Identification of *S*-genotypes in almond progenies by NEPHGE and PCR

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Abstract. Almond *(P. amygdalus* Batsch) shows a gametophytic self-incompatibility system controlled by a multiallelic locus, known as the locus *S*. Self-compatibility has been related to *Sf* allele presence and this trait has become a priority in the main almond breeding programs and the search for new cultivars is focused on the evaluation of desirable traits in autogamous seedlings. Traditionally, self-compatibility has been assessed in almond by laborious and time consuming methods, such as determination of fruit set in bagged branches or microscopic observation of pollen tube growth after self-pollination in laboratory conditions. Recently, molecular methods have been developed to assess the *S*-genotype, such as the identification of stylar *S*-RNases by NEPHGE (non equilibrium pH gradient electro focusing) and the use of conserved and specific PCR primers for the amplification of fragments from the different *S* alleles. In this research we applied these molecular methods for the *S*-genotype assessment in two almond breeding progenies, from the crosses of the self-compatible elite selection 'G-2-25' ($S_{11}S_f$) of the CITA, as female parent, with two self-incompatible cultivars, 'Desmayo Largueta' (S_1S_{25}) and 'Marcona' ($S_{11}S_{12}$), as male parents. Although no discrepancies were found between the two methods, PCR was more suitable than NEPHGE for *S*-genotype assessment. PCR is easier to optimize, cheaper, more precise and reliable. It is also possible to assess the genotype sooner than with NEPHGE, as flowers are not required for the determination, allowing an earlier elimination of the seedlings. In the 'G-2-25' ($S_{11}S_f$) x 'Marcona' ($S_{11}S_{12}$) progeny, the ratio of genotypes was 42% $S_{11}S_{12}$ and 58% S_fS_{12}, approaching the Mendelian laws of transmission in spite of the slightly higher proportion of self-compatible seedlings. In the other family, 'G-2-25' ($S_{11}S_f$) x 'Desmayo Largueta' (S_1S_{25}), four *S*-genotypes are possible, but the ratios obtained were 21% S_1S_{11}, 53% S_1S_f, 0% $S_{11}S_{25}$, and 26% S_fS_{25}. These results showed that pollen carrying the S_{25} allele had only a 26% fertilization success as compared to 73 of pollen carrying the S_1 allele. The absence of $S_{11}S_{25}$ seedlings may imply the expression of a homozygous lethal trait in these zygotes and the distortion of the Mendelian ratios.

Keywords. Self-compatibility – PCR Method – NEPHGE Method.

Identification de génotypes-S chez les descendances d'amandiers par les méthodes NEPHGE et PCR

Résumé. *L'amandier (*P. amygdalus *Batsch) montre un système d'auto-incompatibilité gamétophytique contrôlé par un locus multiallélique, connu comme locus* S*. L'auto-compatibilité a été reliée à la présence de l'allèle* Sf *et cette caractéristique est devenue une priorité pour les principaux programmes d'amélioration de l'amandier, et ainsi la recherche de nouveaux cultivars se focalise sur l'évaluation des caractères désirables pour les plants autogames. Traditionnellement, l'auto-compatibilité a été évaluée chez les amandiers par des méthodes laborieuses et prenant beaucoup de temps, telles que la détermination de la nouaison pour les branches ensachées ou l'observation microscopique de la croissance des tubes de pollen après auto-pollinisation en conditions de laboratoire. Récemment, des méthodes moléculaires ont été développées pour évaluer le génotype-*S*, telles que l'identification de* S*-RNases stylaires par NEPHGE (non equilibrium pH gradient electro-focusing) et l'utilisation d'amorces PCR conservées et spécifiques pour l'amplification de fragments provenant de différents allèles-*S*. Pour cette recherche nous avons appliqué ces méthodes moléculaires pour l'évaluation du génotype-*S *chez les descendances améliorées de deux amandiers, à partir des croisements d'une sélection d'élite auto-compatible 'G-2-25' (*$S_{11}S_f$*) du CITA, comme parent femelle, avec deux cultivars auto-incompatibles, 'Desmayo Largueta' (*S_1S_{25}*) et 'Marcona' (*$S_{11}S_{12}$*), comme parents mâles. Bien que l'on n'ait pas trouvé de divergences entre les deux méthodes, la PCR était plus adéquate que NEPHGE pour l'évaluation du génotype-S. La PCR est plus facile à optimiser, moins onéreuse, plus précise et fiable. Il est également possible d'évaluer le génotype plus tôt qu'avec NEPHGE, étant donné qu'il n'est pas nécessaire d'obtenir des fleurs pour la détermination, permettant ainsi une*

