

Identification and *in silico* Analysis of NADPH Oxidase Homologues Involved in Allergy from an Olive Pollen Transcriptome

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Abstract. Reactive oxygen species generated by pollen NADPH oxidases are present in numerous allergenic pollen species. The superoxide generated by this enzyme has been suggested as a key actor in the induction of allergic inflammation. However, this enzyme has been characterized in *Arabidopsis thaliana* pollen only, where two pollen-specific genes (*RbohH* and *RbohJ*) have been described. The olive (*Olea europaea* L.) pollen is an important source of allergy in Mediterranean countries. We have assembled and annotated an olive pollen transcriptome, which allowed us to determine the presence of at least two pollen-specific NADPH oxidase homologues. Primers were designed to distinguish between the two homologues, and full-length sequences were obtained through a PCR strategy. Complete *in silico* analysis of such sequences, including phylogeny, 3-D modeling of the N-terminus, and prediction of cellular localization and post-translational modifications was carried out with the purpose of shed light into the involvement of olive pollen-intrinsic NADPH oxidases in triggering allergy symptoms.

Keywords: allergy, NADPH oxidase, pollen, Rboh, ROS, superoxide.

1 Introduction

One of the most important causes of seasonal respiratory allergy in the Mediterranean area is olive pollen [1]. Allergens are proteins significantly expressed in pollen, many of them showing essential roles in pollen physiology. In the case of olive, twelve allergens have been identified and characterized to date [2].

Together with the classic view of the allergic inflammatory response triggered by pollen allergens, several studies have shown that pollen is also able to induce IgE-independent mast cell degranulation mediated by reactive oxygen species (ROS) [3].

Moreover, pollen-released soluble molecules such as superoxide, nitric oxide and nitrite seem to contribute to the allergic response [4-7].

NADPH oxidases (NOX or DUOX [dual NADPH oxidases] in animals, also called respiratory burst oxidase homologs [Rbohs] in plants [8]), are transmembrane enzymes which catalyze the generation of superoxide radical $O_2^{\bullet -}$ in the apoplast [9], leading to extracellular ROS increase [8]. This protein family shares 6 transmembrane central domains, two heme-binding sites, and a long cytoplasmic C-terminal owning FAD and NADPH binding domains. In addition, plant NOXs as well as animal NOX5 and DUOX possess Ca^{2+} -binding EF-hands motifs in the N-terminus [10]. In *Arabidopsis thaliana*, ten Rboh are encoded [11, 12], with two forms (RbohH and RbohJ) specifically expressed in pollen [13]. Rboh activity is essential for proper pollen tube growth to the female gametophyte, leading to fertilization [14]. Although pollen NOXs are expected to be located in the plasma membrane, a cytoplasmic localization has also been described [15].

NADPH oxidase enzymes are present in a wide number of allergenic pollens [4]. Superoxide generated by this enzyme activity locates in particular pollen compartments depending on the plant family: surface, cytoplasm, the outer wall or even in subpollen particles (SPPs) released during hydration [7]. These SPPs owning NADPH oxidase activity can reach the lower airways because their size is in the respirable scale [5] and this activity has been involved in the activation of dendritic cells leading to induction of adaptive immune responses [16].

Boldogh *et al.* [4] demonstrated that pollen intrinsic NADPH oxidase activity generates oxidative stress in the airway-lining fluid and epithelium, which in turns facilitates pollen antigen-induced allergic airway inflammation. The authors suggest that this ROS induction is independent of the adaptative immune response. Therefore, in the proposed '2-signal model', pollen NADPH oxidase activity promotes allergic inflammation induced by pollen antigens. Regarding allergic conjunctivitis, NOX-produced ROS from ragweed pollen were able to intensify the responsiveness of patients [17]. However, it has been recently suggested that this ROS-producing activity in pollen is not involved in either sensitization to pollen or in the allergic airway disease [18]. On the basis of the presence of contradictory results, further studies are necessary to determine how pollen intrinsic NADPH oxidase-produced ROS are acting in the allergic response.

An olive pollen transcriptome has been recently generated from cDNA by using a 454/Roche Titanium+ platform (Carmona *et al.*, in preparation). The annotated information is highly valuable to determine the presence of putative new allergens, to elucidate the mechanisms governing the allergy process, and the implication in the olive pollen metabolism through germination, stigma receptivity, and the interaction pollen-stigma. We have screened such transcriptome to identify the presence of Rbohs in the olive pollen, and used the retrieved sequences for further sequence confirmation and bioinformatic analysis.

