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Gene Expression and Sporophytic Self-Incompatibility in Hazelnut

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Abstract

The European hazelnut (*Corylus avellana* L.) is a monoeciuos tree and exhibits sporophytic self-incompatibility. Self-incompatibility is a genetic system that prevents the self-fertilization allowing the pistil to reject the pollen of genetically close individuals. Self-incompatibility is controlled by a single multi-allelic locus, the S locus.

Sporophytic self-incompatibility (SSI) has been reported in: Asteraceae, Betulaceae, Brassicaceae, Caryophillaceae, Convolvulaceae and Sterculiaceae. The involved molecular mechanisms are still partly unknown but in the Brassicaceae. Studies on gene regulation of fertility, pollination and fertilization in hazelnut are very few; so with this research we propose to contribute to the knowledge about the mechanism of sporophytic self-incompatibility in hazelnut.

The Differential Display technique was applied for the study of the female determinant of self-incompatibility. Two developmental stages of styles/stygmas were compared: before emergence from the bud and at full bloom. The results allowed to isolate partial sequences that showed an interesting homology degree with transmembrane serine-threonine kinase receptor of *Brassica oleracea.* Believing that the female determinant of self-incompatibility in hazelnut is very likely a membrane receptor, the efforts for getting differentially expressed sequences of this type were increased. Primers were designed on conserved regions of serine-threonine kinase receptors. Four differentially expressed fragments were isolated from stigmas at full bloom: after blasting in TIGR and NCBI databases, one was homologous to a gene for a kinase receptor, three were homologous to kinase proteins. The isolated sequences are being studied to check their expression in different tissues and style developmental stages.

INTRODUCTION

The genus *Corylus* is a member of the *Betulaceae* family of the order *Fagales* and includes several species, among which the European hazelnut (*Corylus avellana* L.) provides the most important cultivated varieties. *Corylus avellana* L. is a wind-pollinated, monoeciuos species that exhibits sporophytic self-incompatibility (Thompson, 1979; Me *et al.,* 1983; Zannini *et al.,* 1983; Me *et al.,* 2000). Cultivars thus need to be cross-pollinated by pollinizer trees planted in the orchard in order to ensure good nut set. In addition, the frequent cross-incompatibility between cultivars

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greatly reduces the choice of varieties that can be used as pollinators. This problem is increased by the fact that pollinators in orchards should have similar commercial value and the same ripening time of the main cultivar. Therefore, self-incompatibility in this species is an important aspect considered in breeding programs for orchard planting.

Self-incompatibility (SI) is a genetic system that promotes outbreeding, prevents the self-fertilization allowing the pistil to reject the pollen of genetically close individuals, maintaining, therefore, a high heterozigosity level.

Tipically in the sporophytic system, SI is controlled by a single multi-allelic genetic locus, the S locus (sterility locus); when an allele is shared between pollen and pistil, then an incompatible response will follow. At present, 28 S-alleles have been identified in hazelnut (Bassil *et al.*, 2001).

Sporophytic self-incompatibility (SSI), has been reported in the families: *Asteraceae, Betulaceae, Brassicaceae, Caryophillaceae, Convolvulaceae* and *Sterculiaceae*. The SI system found in the *Braassicaceae*, however, is currently the best understood mechanism with both the primary male and female determinants being characterized (Hiscock and McInnis 2003). The studies on this family can generate a possible model of sporophytic self-incompatibility for the study of other plant families.

The *Brassica* S Locus is constituted by three highly polymorphic genes, encoding for three proteins: SRK (*S locus receptor kinase*) encoding for a plasma membrane-anchored S receptor kinase responsible for the pollen recognition on the stigma; SLG (*S locus glycoprotein*) encoding for a glycoprotein synthesized in the stigma which seems to play an accessory role in the incompatibility system; SCR (*S locus cysteine-rich protein*) encoding for a small cysteine-rich protein expressed in anther determining the pollen phenotype (Takasaki *et al.*, 2000; Shiba *et al.*, 2001; Takayama and Isogai, 2003, Fobis-Loisy *et al.*, 2004, Okamoto *et al.*, 2004; Sato *et al.*, 2004).