élimination précoce des plants. Dans la descendance de 'G-2-25' ($S_{11}S_f$*) 'Marcona'* ($S_{11}S_{12}$*), le quotient des génotypes était de 42%* $S_{11}S_{12}$ *et de 58%* S_fS_{12}*, se rapprochant des lois mendéliennes de transmission malgré la proportion légèrement plus élevée de plants auto-compatibles. Pour l'autre famille, 'G-2-25'* ($S_{11}S_f$*) 'Desmayo Largueta'* (S_1S_{25}*), quatre génotypes-*S *sont possibles, mais les quotients obtenus étaient de 21%* S_1S_{11}*, 53%* S_1S_f*, 0%* $S_{11}S_{25}$*, et 26%* S_fS_{25}*. Ces résultats montrent que le pollen portant l'allèle* S_{25} *avait une réussite de fertilisation d'uniquement 26% comparée à 73 pour le pollen portant l'allèle* S_1*. L'absence de plants* $S_{11}S_{25}$ *pourrait faire penser à l'expression d'un caractère létal homozygote chez ces zygotes et à la distortion des quotients mendéliens.*

Mots-clés. *Auto-compatibilité – Méthode PCR – Méthode NEPHGE.*

I – Introduction

Almond (*Prunus amygdalus batch* syn. *Prunus duclis* [Mill] D.A. Webb) is one of the most important nut trees in the world. Main traditional almond cuctivars are self-incompatible, However, there are some self compatible such as 'Touno', 'Supernova', 'Ginco', etc. (Raynaud and Grassely, 1985).

Self-incompatibility in almond is gametophytic and controlled by a single *S*-locus with multiple codominant alleles (Socias i Company *et al.*, 1976).

This trait is expressed in the style by special glycoproteins (*S*-RNases) that arrest the growth of pollen tubes in self-incompatible cultivars (Socias i Company *et al.,* 1976; Boskovic *et al.,* 1977).

Nowadays, using molecular methods is very important for identificafion of self-compatible and self-incompatible cultivars. In addition these methods are very reliable for determination of *S*-genoyypes in almond. So far, it has been was shown that there are about 27 self incompatible alles (S_1, S_2,...S_{27}) and one self- compatible allel (S_f).

Analysis of style ribonucleases (NEPHGE) and PCR methods are two important molecular methods to assess self compatibility in almond. The purpose of this research was the determination of *S*-genotypes in almond progenies, by using PCR and NEPHGE methods.

II – Material and methods

1. Plant material

Thirty one almond progenies obtained from crossing of 'G-2-25' as a self-compatible cultivar with 'Desmayo Larqueta' and 'Marcona', both as self-incompatible cultivars, were studied.

'G-2-25' is one of the most late blooming cultivars in Spain, as well as 'Desmayo Larqueta' and 'Marcona' which are commercial early blooming spanish cultivars.

2. PCR method

We used several specific and conserved primers for identification of *S*-alleles in the almond progenies as follows:

(i) *S_fF-S_fR* (Forward, GTGCCCTATCTAATTTGTTGAC) and (Reverse, GACATTTTTTTAGAAAGAGTG3) (Channuhtapipat *et al.,* 2003).

