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Isolation of a gene encoding for a class III peroxidase in female flower of *Corylus avellana* L.

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Abstract
Hazelnut is a monoecius species characterized by mid-winter blooming and sporophytic incompatibility. The molecular mechanisms at the basis of the female flower development and of the pollen-stigma interaction are little known, although pollination in this species is a critical factor to ensure good yield. Differential Display technique was used to study genes expressed during the female flower development, comparing styles before emergence from the bud and styles at full bloom. The full-length cDNA clone, designated CavPrx (*Corylus avellana* peroxidase) and isolated in mature styles, was characterized as a sequence encoding for a 330 amino acids protein, containing all the conserved features of class III peroxidases. CavPrx resulted expressed only in styles, with a peak in mature styles pollinated with compatible pollen. Class III peroxidases are expressed in several different plant tissue types and are involved in a broad spectrum of physiological processes. Until now, four peroxidases expressed in the stigma were identified in *Arabidopsis thaliana* and *Senecio squalidus*: they were assumed to be possibly involved in pollen-pistil interaction, pollen tube penetration/growth and/or in defence against pathogens. CavPrx is the first gene for a floral peroxidase isolated in hazelnut and its expression pattern suggests a possible role in the pollination process.

Key words hazelnut · flower biology · CavPrx peroxidase · gene expression

Introduction

Peroxidases (PRXs) are heme-containing glycoproteins that may have roles in both the production and scavenging of reactive oxygen species (ROS). In fact they catalyze oxidoreduction between H_2O_2 and various organic and inorganic reducing compounds [1, 2]. Peroxidases have been isolated from a variety of plant, animal, fungal and bacterial sources.
Plant peroxidases belong to a superfamily, divided into three classes. Class I comprises intracellular peroxidases, found in many organisms, except animals, and are divided into three groups: ascorbate peroxidases, cytochrome *c* peroxidases and catalase peroxidases. They have a principal role in protection against excess H_2O_2. Class II contains extracellular peroxidases, secreted by fungi, such as lignin peroxidases and manganese peroxidases. They are involved in soil debris. Finally class III includes the large family of secreted peroxidases in plant [3].
Class III peroxidases are expressed in many types of plant tissues and are often targeted outside of the plant cell or to the vacuole, via the endoplasmic reticulum (ER). PRXs present a large number of isoform, suggesting their involvement in a broad spectrum of physiological processes [4]. They play different roles in metabolism and physiological processes, such as oxidative stress response, salt tolerance, lignification, suberization, auxin catabolism, defence against pathogen attack, cell wall metabolisms, developmentally related processes [1, 5] and recently are also considered as potentially important components of plant signal transduction pathways [6].
Class III PRXs constitute a large family of genes in plants: in fact 73, 138 and 101 peroxidase genes were found in *Arabidopsis thaliana* (L.) Heynh [5], *Oryza sativa* L. [2] and *Populus trichocarpa* Torr. & A. Gray (PeroxiBase, http://peroxibase.toulouse.inra.fr/organism.php), respectively.
Class III peroxidases generally do not have a high level of tissue specificity [7, 8], but in recent years plant peroxidases with organ/tissue specificity were identified [3, 9-11]. About flower specificity, a class III peroxidases was identified in *Gossypium hirsutum* L. (GhPrx37) as specific for male flower structures and pollen [12]. Other three peroxidases in Arabidopsis (AtPrx28, AtPrx39 and AtPrx58) are demonstrated to be specifically expressed in stigmas and not in other floral tissues/organs and to be involved during interaction between pollen and pistil [13, 14]. In *Senecio squalidus* L. a stigma-specific class III peroxidase (SspPrx01) was identified and it could be involved in defense against pathogens or in pollen-stigma signaling [6].
In flowering plants a correct communication and coordination between pollen (containing sperm) and pistil (containing ovule) is necessary to have successful pollination. Only in this case the pollen tube produced by the pollen grain can penetrate the stigmatic surface and correctly grow to reach the ovary [15, 16]. Moreover in many species the stigma is also capable to recognize between compatible pollen grains, which are able to lead to successful pollination, and incompatible pollen grains [17].
One of this case is the sporophytic incompatibility (SI), a mechanisms that prevents self- and genetically close individual- fertilization [18]. SI is controlled by a single multi-allelic locus, the S locus, comprising genes for the male and female determinants. These genes codify for proteins, expressed in the stigma surface (female determinant) and in the tapetum (sporophyte). The tapetal protein is deposited onto the exin of the pollen grains. When pollen interacts with a stigma bearing the protein expressed by the same S allele it is recognised as self and rejected [19]. This occurs without pollen tube penetration in the style, as it happens instead in the gametophytic incompatibility.
The identification of the genes involved in the pollen-pistil interaction, together with genes involved in structural flower development, is an important step to improve plant breeding techniques. Hazelnut is a monoecius species with a particular flower biology characterized by mid-winter blooming and SI. The molecular mechanisms at the basis of the female flower development and of the interaction between pollen and stigma are little known, although pollination in this species is a critical factor to ensure good yield. At present, only a limited number of ESTs (Expressed Sequence Tag) sequences of hazelnut are deposited in databases, the most part of them encoding for allergenic molecules.
In this study a class III peroxidase, predominantly expressed in mature styles after pollination with compatible pollen, was identified and fully characterised in hazelnut (*Corylus avellana* L.). The gene was selected among sequences rescued by Differential Display comparing gene expression in mature and immature styles with the aim of isolating genes involved in the development of the female inflorescence and in the SI mechanism [20]. This is the first class III peroxidase identified and described in hazelnut.

