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Comparative proteomics of pistils and anthers from self-incompatible and self-compatible almonds by iTRAQ and 2D-nano-LC ESI-MSMS

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Abstract. It is known that the cultivated almond [*Prunus dulcis* (Miller) D.A. Webb] exhibits gametophytic self-incompatibility controlled by the *S* locus. This locus contains two genes that codify for the S-RNase and SFB proteins, which seem to determine the specificity of incompatibility. However, recent studies support the involvement of other unidentified proteins in the incompatibility system of this species. A few proteomic studies have tried to identify candidates to modifier factors of the incompatibility system in *Prunus* sp. using comparative proteomics of pollinated pistils. However, there are no studies in which pollen and un-pollinated pistils from self-incompatible and self-compatible individuals are compared. To identify proteins differentially expressed in the pistils and anthers of almonds with an identical *S*-haplotype but different incompatibility phenotypes, iTRAQ and 2D-nano-LC ESI-MSMS were carried out. Seventeen and 23 proteins were identified as differentially expressed in anthers and pistils, respectively. Most of these proteins had a metabolic or stress resistance and defence function, and some of them had been associated to pollen development, pollen dynamics or to pollen-pistil interactions. These results provide proteomic profiles of differential expression in mature pistils and pollen, and could also serve as a reference for other comparative proteomic studies in almond and in other species with the same incompatibility system.

Keywords. *Prunus dulcis* – Self-incompatibility – iTRAQ – 2D-nano-LC ESI-MSMS.

Protéomique comparative des pistils et anthères d'amandes auto-incompatibles et auto-compatibles par iTRAQ et 2D-nano-LC-ESI MSMS

Résumé. *Il est connu que l'amande cultivé [*Prunus dulcis *(Miller) D.A. Webb] présente une auto-incompatibilité gamétophytique contrôlée par le locus S. Ce locus contient deux gènes qui codifient les protéines S-RNase et SFB, qui semblent déterminer la spécificité d'incompatibilité. Cependant, des récentes études soutiennent l'implication d'autres protéines non-identifiés dans le système d'incompatibilité de cette espèce. Certaines études protéomiques ont essayé d'identifier des candidats facteurs-modificateur du système d'incompatibilité dans* Prunus *sp. en utilisant la protéomique comparatives de pistils pollinisés. Cependant, il n'existe aucune étude dans laquelle le pollen et les pistils non pollinisés, appartenant à des auto-incompatibles et auto-compatibles, sont comparés. Pour identifier les protéines différentiellement exprimées dans les pistils et les anthères d'amandiers avec un S-haplotype identiques mais ayant différents phénotypes d'incompatibilité, iTRAQ et 2D-nano-LC-ESI MSMS ont été réalisées. Dix-sept et 23 protéines, exprimées de manière différentielle, ont été respectivement identifiés dans les anthères et les pistils. La plupart de ces protéines avaient une fonction métabolique ou de résistance au stress et un rôle de défense. Certaines d'entre elles avaient été associées au développement du pollen, de la dynamique de pollen ou aux interactions de pollen pistil. Ces résultats fournissent des profils protéomiques d'expression différentielle dans les pistils matures et le pollen, et pourraient également servir de référence pour d'autres études protéomiques comparatives pour l'amande et d'autres espèces avec le même système d'incompatibilité.*

Mots-clés. Prunus dulcis – *Auto-incompatibilité – iTRAQ – 2D-nano-LC ESI-MSMS.*

I – Introduction

The cultivated almond [*Prunus dulcis* (Miller) D.A. Webb] exhibits gametophytic self-incompatibility (GSI) controlled by the *S*-locus (Tao and Iezzoni, 2010). However, some almond cultivars from Apulia (Italy) were found to be self-compatible, which have been used in breeding programmes to obtain other self-compatible cultivars. Newly characterised almond accessions with the S_f haplotype, traditionally associated with self-compatibility, have been found to be phenotypically self-incompatible (Martínez-García *et al*., 2011). This finding supports the involvement of modifier factors coded outside the *S*-locus in the GSI system. Several studies intended to identify these factors in *Prunus* species using different variants of 2D electrophoresis after self-incompatible and self-compatible pollen-pistil interactions (Martínez-García *et al*., 2015). However, comparative proteomic analyses of pollen or un-pollinated pistils have not yet been reported in *Prunus*.

The aim of this work was to identify proteins differentially expressed in pistils and in anthers of self-compatible and self-incompatible almonds with the S_f-haplotype. To achieve this goal, the differential protein expression in mature anthers and pistils was analysed using iTRAQ and 2D-nano-LC ESI-MSMS.

II – Materials and methods

Flower buds from the almond selections A2-198 (homozygous self-compatible, S_fS_f), and ITAP-1 (heterozygous self-incompatible, $S_{11}S_f$) were collected at 'D-E' developmental stage. Pistils and anthers samples were taken from the buds and frozen separately at -80ºC.

Proteins were extracted from 0.5 g of pistils and anthers samples following the protocol described in Martínez-García *et al.* (2015) with some modifications. A total of 40 µg of protein from each condition was precipitated for digestion by the methanol/chloroform method. Digested pistil and anther samples were labelled with an iTRAQ Reagents Multi-plex kit, using a 2-plex design for each studied condition. A 2.5 µg aliquot of the resulting mixture was subjected to LC ESI-MSMS analysis using a nano liquid chromatography coupled to a high speed Triple TOF 5600 mass spectrometer with a duo spray ionization source. Mass spectrometry and MS/MS data obtained were processed using Analyst® TF 1.5.1 Software (AB SCIEX). Raw data file conversion tools generated mgf files which were also searched against the UniProtKB/SwissProt database from *Prunus persica* (taxon identifier: 3,760).