2 Materials and Methods

2.1 Screening and Identification of NADPH Oxidase Transcripts in the Olive Transcriptome

Different strategies were defined to select such transcripts. Searches were performed by definition using GO, EC, KEGG and InterPro terms and codes, orthologues and gene names in the annotated transcriptome. BLAST searches were also performed using heterologous sequences available in public databases and well- established bibliography resources as TAIR and GenBank.

2.2 Olive Pollen *Rboh* Cloning and Sequencing

Olive pollen transcriptome retrieved sequences corresponding to *Rboh*-homologues (partial, internal sequences) were used to design primers in order to amplify the known sequence from pollen cDNA. Plant material was obtained according to [19]. Total RNA from mature pollen was extracted using the RNeasy Plant Total RNA kit (Quiagen, U.S.A.) and first-strand cDNA was synthesized with oligo(dT)19 primer and M-MLV reverse transcriptase (Fermentas). Standard PCR were carried out using Taq polymerase (Promega) and Pfu (Promega) to obtain or confirm nucleotides sequences. Full sequences were obtained by means of both 3'- and 5'-RACE (smarter RACE, Clontech), following manufacturer's specifications. pGEMT-easy (Promega) was used for cloning purposes. Sanger sequencing was achieved in the facilities of the EEZ-CSIC institute in Granada.

2.3 *In silico* Analysis of the Sequences

Nucleotide sequences were aligned using CLUSTAL OMEGA multiple alignment tool with default parameters [20]. Phylogenetic trees were constructed with the aid of the software Seaview [21] using the maximum likelihood (PhyML) method and implementing the most probable nucleotide substitution model (GTR) previously calculated by JmodelTest2 [22]. The branch support was estimated by bootstrap resampling with 100 replications.

For the prediction of protein cell localization, the software Plant-mPloc [23] was used. PhosPhAt [24] and PlantPhos [25] were used to predict serine, threonine and tyrosine phosphorylation. GPS-SNO 1.0 was used for prediction of S-nitrosylation sites [26]. TerminiNator was used to predict N-terminal methionine excision, acetylation, myristoylation or palmitoylation [27].

Structure prediction of OeNOX1 N-terminal region was modeled by using the fold recognition-based Phyre2 server [28], with c3a8rB [29] as the template and a 100% confidence. Ligand binding sites were predicted with the server 3DLigandSite [30].

3 Results

3.1 Retrieval and Alignment of NADPH Oxidase Sequences from the Olive Pollen Transcriptome

Transcriptome mining led us to pick seven entries identified as internal, partial sequences corresponding to NADPH oxidases (Table 1) by BLASTing each individual sequences against the GenBank database.

Table 1. Output sequences identified as NADPH oxidase after screening. The lengths of the assembled sequences are indicated.

Sequence No.	Sequence name	Lenght (bp)
1	input_mira_c5325	2032
2	input_mira_c12648	463
3	HVRC56C02JI550_RL3	459
4	input_mira_c8822_split_1	610
5	HVRC56C02GU1BZ_RL1	370
6	input_mira_c5061	643
7	input_mira_c8822_split_0	281

Figure 1 displays a simplified representation of a multiple sequence alignment between the transcripts obtained and well-characterized plant NOXs available at public databases. Such alignment, together with the phylogenetic analysis displayed in Fig. 2, led us to conclude that the 7 outputs identified in the annotated transcriptome belong to at least two different coding sequences, that we named OeNOX1 and OeNOX2.

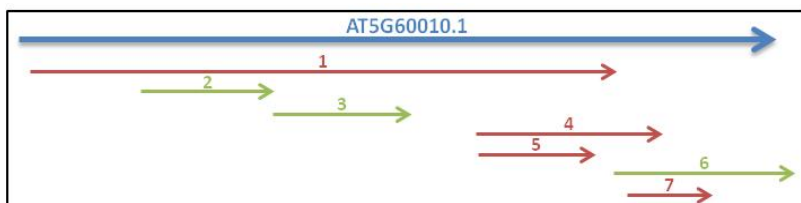


Fig. 1. Sequences alignment using *AT5G60010.1* (*Arabidopsis thaliana* pollen-specific *RbohH*) as the reference (blue arrow). Green arrows: OeNOX1; red arrows: OeNOX2.