Materials and Methods

Plant material

Plant material was collected from hazelnut bushes of the cultivar 'Tonda Gentile delle Langhe' ('TGdL'). The plants were located in a hazelnut germplasm collection established by the University of Torino in Cravanzana (Cuneo province, Italy). *C. avellana* is a species blooming in mid-winter and the pistillate inflorescence consists

of cluster of 4 to 14 flowers born at the apex of a compound bud. Stigma in hazelnut is not a distinct structure from the style which becomes receptive soon after emergence from the bud.
For the Differential Display immature styles were collected in December, before the red-dot stage, when the styles were not exposed yet (Fig. 1). At the same time branches were emasculated and then covered with pollen-barrier bags, to prevent cross pollination. In February, at flowering time, mature fully expanded styles were collected from the bagged inflorescences (Fig. 1).
For testing gene expression in different tissues and organs by Real Time PCR, young leaves were collected in spring and young catkins were harvested at three different times: in mid-August (stage I), at the end of August (stage II) and in mid-September (stage III) when pollen grains complete their formation. Moreover, in December, branches bearing catkins from cultivars 'Culplà' and 'TGdL' were collected and let flower in greenhouse in order to collect compatible and incompatible pollens for 'TGdL' female flowers. These pollens were stored at -20°C and used in February to pollinate 'TGdL' flower buds. Pollinated styles were sampled after 4 hours from pollination. All sampled plant material was immediately deep-frozen and stored at -80°C.

Differential Display

The comparison by Differential Display was carried out between 2 developmental stages of styles: before red-dot stage (material collected in December) and at full bloom (fully expanded styles, receptive but not pollinated styles collected in February).
RNA from mature and immature styles was extracted using the protocol by Chang et al. [21] and was cleaned up with RNeasy Mini Protocol for RNA Cleanup (Qiagen, Hilden, Germany). Residual DNA was removed by a DNase digestion with RNase-Free DNase Set (Qiagen). cDNA was synthesized through a RT-PCR (reverse transcriptase PCR), using SuperScript™ Reverse Transcriptase (Invitrogen, Carlsbad, California, USA) in combination with one-base anchored oligo(dT) primers.
One-base anchored oligo(dT) primers $H(T)_{11}A$ was used with arbitrary primer H-AP34 (5'-AAGCTTCAGCAGC-3') for the Differential Display PCR (RNAimage kits, GenHunter Corporation, Nashville, Tennessee, USA).
The PCR was performed in a final volume of 20 µl containing 1x PCR buffer, 3 mM $MgCl_2$, 20 µM dNTPs, 0.2 µM primers, 1U of Taq polymerase (Bioline, Taunton, Massachusets, USA) and approximately 25 ng of template cDNA with the following steps: 40 cycles of 94°C for 30 s, 40°C for 2 min, 72°C for 30 s, followed by one final elongation step of 72°C for 7 min. Two PCR replications per thesis were carried out and a negative control was introduced, using H_2O instead of template.
Samples were mixed with a loading dye, containing formamide, bromophenol blue and xylene cyanol, then denaturated at 95°C for 10 min and chilled on ice.
Amplicons were separated by electrophoresis on polyacrilamide gel (5%) and stained with silver nitrate [22] (with modifications). Differentially expressed bands were extracted from the gel, according to the protocol by Basak et al. [23], and reamplified and cloned in *E. coli* (Subcloning Efficency™ DH5α™ Competent Cell, Invitrogen) after ligation in a plasmid vector (Kit Topo TA Cloning®, Invitrogen). Differentially expressed fragments were sequenced by capillary electrophoresis on 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA), using a ABI PRISM® BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems); their sequence was aligned with sequences deposited in NCBI database by BLAST program [24].

