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, gametophytic (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

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 distyloous 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
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 targeted MLPK gene was purified (PCR Purification Kit, Qiagen), ligated with deoxadenine 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-aided analysis**

The obtained partial MLPK nucleotide sequence published in this paper was deposited at the NCBI.
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 MLPK2 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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**REFERENCES**


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 две 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 диплидним 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 молекуарном 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 молекулярних 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 узгађање SI врсте могу поседовати исте или сличне предаче SI гене, ми смо одлучили да испитамо присуство ортолога ових гене 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 ортолого ових генов 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 и 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 ортологи који би могли да имају и сличну улогу. Наш следећи корак је добијање цело нуклеотидне секвенце MLPK хељде уз испитивање постојања альтернативних места искрлања и одређивање нивоа експресије по ткима, 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 SSI система биљака цветница. Најзад, расветљавањем SSI система хељде, која се користи у исхране, биће могуће генетички контролисати укрштање хељде и добијање линија са жељеним хранљивим и/или физиолошким особинама.