3.2 Cloning of OeNOX1 and OeNOX2 and Phylogenetic Analysis

PCR amplification of cDNA enabled us to obtain the whole sequence of the coding region of OeNOX1 (2502bp) and a large proportion of the coding region of OeNOX2 (2094bp). The nucleotide sequences of both genes were subjected to a phylogenetic analysis including a representation of NADPH oxidases identified in different

taxonomical groups (Fig. 2). Different clusters are easily differentiated. Ancestral-type NOXs are presents in humans (NOX1-4) as well as in fungus (*Fusarium graminearum*: NOXA and NOXB) (yellow cluster). Animal NOX5 and DUOX1-2 share EF-hands motifs with plants NADPH oxidases, and all of them are considered calcium-dependents (green cluster). OeNOX1 and OeNOX2 proteins belong to a family of highly related proteins in plants (blue cluster) with ten orthologues in *Arabidopsis*. Olive pollen NADPH oxidases are gathered in a subgroup together with the three pollen specific NOXs identified up to now (AtRbohH and J from *Arabidopsis* and NtNOX from *Nicotiana*) (purple cluster) [31, 32].

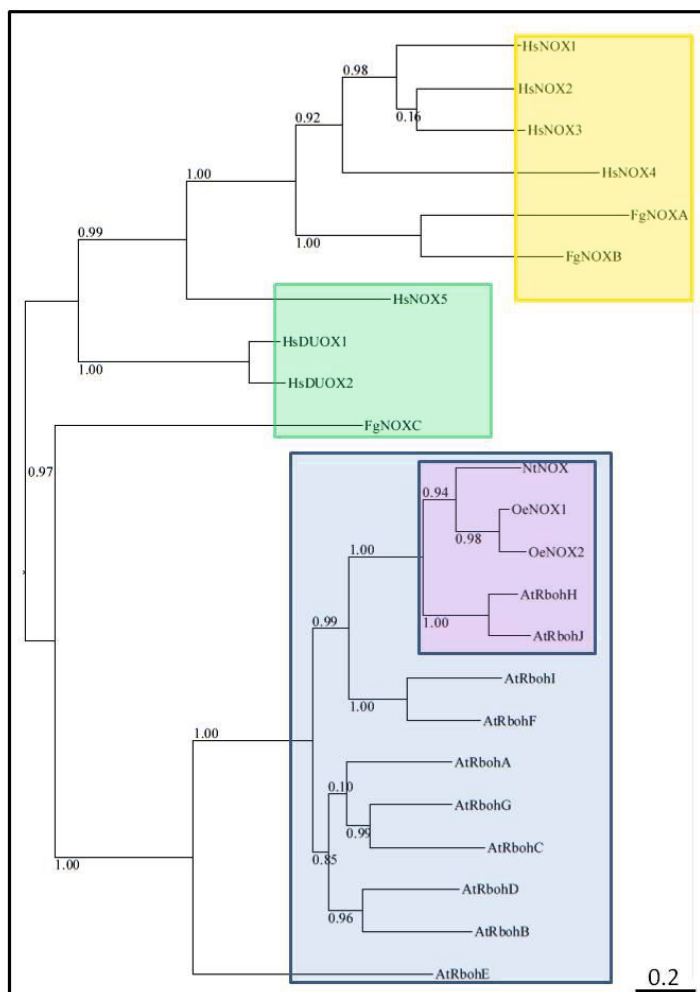


Fig. 2. Phylogenetic relationships between olive pollen NADPH oxidases OeNOX1 and 2 and their homologues in vegetative and reproductive tissues from *Arabidopsis* (AtRbohA-J) and other taxonomically distant NOX proteins.

3.3 *In silico* Prediction of Cellular Localization and Post-translational Modifications

Plant-mPloc software allowed to predict OeNOX1 and OeNOX2 cellular localization in plasma membrane, as it occurs with AtRbohH from *Arabidopsis*. Nevertheless, cytosolic localization was predicted for the *Arabidopsis* homologue AtRbohJ.

PhosPhAt and PlantPhos allowed us to predict 23 putative phosphorylation (Table 2). Potential S-nitrosylation sites were identified in 2 Cys-containing peptides by using the software GPS-SNO, as described in Table 3. Moreover, the N-terminus methionine of the mature protein was predicted to be acetylated with a 100% probability by using the prediction tools cited in material and methods. On the contrary, the presence of neither myristoylation nor palmitoylation sites was predicted.