Isolation of the full-length cDNA

A specific primer pair (F-GSP: 5'-GCAGCAACCAGACGGCTTTCTTTGA-3'; R-GSP: 5'-CAGTCCGACCGAATCTCCCCGTTG-3') was designed on the sequence of the putative peroxidase. The primers were initially used to perform PCRs on cDNAs from leaves, catkins, mature and immature styles of 'TGdL' to check the accuracy of the product obtained and the expression profile in different tissues of the plant. The same cDNAs were amplified with actin gene-specific primers, as control. PCRs were performed in 20 µl containing 1x PCR buffer, 2.5 mM $MgCl_2$, 20 µM dNTPs, 0.05 µM of each primer, 1U of Taq polymerase (Bioline) and approximately 25 ng of template cDNA in a iCycler thermal cycler (Biorad, Hercules, California, USA) with the following steps: 1 cycle of 95°C for 3 min, 30 cycles of 95°C for 30 s, 65°C for 45 s, 72°C for 1 min 30 s, followed by one final elongation step of 72°C for 10 min.
R-GSP primer, designed as described in the SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, California, USA), was also used to perform the RACE (rapid amplification of cDNA ends) and obtain the full-length cDNA.
The elongated fragment was visualized on agarose gel and was excised and purified using a Nucleo Trap Gel Extraction Trial Kit (Clontech). It was cloned by a Topo TA Cloning® Kit (Invitrogen) and sequenced by capillary electrophoresis on 3130 Genetic Analyzer (Applied Biosystems).

Real time PCR

Total RNA was extracted from 2 g of the sampled plant materials (leaves, not pollinated mature and immature styles of 'TGdL', compatible and incompatible pollinated styles, three different stages of developmental catkins) using the protocol by Chang et al. [21]. In order to remove contaminant DNA from the RNA samples, the nucleic acid extract was treated with DNaseI (Fermentas, Burlington, Ontario, Canada), according to the manufacturer's instructions. Five μg of each RNA sample was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. Quantitative PCR was performed with StepOne Plus Real Time PCR system (Applied Biosystems) in a reaction volume of 20 μl containing 10 μl of SYBR Green Mix (Applied Biosystems), 0.6 μM primer and 2 μl of cDNA. The PCR cycle profile was as follows: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplification data were collected during the step at 60°C. Melt curve analyses were made by elevating the temperature from 60°C to 95°C at a rate of 0.3°C s^{-1}. Only a single peak with a characteristic melting point was observed for each sample, indicating that the product was specific to the primers.
Normalization was done on the basis of the expression of *VvUbiquitin1* [25] (F-Ubi: 5'-TCTGAGGCTTCGTGGTGGTA-3' and R-Ubi: 5'-AGGCGTGCATAACATTTGCG-3') and on the expression of *VvACT1* (Genoscope accession number: GSVIVT00034893001; F-Act1: 5'-GCCCCTCGTCTGTGACAATG-3' and R-Act1: 5'-CCTTGGCCGACCCACAATA-3'). The peroxidase-specific primers were designed based on the isolated sequence (F-CavPrx: 5'-CTCGAGGGTTTGACGTTGTTG-3' and R-CavPrx: 5'-GCTTCAGCAGCAAGGGCTAGA-3').
Relative expression of the peroxidase gene in the mature and pollinated styles, catkins and leaves was calculated by comparison with expression in the reference sample immature styles using a ΔCt method, after normalization with housekeeping genes *VvUbiquitin1* and *VvACT1*. Data represent the average of three technical replicates (±SD).
F-CavPrx and R-CavPrx primers, designed for Real Time PCR, were also used to perform a PCR reaction in 20 μl containing 1x PCR buffer, 2.5 mM $MgCl_2$, 20 μM dNTPs, 0.5 μM of each primer, 1U of Taq polymerase (Bioline) and approximately 300 ng of template cDNA. The PCR was performed in a iCycler thermal cycler (Biorad) with the following steps: 1 cycle of 95°C for 3 min, 30 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 1 min 30 s, followed by one final elongation step of 72°C for 10 min. The obtained fragment was directly sequenced by capillary electrophoresis on 3130 Genetic Analyzer (Applied Biosystems), in order to check the correct identity of the amplified product.