Studies about gene regulation of fertility, pollination and fertilization are very few in hazelnut and thus the objective of this work was to investigate the mechanism of self-incompatibility in *Corylus avellana*. The Differential Display technique, already successfully used to isolate the male determinant of self-incompatibility in *Brassica* (Takayama *et al.*, 2000) was chosen to investigate the SI female determinant, to isolate sequences differentially expressed in hazelnut flowers buds at 2 stages of stigma development.

The basic principle of Differential Display technique is to amplify the cDNA obtained from mRNA extracted from 2 tissues/organs which are maintained in the same conditions except for one single factor, that is supposed to make a difference in term of gene expressions. The cDNA is amplified using an anchored oligo(dT) and primers of arbitrary sequence; the PCR yield hundred different amplicons that will subsequently be separated by electrophoresis.

MATERIALS AND METHODS

In this research, a comparison by Differential Display was carried out between two developmental stages of female flowers, i.e. before red-dot stage, collected in the month of December, and at full bloom (fully expanded styles, receptive but not pollinated stigmas), collected in the second half of February.

RNA was extracted using the protocol by Chang *et al.* (1993). cDNA was synthesized through a RT-PCR (*reverse transcriptase PCR*), using SuperScript^{TM} Reverse Transcriptase kit.

Differential Display was carried out using 2 strategies.

In a first stage of the work, two base anchored oligo(dT) primers ($(T)_{12}AA$, $(T)_{12}GA$, $(T)_{12}CA$, $(T)_{12}AG$, $(T)_{12}GG$, $(T)_{12}CG$ $(T)_{12}AC$, $(T)_{12}GC$, $(T)_{12}CC$) were used with 42 decamer primers (Kit Operon) for the amplification PCR-DDRT (Differential Display-Reverse Transcription)

In a second stage, believing that the female determinant of self-incompatibility in hazelnut is very likely a membrane receptor, the efforts for getting differentially expressed sequences of this type were increased. Therefore, 10 degenerate primers on conserved regions of serine-threonine kinase receptors were designed. These primers were used with two-base anchored oligo(dT) primers ($(T)_{12}$AA, $(T)_{12}$GA, $(T)_{12}$CA, $(T)_{12}$AG, $(T)_{12}$GG, $(T)_{12}$CG $(T)_{12}$AC, $(T)_{12}$GC, $(T)_{12}$CC) for the amplification PCR-DDRT.

For each amplification a repetition was carried out and for each primer combination a negative control was introduced.

The amplicons were, then, separated by electrophoresis on polyacrilamide gel (5%) and visualized by silver staining (Caetano-Annoles and Gresshoff 1994, with some modifications). Differentially expressed bands were extracted from gel and sequenced by capillary electrophoresis on ABI 3130 Genetic Analyzer (Applied Biosystems). Their sequences were aligned with sequences deposited in NCBI and TIGR database (http://www.ncbi.nlm.niih.gov; http://tigrblast.tigr.org/tgi).

RESULTS AND DISCUSSION

The Differential Display technique was chosen to investigate the female determinant of self-incompatibility. From the two different approaches, 80 bands differentially expressed were obtained, sequenced with success and aligned with sequences deposited in NCBI and TIGR database.

Based on results obtained by the alignments, the sequences were divided in four main groups, which represent the type of process in which they might be involved and their possible role.

1) sequences that could be involved in the mechanisms of self-incompatibility system, as membrane receptors, or in the signal transduction;
2) sequences involved in the flower development;
3) sequences involved in general metabolism, cell development, in nucleic acid synthesis, photosynthesis, or coding for transporters or transposons;
4) sequences coding for hypothetical proteins, whose function is still to be defined.