(ii) *conF-conR* (Forward,GTGCAACAATGGCCACCGAC) and (Reverse,TACCACTTCATGTAACAACTGAG) (Channuhtapipat *et al., 2001,* Tamura *et al.,* 2000).

(iii) *ASIII-AmyCSR*(Forward,TATTTTCAATTTGTGCAACAATGG) and Reverse,CAAAATACCACTTCATGTAACAAC) (Gómez and López, 2004).

3. PCR condition

Genomic DNA was extracted from 1 gr fresh leaves according to Gepts and Clegg method (1989). Long genomic DNA parts were amplified in a 25 µl reaction containing: Mmol (tris-HCl, pH=8), 100 Mm of each *dNTPs*, 1.5 mM $MgCl_2$, 0.25 mM of each primers and 5 unit of *Taq DNA* polymerase. PCR program for *conF-conR* and S_fF-S_fR was an initial denaturation time of 3 min. at 94°C, followed by 34 cycles of 30 sec. denaturation at 95°C, 45sec annealing at 55°C and 1 min at 72°C with a final extention step of 10 min at 72°C. Cycling program for the *ASIII* and *AmyC5R* were: 5 min initial denaturation at 94°C, 30 cycles of 1 min denaturation at 94, 1 min of annealing at 53°C and 2 min of extension at 72°C, and then a 5 min final extention.

4. Gel electrophoresis

PCR product were run in 2% normal agaros with TAE Buffer at 100 V for 90 min, then gel stained by ethidoum bromid (1mg ml^{-1}) and size of PCR products were determined with 1,2, 3 bp ladder.

NEPHGE (Non equilibrum pH gradient Elecro focusing): in this method, first of all, 120 styles were collected for each progenies at D stage (Boskovic *et al*., 1997) , were put in 4 small tubes (1.5 ml Ependorf) 30 samples per each and stored at -80°C until next step (Boskovic *et al*., 1997).

Extracted proteins from styles were separated electrophoretically on poly-acrylamide gel with 10 and 12% concentrations. It was run for 20 min at l00 V, 1 h at 150 V, 1 h at 100 V and 3 h at 400 V.

Later, gel were stained by toluiden blue for about 20 min. It was followed by immersing gel in distillated water for 12 h. Finally zymograms were compared (Boskovic *et al*., 1997).

III – Results and discussion

Results showed that both PCR and NEPHGE methods are suitable for identificatlon of *S*-genotypes in almond. However, PCR method is easier. Regarding the use of several primers, we could identify all self-compatible genotypes from self-incompatible genotypes by using S_fF-S_fR primer, which produced 459 bp band for S_f allel. *conF- conR* primer could identify S_{ll} and S_1 alleles, whearas *ASIII* and *AmyC5R* could amplifiy S_1, S_{11} and S_{12} alleles. However *conF-conR* and *ASIII* and *AmyC5R* could amplificate S_f alleles, but S_1 and S_{25} alleles were only amplified with *ASIII* and *AmyC5R* primers. These results are in the same line as López *et al.* ,2004. Results obtained also showed that *conF-conR* could not amplify S_{25} allele but it amplified S_1 allele.

The results obtained from NEPHGE method showed that this method needs more time compared to PCR. This method is more difficult and more expensive. However, its precision and accuracy are very high.

The ratio of *S*-genotypes obtained from crossing between 'Marcona' and 'G-2-25' cultivars, were according to Mendelian law. However, it was not exactly true for the crossing between 'Desmayo Larqueta' and 'G-2-25' cultivars, in wich $S_{11}S_{12}$ genotype was absent (21% S_1S_{11}, 53% S_1S_f, 0% $S_{11}S_{25}$ and 26% $S_{25}S_f$).

These results showed that pollen carrying the S_{25} allele had only a 26% fertilization success as compared to 73% of pollen carrying the S_1 allele.

In addition, the absence of $S_{11}S_{25}$ seedlings may imply the expression of a homozygous lethal trait in these zygotes and the distotion of the Mendelian ratios.

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