Characterization of introns

Two couples of primers (CavPrxF1:5'-AAGCAATGGGCTCTCCTACA-3'; CavPrx R1:5'-TGTGTGAGCACCCGATAAAG-3'; CavPrxF2:5'-ACCTCCATTCCCTCTCCTGT-3'; CavPrxR2:5'-CCTCCATTAACCTTCTTACAGTCC-3') were designed on the CavPrx expressed sequence and used for amplification on DNA extracted from mature styles of TGdL. DNA was extracted using the protocol by Thomas et al. [26] with some modifications. PCR reactions were performed in a total volume of 20 μl containing 1x PCR buffer, 2.5 mM $MgCl_2$, 20 μM dNTPs, 0.5 μM of each primer, 1U of Taq polymerase (Bioline) and approximately 50 ng of template DNA. Reactions were carried out with the following steps: 1 cycle of 95°C for 3 min, 30 cycles of 95°C for 30 s, 54°C for 45 s, 72°C for 1 min 30 s, followed by one final elongation step of 72°C for 10 min. Fragments were run on 1.2% agarose gel, excised, cloned and sequenced as described before.
Sequences obtained were aligned with the expressed sequence and analyzed by FgeneSH (http://mendel.cs.rhul.ac.uk/mendel.php?topic=fgen) to identify the introns.

Bioinformatics and phylogenetic analysis

The amino acid sequence of the isolated full-length cDNA was generated by software freely available at the ExPASy proteomics server of the Swiss Institute of Bioinformatics (http://www.expasy.org/). BLASTp in NCBI was used for identification of the isolated sequence.
Functional and conserved domains typical of class III peroxidases were predicted by ProSite [27] (http://www.expasy.ch/prosite/), while peptide signal was individuated by SignalP v3.0 [28] (http://www.cbs.dtu.dk/services/SignalP/).
A BLASTp in PeroxiBase (http://peroxibase.isb-sib.ch/index.php) [29] was used for the collection of phylogenetically related sequences. Sequences were aligned using MEGA version 3.1 [30]. Phylogenetic tree was generated with MEGA version 3.1, using default settings, except for bootstrap calculations that were set at 2000 resampling iterations.

Results

Isolation of a gene sequence predominantly expressed in mature styles

The Differential Display technique was chosen to investigate genes expressed in female flowers of hazelnut and related to the development of the reproductive organ.
cDNAs from immature and mature styles of hazelnut were analyzed.
Several fragments generated by Differential Display PCR resulted differentially expressed in mature styles compared with immature styles. One of the fragments predominantly expressed in the mature styles was identified as being from a putative peroxidase gene that was fully characterised. The original fragment was 260 bp long and was obtained by PCR with H-AP34 primer (RNAimage Kit 4, GenHunter Corporation) and the oligo(dT) primer H-T_{11}A. It showed a Poly A tail, with a T nucleotide upstream, as we expected using H-T_{11}A oligo(dT) primer.
After alignment with sequences deposited in NCBI database, the fragment showed a high level of identity (85%) and similarity (92%) with the gene AtPrx53 encoding for a class III peroxidase of *A. thaliana* expressed in flower tissues [31].