The confidence interval for protein identification was set to ≥ 95%, and only peptides with an individual ion score above the 5% False Discovery Rates (FDR) threshold were considered as correctly identified. Only proteins having at least two quantitated peptides were considered in the quantitation. Finally, the identity of each protein was assessed after a BLAST search in the UniProtKB/SwissProt database.

III – Results and discussion

A total of 1,667 proteins were identified in pistils and 1,391 in anthers by iTRAQ and mass spectrometry. Of these proteins, 945 in pistils and 844 in anthers had at least two quantified peptides and were thus considered in protein quantitation. Seventeen and 23 proteins could be identified as differentially expressed in anthers and pistils, respectively (Table 1). Two of the down-regulated proteins and three of the up-regulated proteins in pistils were found to be differentially expressed in the same way in anthers (Table 1).

Table 1. Differentially expressed proteins in the pistils and anthers of ITAP-1 (self-incompatible, SI) and of A2-198 (self-compatible, SC) almonds

Pistils			Anthers		
UniProt number	Protein name	Ratio SI/SC	UniProt number	Protein name	Ratio SI/SC
Down-regulated in A2-198					
M5W1Q5	Pathogenesis-related protein PR-4	6.67	M5VQU4	Glucan endo-1.3-beta-glucosidase	4.20
M5VQU4	Glucan endo-1,3-beta-glucosidase	2.15	M5X1U2	Annexin	3.11
M5WI14	Mitochondrial fission 1 protein	2.13	M5VGZ1	GDSL esterase/lipase	2.24
M5Y240	Polygalacturonase	2.07	I2BF37	Chalcone synthase (CHS)	2.21
M5XB01	Uncharacterised protein	2.04	M5W1Q5	Pathogenesis-related protein PR-4	1.92
M5XD05	DEAD box RNA helicase RH2a	1.93	M5X5T2	Quinone oxidoreductase	1.73
M5XNQ1	Ribulose bisphosphate carboxylase	1.89			
M5Y9J3	Glycine-rich RNA-binding protein	1.84			
M5WDV5	Uncharacterised protein	1.79			
M5XMY7	Glutathione S-Transferase	1.76			
Up-regulated in A2-198					
M5WT96	PR thaumatin-like protein	0.36	M5XDU4	(R)-mandelonitrile lyase 2	0.14
M5XJV8	Amine oxidase	0.38	M5XCQ5	Class IV chitinase	0.38
M5XDU4	(R)-mandelonitrile lyase 2	0.40	M5XJJ3	Enolase	0.39
M5WWB9	Epoxide hydrolase 3	0.47	M5WGH9	Plastid lipid-associated protein	0.43
M5W7R0	Uncharacterised protein	0.51	M5XF62	GTP-binding nuclear protein	0.46
M5Y9F2	Pleckstrin homology domain	0.53	M5WD64	Patatin	0.49
M5VPU2	Class V chitinase	0.53	M5WC10	40S Ribosomal protein S7	0.52
M5XLQ7	Periplasmic beta-glucosidase	0.54	M5XS55	Serine carboxypeptidase	0.54
M5WXM6	Uncharacterised protein	0.55	M5WGA1	Uncharacterised protein	0.56
M5XJJ3	Enolase	0.55	M5WRJ6	Pollen coat-like protein	0.57
M5W0M7	Histone H2B	0.57	M5XQ02	Uncharacterised protein	0.61
M5Y2Y6	Mandelonitrile glucosyltransferase	0.58	M5WX95	Uncharacterised protein	0.61
M5XIU5	Uncharacterised protein	0.58	M5X4I0	Malate dehydrogenase	0.61
M5W248	Endo-1.4-beta-glucanase	0.58	M5W0M7	Histone H2B	0.64
I1U4K7	Polyphenol oxidase II	0.59			
M5W266	Prunasin hydrolase	0.60			
M5WP60	Ribosomal protein L2	0.61			
M5VMV1	Putative p23 co-chaperone	0.61			

Functional classification of differentially expressed proteins according to their role in cellular pathways indicated that metabolism and stress resistance and defence were the predominant categories in both conditions. Similar results were obtained in Chalivendra *et al*. (2012), who indicated that lipid metabolism and cell-wall loosening and defence proteins are characteristic of pistil mature stages.

Among all the proteins indicated in Table 1, thaumatine-like protein, glucan endo-1,3-beta-glucosidase and glutathione S-transferase deserve a special mention because they have been linked to pollen-pistil interactions (Wang *et al*., 2014). In the case of proteins differentially expressed in anthers annexin, chalcone synthase, GDSL esterase/lipase and serine carboxypeptidase should be highlighted since they have been linked to pollen development, pollen dynamics or to pollen-pistil interactions (Taylor and Jorgensen, 1992; Dai *et al*., 2006; Updegraff *et al*., 2009).

These results provide proteomic profiles of differential expression in mature pistils and anthers, and could also serve as a reference for other comparative proteomic studies of pollinated pistils in almond and in other species with GSI.

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