутивно удаљене SI врсте могу поседовати исте или сличне предачке SI гене, ми смо одлучили да испитамо присуство ортологих гена укључених у SI одговоре *Brassica* и *Prunus* у геному хељде.

Употребом изрођених прајмера дизајнираних на основу еволутивно очуваних региона *SRK*, *SLG*, *SP11* и *MLPK* секвенци *Brassica rapa*, као и S-*RNaza* и *SFB* гена рода *Prunus,* доступних у NCBI бази података, испитано је присуство ортолога ових гена у геному хељде. Такође је присуство *S-RNaza* испитано у протеинским изолатима неопрашених и компатибилно и инкомпатибилно опрашених тучкова хељде оба морфа.

Резултати су показали да нема ортолога *SRK*, *SLG*, *SP11*, као ни *S-RNaza* и *SFB* у геному хељде, али да постоји *MLPK* ортолог код хељде. Изведена аминокиселинска секвенца показала је 80 % сличности са *MLPKf2* секвенцом *Brassica rapa* и APK1A *Arabidopsis thaliana*, потврђујући да су у питању ортолози који би могли да имају и сличну улогу. Наш следећи корак је добијање целе нуклеотидне секвенце *MLPK* хељде уз испитивање постојања алтернативних места искрајања и одређивање нивоа експресије по ткивима, као и испитивање могуће улоге у SI одговору хељде.

Ови одговори омогућиће боље упознавање хетероморфних SSI система који су још увек у својој најранијој фази истраживања и обезбедиће податке нужне за увид у еволуцију SI система биљака цветница. Најзад, расветљавањем SSI система хељде, која се користи у исхрани, биће могуће генетички контролисати укрштање хељде и добијање линија са жељеним хранљивим и/или физиолошким особинама.