Isolation of CavPrx full-length cDNA

Specific forward and reverse primers (F-GSP and R-GSP) were designed on the fragment sequence and used to perform a PCR on cDNA in order to check the accuracy of the product and to have a preliminary expression profile. On agarose gel a single band of 104 bp was obtained, as predicted by primer design. It was absent in leaves and catkins, while it was present in both immature and mature styles. Yet, the band was more evident in the mature style than in the immature one, suggesting a different level of expression between the two stages.
In order to obtain the full-length cDNA, a 5'-RACE was performed on cDNA from mature styles. A single band of about 1100 bp was obtained, cloned and sequenced. Nucleotide sequence alignment of this product with the fragment obtained by Differential Display PCR showed 100% identity in the overlapping regions. The full-length cDNA obtained was translated in amino acid sequence and analyzed by BLASTp search; it revealed a high level of identity with sequences of class III peroxidises, including AtPrx53 (75% identity and 86% similarity). Consequently, the sequence was called CavPrx (*Corylus avellana* peroxidase).
CavPrx sequence when translated, revealed a putative 330 amino acid protein with typical characteristics of plant peroxidases. Protein sequence contained eight conserved cysteine residues (C35, C68, C73, C116, C122, C201, C233, C324), which yielded four disulfide bridges (C35-C116, C68-C73, C122-C324, C201-C233). It also contained active site residues (57-68: GAslIRLhFHDC), proximal heme-ligand signature (186-196: DLVALSGAHTF) and two conserved calcium binding sites (group1: D67, V70, G72, D74, S76; group2: T195, D246, T249, D254). S121, D124, G147 and R148 residuals, reported as being important to form a salt bridge motif in all peroxidases [32], resulted conserved in the sequence isolated. Other amino acid residues that are considered of importance for the integrity of protein structure were present (Fig. 2) [5]. All these characteristics are common to most class III peroxidases.
Analysis of CavPrx sequence with SignalP v3.0 software identified a putative 24 amino acid signal peptide (1-24: MGSPTSLAVATIFVAVIMLYESNA) cleaved between A24 and Q25 and located at the start of the mature protein (Fig. 2). The presence of the signal peptide suggested that CavPrx could be a secreted protein.
As well as HRPC (Horseradish peroxidase C), CavPrx revealed 13 alpha-helices (A:A38-S52; B:I56-C68; C:F102-S115; D:C122-S137; D':Q156-S162; E:L170-A179; F:T184-L190; F':I205-L208; F'':N222-Q231; G:G256-Q262; H:Q272-S276; I:V283-S291; J:Q293-M307), common to most of class III peroxidases in plants (Fig. 2).
To study the expression level of CavPrx in detail, a Real-Time PCR analysis was performed on 'TGdL' leaves, three different stages of developmental catkins, immature styles and mature styles not pollinated and pollinated with compatible or incompatible pollen. The expression level of CavPrx in mature styles was 3-fold more abundant than that of immature styles, while it was almost absent in leaves and catkins. Moreover the expression level was comparable in mature not pollinated styles and in styles pollinated with incompatible pollen, while it was significantly higher (almost 25%) in styles pollinated with compatible pollen (Fig. 3). The fragment amplified with the primers used for Real Time PCR was sequenced and confirmed to be from CavPrx.
PCR amplifications of genomic DNA produced a sequence of 2190 bp with a classical class III gene structure: four exons (213, 195, 166 and 419 bp) separated by three introns. The first and the second introns were 94 and 89 bp in length, while the third one was 801 bp long.

Phylogenetic analysis of CavPrx

In order to analyze the relationship between CavPrx and other class III peroxidases, a phylogenetic analysis was performed using MEGA version 3.1 with Neighbour-Joining method. Two thousand bootstrap replicates were

carried out. Sequences of other hazelnut peroxidases were not available in the databases, consequently the analysis was performed considering the class III peroxidases with the greatest sequence similarity to CavPrx, after alignment with BLASTp using the PeroxiBase database. For these peroxidases, information about expression pattern and localization in plants were get from literature or from the PeroxiBase database (Table 1).
Five major groups (A, B, C, D, E), with two of them divided in two sub-groups (A1 and A2; E1 and E2), were resolved. It can be noticed that peroxidases from the same species did not always cluster in the same group; at the same way peroxidases with similar plant localization or function were not always grouped together (Fig. 4). CavPrx clustered in the group B: the other peroxidases of the group B were not expressed in the flower, but in other organs, such as roots, leaves, stems and fruits (Table 1). The other peroxidases in the phylogenetic tree with some degree of expression in flowers were AtPrx53 of *A. thaliana* in sub-group A2, NtPrx09a of *N. tabacum* in group D, VvPrx17 of *V. vinifera*, GhPrx10 of *G. hirsutum* and CsPrx05, CsPrx12 and CsPrx10 of *C. sinensis* in sub-group E2.