Among the sequences of the first group, the most significant for this research, 3 sequences isolated in mature stigmas were found to have high homology (75%, 65% and 61% respectively) with the sequences of kinase receptors. In particular, 1 fragment showed an interesting homology (61%) with a transmembrane serine-threonine kinase receptor of *Brassica oleracea*. This type of protein belongs to one of 12 subfamilies of kinase proteins: RLK (Receptor-like Kinases). In this sub-family, there are various kinase proteins, including RLK with S domain, similar to SRK of *Brassica*. In fact, SRK proteins have an extracellular domain similar to the S-locus glycoprotein (SLG) of *Brassica*, a transmembrane domain, and a serine-threonine kinase domain. In 1993 Stein and Nasrallah showed serine-threonine kinase activity of SRK_6, expressing the gene into *Escherichia coli*. Other 3 sequences, have been aligned with sequences of kinase proteins but their role is still to be defined and could be related to the transduction of the signal.

Three sequences expressed both in style/stigmas before red-dot stage and at full bloom, were aligned (72% of homology degree) with protein containing a thioredoxin domain and belonging to the thioredoxin family. In *Brassica,* was shown that H-thioredoxin proteins are negative regulators in the self-incompatibility system, preventing autophosphorylation of SRK (Mazzurco *et al.*, 2001) and therefore this homology could suggest a similar role in hazelnut.

In the second group of sequences, including those involved in flower development, one sequence, expressed in the style/stigma before the red-dot stage, was aligned with a MAF protein (MADS Affecting Flowering) of *Arabidopsis thaliana*. MAF genes are part of MADS box genes and are considered the main factor responsible for the vernalization response (Ratcliffe *et al.*, 2003). Another sequence was aligned with a MADs-box gene thought to be involved in the development and identity of floral organs, although its precise role is still under investigation.

CONCLUSION

The objective of this research was to contribute to the knowledge about the mechanism of sporophitic self-incompatibility in hazelnut. Since the self-incompatibility reaction takes place on the stigma surface, and following a release of a recognition factor, it is very likely the presence of a membrane receptor in the stigma, allowing the signal translation afterwards the contact with the incompatible pollen.

The isolated sequences are currently studied to obtain the cDNA full length by RACE (Rapid amplification of cDNA) technique and to verify their identity through the amplification of cDNA and by Northern blotting comparing the gene expression in different tissues and developmental stages.

Moreover, in order to facilitate the identification of the self-incompatibility protein, two dimensional electrophoresis analysis (2D-PAGE) will be used to recognize differentially expressed proteins in the stigma and in the pollen of hazelnut, following the work by Hiscock *et al.* (2003) and McInnis *et al.* (2005) carried out for *Asteraceae*.

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Literature cited

Bassil, N. and Azarenko, A.N. 2001. RAPD markers for self-incompatibility in *Corylus avellana.* Acta Horticulturae 556:537-543.

Caetano-Anollés, G. and Gresshoff, P.M. 1994. Staining Nucleic Acids with Silver: An Alternative to Radioisotopic and Fluorescent, Labeling Promega Notes Magazine N° 45:13.

Chang, S.J., Ruryear, J. and Gairney, J. 1993. A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11(2):113-116.

Hiscock, S.J. and McInnis, S.M. 2003. Pollen recognition and rejection during the sporophytic self-incompatibility response – Brassica and beyond. Trends Plant Sci. 8:606-613.

Hiscock, S.J., McInnis, S.M., Tabah, D.A., Henderson, C.A. and Brennan, A.C. 2003. Sporophytic self-incompatibility in *Senecio Squalidus* L. (Asteraceae) - the search for S. Journal of Experimental Botany, vol 54, n°380, Plant Reproductive Biology Special Issue, pp. 169-174, January 2003.

Fobis-Loisy, I., Miege, C. and Gaude, T. 2004. Molecular evolution of the S locus controlling mating in the Brassicaceae. Plant Biology 6(2): 109-118.