Table 2. Amino acids prone to phosphorylation within the OeNOX1 sequence as predicted by PhosPhAt/PlantPhos

Serine	Threonine	Tyrosine
14	90	231
49	91	253
75	92	269
189	246	514
689	271	527
691	521	628
804	578	645
	625	736

Table 3. Cys-containing peptides prone to S-nitrosylation (B) within the OeNOX1 sequence as predicted by GPS-SNO

Position	Sequence
347	ALILLPVCRRTLTKL
815	KPLKQLCQELSLTS

3.4 3D Modeling of the Structure and *in silico* Analysis of the Function

The structure prediction served used confirmed that the corresponding translated amino acid sequences of OeNOX1 and OeNOX2 were consistent with available 3D models developed by using folding-recognition software. However such available models included partial structures only. N-terminus of OsNOX1/2 was modeled from Met73 to Leu241 with 100% confidence with the template corresponding to the (X-ray) structure of the N-terminal regulatory domain of a plant NADPH oxidase [29].

The most interesting feature was the presence of Calcium binding sites predicted at residues Asp181, Asn183, Asp185 and Met187, (Fig.3).

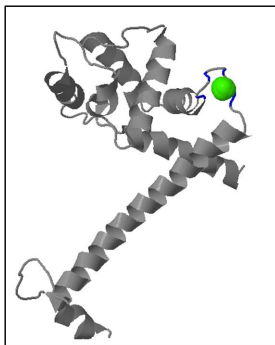


Fig. 3. 3D modeling of the N-terminus of OeNOX1/2. Predicted Ca-binding sites are displayed in blue and Calcium ion is displayed in green.

4 Discussion

In this paper we have carried out a bioinformatic screening of exome sequences of mature olive pollen. This allowed us to detect the existence of at least two *Rboh* genes, homologous to other pollen *Rboh* genes described previously in tobacco and *Arabidopsis*. Subsequent PCR and RACE experiments confirmed such expression in olive pollen and drove to the obtention of full sequence of one of the two genes, namely OeNOX1. The overall filtering of transcriptome outputs to identify the two NOX sequences obtained resulted to be a challenge due to the high homology between these two genes, which required not only a knowledge of bioinformatics tools but previous experience in these protein families. The phylogenetic analysis carried out clustered OeNOX1 and OeNOX2 within a pollen-specific NOXs subgroup, thus suggesting distinctive features for these *Rboh* homologous in pollen.

ROS production by *RbohH* and *RbohJ* is essential for proper pollen tube tip growth [14], acting as a speed control to dampen growth rate oscillations during polarized cell growth [15]. Furthermore, the activity of these enzymes has been demonstrated to be subjected to a complex regulation by multiple elicitors including Ca^{2+} , signaling phospholipids and Rac/Rop GTPases [33]. Although the key involvement of NADPH oxidases in olive pollen physiology has been determined as well [33], little is known as regard to the involvement of these enzymes in the development of allergy. Olive pollen generates superoxide which can be detected by using confocal microscopy in combination with the use of dihydroethidium [34]. Still many aspects have to be elucidated, for example putative release of sub-pollen particles from olive pollen upon hydration, superoxide dismutase inhibition of both pollen NOX activities, their presence in insoluble fractions which could facilitate the exposure of tissues to ROS generated by these enzymes etc. These many aspects may contribute to processes like sensitization and pathogenesis of allergic inflammation [7].

Concerning bioinformatic predictions of post-translational modifications, caution should be taken, considering the *in silico* nature of the outputs. However, some of the results agree with experimental data previously published in plants other than olive. This is the case of the highly conserved Cys815, which is a predicted candidate for S-nitrosylation. This residue was previously disclosed to be nitrosylated in *Arabidopsis thaliana*, leading to NADPH oxidase activity regulation during the immune response in plants [35]. Considering the proposed involvement of pollen-generated nitric oxide (NO) in the allergic response as well [6] we also suggest another possible interconnection of these events with pollen-induced allergy via NOX activity. Unfortunately, the available 3D templates did not enable us modeling the protein portion including such cysteine liable to be S-nitrosylated. Concerning other modifications, NADPH oxidases in plants have been previously described to be modified by phosphorylation, probably in a synergistic way with Ca^{2+} [36, 37]. OeNOX1 has been predicted to be acetylated at the N-terminus, as it occurs for almost 80% of eukaryotic proteins [27]. Finally, the expected plasma membrane localization reported is also in good agreement with determinations made by other authors [15].

Here we show for the first time the presence of two NADPH oxidase forms in a highly allergenic pollen like that of *Olea europaea*. Predictions of sequence, structure, location and post-translational modifications have been made in order to initiate a valuable characterization of this enzyme which help to further assess its implication in allergy.

5 Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

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