Discussion

The Differential Display technique was used to investigate gene expression in the female flower of hazelnut. This technique was used also by Takayama et al. [33] to identify the male determinant of SI in *Brassica campestris* L. and by Chen et al. [12] to isolate a flower-specific class III peroxidase gene in *G. hirsutum*.
Among the differential expressed sequences that were isolated, one was identified as being from a putative class III peroxidase gene that was eventually fully characterised. The presence of highly conserved amino acids characterize the plant PRX protein sequences. Two histidine residues (distal and proximal histidines) and eight cysteine residues are very important for the interaction with the heme group and the formation of disulphide bridges, respectively. In particular the distal histidine is required for the catalytic activity. Other amino acid residues are important to maintain the peroxidase structure and function and to target it to the outside of plant cell or to the vacuole. These proteins show a constant molecular organization characterized by the presence of 13 α-helices [32, 34]. The peroxidase sequence of hazelnut contains eight conserved cysteine residues, conserved distal and proximal heme binding sites, two conserved calcium binding sites and other active site residues, as typical of class III peroxidases family. It presents 10 alpha-helices common to the peroxidase superfamily and also the three specific alpha-helices typical of class III peroxidases [34] (Fig. 2). In fact, it is known that protein structure, amino acid residues and protein size are conserved in all class III peroxidases [5, 35].
CavPrx showed a “three intron” structure that is a common feature of the most of class III peroxidases in rice and Arabidopsis [2, 7].
This is the first class III peroxidase isolated in *C. avellana* and Real Time analysis showed that it is expressed in the styles, with a peak of expression in mature styles; in addition its level of expression increased significantly in styles pollinated with compatible pollen. Despite most of plant peroxidases are active in all part of the plant [5, 7] some example of localized peroxidases in particular tissue/organ exist in literature, as reported in the introduction. In particular, five peroxidases were identified to be expressed in particular parts of the flower: GhPrx37 in *G. hirsutum* resulted expressed only in stamen and pollen [12]; SsqPrx01 in *S. squalidus* was demonstrated to be localized in the stigmatic papillae and expressed only in stigmas with maximal level at anthesis [6]; AtPrx28, AtPrx39 and AtPrx58 in *A. thaliana* resulted expressed in stigmas [13, 14].
Phylogenetic analysis was carried out in order to understand the relation among CavPrx and other peroxidases with the greatest sequence similarity. It was noticed that peroxidases of the same species not always clustered together. A high variability in peroxidase sequences within single plant species was thus highlighted since total amino acid sequence identity was sometimes lower than 35% [36] (Fig. 4).
CavPrx clustered in the group B and resulted most closely related to the PpePrx89 of *P. persica*, expressed in buds, fruits and mesocarp (PeroxiBase database). From literature information, the other peroxidases in the group B resulted expressed in vegetative tissues, roots, fruits, seeds and cell culture [37, PeroxiBase database]. About peroxidases with some degree of expression in flower in the phylogenetic tree, the most related ones was AtPrx53 of *A. thaliana* in sub-group A2 that shared 75% identity and 86% similarity with CavPrx. AtPrx53 was described to be expressed in the stamen abscission zone by microarray analysis [31]. This peroxidase is not flower-specific because it resulted expressed also in vascular bundles [35] and it could be putatively involved in lignification [3]. Other peroxidases expressed in flowers were NtPrx09a of *N. tabacum* (63% identity and 78% similarity with CavPrx), VvPrx17 of *V. vinifera* (67% identity and 80% similarity with CavPrx), GhPrx10 of *G. hirsutum* (62% identity and 74% similarity with CavPrx), CsPrx05 (64% identity and 76% similarity with CavPrx), CsPrx12 (64% identity and 77% similarity with CavPrx) and CsPrx10 (60% identity and 73% similarity with CavPrx) of *C. sinensis*. Their function is unknown except for VvPrx17 and CsPrx05 that could be induced by senescence and insect damages, respectively (Peroxibase database).
It was not possible to find information in literature about expression pattern for all the peroxidases phylogenetically analyzed. Despite of this, it seems that the peroxidase sequences analyzed are not always grouped together based on reported expression pattern or function. For instance, peroxidases expressed in roots,

flowers, xylem or involved in lignification and pathogen/stress disease are dispersed in the dendrogram. It seems that it is not possible to infer a putative role of a peroxidase based on sequence similarity or phylogenetic position. This observation was highlighted in previous works. Delannoy et al. [38] carried out a molecular analysis on 12 peroxidases of cotton and, after phylogenetical analysis, four peroxidases (including GhPrx10) with the same expression profile resulted dispersed in the dendrogram. On the other hand an extensive study on *Arabidopsis* peroxidases showed that very similar genes have not similar biological role or expression pattern [5, 8]. This could be due to translational regulations, which peroxidases are subjected to, for regulating the amount of protein in a particular tissue/organ and so its activity [8, 39]. These observations indicate that genes could have different roles and functions, even if closely related, and so it is difficult to predict role on the base of similarity or phylogenetical relations [3, 12]. At the same time genes with a similar expression pattern could have a low sequence similarity [40]. For instance, the peroxidases of *A. thaliana* (AtPrx28, AtPrx39, AtPrx58) and *S. squalidus* (SsqPrx01) showed a low identity and similarity among them, even if they are all expressed in stigma.