Mazzurco, M., Sulaman, W., Elina, H., Cock, J.M. and Goring, D.R. 2001. Further analysis of the interactions between the *Brassica* S receptor kinase and three interacting proteins (ARC1, THL1 and THL2) in the yeast two-hybrid system. Plant molecular biology 45:365-376.

McInnis, S.M., Costa, L.M., Gutiérrez-Marcos, J.F., Henderson, C.A. and Hiscock, S.J. 2005. Isolation and characterization of a polymorphic stigma-specific class III peroxidase gene from *Senecio Squalidus* L. (Asteraceae). Plant Molecular Biology 57:659-677.

Me, G. and Radicati, L. 1983. Studies on pollen incompatibility in some Filbert (*Corylus avellana* L.) cvs and selections. In 'Pollen: Biology and Implications for Plant Breeding' Mulcahy and Ottaviano Eds. Elsevier Biomedical, New York, Amsterdam, Oxford:237-242.

Me, G., Radicati, L., Vallania, R., Miaja, M.L., Valentini, N. and Cancheri, G. 2000. Research on the genetics of incompatibility in *Corylus* – Proc. Eucarpia Symp. on Fruit Breed and Genetics Acta Hort. ISHS 538:477-481.

Okamoto, S., Sato, Y., Sakamoto, K. and Nishio, T. 2004. Distribution of similar self-incompatibility (S) haplotypes in different genera, Raphanus and Brassica. Sexual Plant Reproduction 17(1): 33-39.

Ratcliffe, O.J., Kumimoto, R.W., Wong, B.J. and Riechmann, J.L. 2003. Analysis of the Arabidopsis MADS AFFECTING FLOWERING Gene family: MAF2 Prevent vernalization by short periods of cold. The Plant Cell 15:1159-1169

Sato, Y., Okamoto, S. and Nishio, T. 2004. Diversification and alteration of recognition specificity of the pollen ligand SP11/SCR in self-incompatibility of Brassica and Raphanus. Plant Cell 16(12):3230-3241

Shiba, H., Takayama, S., Iwano, M., Shimosato, H., Funato, M., Nakagawa, T., Che, F., Suzuki, G., Watanabe, M.,. Hinata, K. and Igosai, A. 2001. A pollen Coat Protein, SP11/SCR, determines the pollen S-specificity in the self-incompatibility of Brassica species . Plant Physiology 125:2095-2103.

Stein, J.C.and Nasrallah, J. 1993. A plant receptor-like gene, the S-locus receptor kinase of *Brassica oleracea* L., encodes a functional serine/threonine kinase. Plant Physiol. 101:1103-1106.

Takasaki, T., Hatakeyama, K., Suzuki, G., Watanabe, M., Isogai, A. and Hinata, K. 2000. The S-receptor kinase determines self incompatibility in Brassica stigma. Nature 403:913-916.

Takayama, S., Shibo, H., Iwano, M., Shimosato, H., Che, F., Kai, N., Watanabe, M., Suzuki, G., Hinata, K. and Isogai, A. 2000. The pollen determinant of self-incompatibility in *Brassica campestris*. Proc. Natl. Acac. Sci. 1920-1925.

Takayama, S.and Isogai, A. 2003. Molecular mechanism of self-recognition in Brassica self- incompatibility. Journal of Exp. Botany 54(380):149-156.

Thompson, M. 1979. Incompatibility alleles in *Corylus avellana* L. cultivars. Theor. Appl. Genet. 55:29-33.

Zannini, P., Me, G., Radicati, L., Sacerdote, S. and Vallania, R. 1983. Recenti acquisizioni sulla compatibilità gamica fra le cultivar di nocciolo e loro riflessi in rapporto alla scelta degli impollinatori – Atti del Convegno Internazionale sul nocciolo Avellino (Italia) 22-24 settembre pp.313-319**.**