In order to understand where CavPrx expression was localized, two specific primers, F-GSP and R-GSP, were designed on the fragment sequence obtained by Differential Display and used to perform PCR on cDNA of catkins, leaves, mature and immature styles. Amplifications were detected only in styles while no bands were evident in catkins and leaves. In addition, amplification in mature styles was higher than in immature styles. This putative differential expression profile was better investigated by Real Time PCR on cDNA of leaves, mature and immature styles, styles pollinated with compatible and incompatible pollen and catkins. CavPrx expression in mature styles was 3-fold more abundant than in immature styles and almost absent in leaves and catkins (Fig. 3). This temporal regulation could be noticed also for SsqPrx01 of *S. squalidus*, which expression was absent in small buds and increased with flower development, reaching a maximum level in mature stigmas, receptive to pollen [6]. Moreover the expression level of CavPrx in styles pollinated with compatible pollen was 25% higher than in styles not pollinated or pollinated with incompatible pollen: this level of expression suggests a possible involvement in pollination events.

One of the possible role of peroxidases in the pollen-stigma interaction could be the promotion of pollen tube penetration and growth within the stigma, by loosening stigma cell wall components [41]. Microarray analysis carried out by Tung et al. [14] in stigmas and transmitting tracts of *A. thaliana* revealed the presence of a consistent group of genes predicted to encode proteins with N-terminal signal peptides, as found in CavPrx. Among these, three peroxidases (AtPrx28, AtPrx39 and AtPrx58) were identified as putative cell wall-localized enzymes that could have a role for the stigmatic extracellular matrix modification during pollination and pollen tube penetration. These proteins could act in wall loosening by generating hydroxyl radicals for the cell wall polysaccharides degradation [42]. Their implication in wall loosening is also underlined by the link between stigma receptivity and presence of stigma surface peroxidases in several species [44].

It is well-known that the expression of peroxidases increases with flower development, reaching a peak in mature and receptive styles [41, 43]. Moreover it is known that principal ROS (reactive oxygen species) in stigmas is H_2O_2 [41] but its function is yet unknown. This contemporary presence of high level of H_2O_2 and peroxidases in the mature styles suggests that they could interact, even if the course of action is still unknown [41].

Stigmatic peroxidases could be important for regulating levels of H_2O_2 in stigmas, either by degradation or generation of H_2O_2 [44]. In tobacco Potocký et al. [45] demonstrated that H_2O_2 and other ROS were implied in maintenance of polarization during pollen tube growth, while Foreman et al. [46] individuated H_2O_2 as a positive regulator of cell growth in root hairs, which elongated by tip growth, like pollen tube.

Recent studies on cellular localization of ROS in reproductive tissues during flower development of olive [47] showed maintenance of high level of H_2O_2 during the early stages of development until the maturity of styles, then the level started to decrease when stigmatic receptivity to pollen results enhanced. This decrement of H_2O_2 levels could be related to the raising of peroxidase activity that occurs in Angiosperm stigmas at maturity [48], in order to allow pollination. High level of ROS/H_2O_2 in the stigma in fact could be dangerous for pollen grain and pollen tube growth, but further studies will be necessary to understand if this decrement of ROS/H_2O_2 levels is a common feature of Angiosperm stigmas.

Another hypothesis could be the possible role of stigma-specific peroxidases in the defence of stigma against pathogen attack. In literature it is known that some peroxidases are involved in defence response by induction or up-regulation in relation to stress and hypersensitive response [1]. For instance, a peroxidase of *Capsicum annum* L. CaPrx02 resulted expressed during response against pathogen attack regulating H_2O_2 levels [49], while a research in almond demonstrated the expression of peroxidases in the pistil in relation with pathogenesis response [50], enforcing the idea of a putative role of stigma-specific peroxidases in defence mechanisms, when the pistil is able to receive pollen. Another research revealed that GhPrx37, a floral-specific peroxidases of *G. hirsutum* was predominantly expressed during pollen development and it could be important for the correct development of male reproductive organs, because of its putative role in defence against stress [12]. In addition, high level of H_2O_2, produced by a superoxide dismutase were detected in nectar, with the aim of protecting it

from microbial infections [51]. In the same way the abundant presence of H_2O_2 in the stigmas and the almost total absence of pathogen attack on stigmas could improve the hypothesis about a possible interaction between stigma-specific peroxidases and H_2O_2 with a defence role [6].
In conclusion, CavPrx represents a first putative stigma-specific peroxidase of hazelnut, whose expression profile changes during flower development, with a maximum level in mature stigmas pollinated with compatible pollen. Further studies may carry out a time course analysis of pollinated stigmas during pollen germination and afterwards to highlight more in details the difference between the expression patterns of compatible and incompatible interactions. The identification of this new putative stigmatic peroxidases in hazelnut is a further opportunity to analyze and understand the specific function of stigma-specific class III peroxidases and their role in flower fertility.

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Table and figure captions

Table 1 References for sequence and expression data presented in Fig. 4

Label	Entry ID PeroxiBase	Organs of expression	References
A.rusticana AruPrx04	298	Root	[52]
A.thaliana AtPrx53	219	Whole plant	[35]
		Stamen	[31, 53]
		Leaf	[53]
A.thaliana AtPrx54	220	Callus, hypocotyl, root	PeroxiBase database
B.napus BnPrx54-1	6603	Not determed	PeroxiBase database
B.rapa BrPrx53	6921	Root	UniGene (www.ncbi.nlm.nih.gov)
C.avellana CavPrx		Style	This study
C.sinensis CsPrx05	1373	Flower, fruit, meristem, vegetative tissues, whole plant	PeroxiBase database
C.sinensis CsPrx10	1378	Callus, flower, fruit, ovarie, root, seedling, shoot merisytem	PeroxiBase database
C.sinensis CsPrx12	1380	Ovarie	PeroxiBase database
C.sinensis CsPrx17	1385	Seedling	[54]
E.grandis EgrPrx48	8165	Not determed	PeroxiBase database
E.grandis EgrPrx49	8100	Not determed	PeroxiBase database
E.grandis EgrPrx51	8097	Not determed	PeroxiBase database
G.hirsutum GhPrx10	145	Cotiledon, leaf, flower, root, stem	[38]
G.hirsutum GhPrx22	439	Not determed	PeroxiBase database
G.max GmPrx38	529	Seedling, stem	PeroxiBase database
G.max GmPrx71	570	Root	PeroxiBase database
H.brasiliensis HbPrx01	3452	Leaf	[55]
I.batatas IbPrx05	3807	Cell culture, root, stem	[56]
I.batatas IbPrx15	296	Root	[57]
L.esculentum LePrx05	276	Leaf	[58]
L.esculentum LePrx35	633	Cell culture, fruit, seed	PeroxiBase database
L.japonicus LjPrx44	695	Pod	[59]
M.domestica MdPrx08	7125	Leaf	PeroxiBase database
M.truncatula MtPrx23	349	Pod	PeroxiBase database
N.tabacum NtPrx04a	1864	Cell culture	PeroxiBase database
N.tabacum NtPrx09a	3701	Cell culture, flower, leave, stem	PeroxiBase database
N.tabacum NtPrx09b	1	Cell culture	[60]
N.tomentosiformis NtoPrx01	2142	Not determed	PeroxiBase database
P.alba PalPrx05	4055	Bark, leaf, petiole, shoot, stem, xylem	[37]
P.kitakamiensis PkPrx01	323	Not determed	PeroxiBase database
P.kitakamiensis PkPrx03	54	Xylem	[61]
P.persica PpePrx89	2045	Bud, fruit, mesocarp	PeroxiBase database
P.taeda PtaPrx14	2050	Root, xylem	PeroxiBase database
P.trichocarpa PtPrx01	907	Xylem	[62]
P.trichocarpa PtPrx72	3164	Stem, leaf	PeroxiBase database
P.trichocarpa PtPrx102	3249	Not determed	PeroxiBase database
P.vulgaris PvPrx05	272	Root, stem, leaf	[63]
S.tuberosum StPrx09	1912	Leaf, petiole, sprouting eye, stolon, tuber	PeroxiBase database
S.tuberosum StPrx20	1923	Leaf, sprouting eye	PeroxiBase database
V.vinifera VvPrx05	297	Berry	PeroxiBase database
V.vinifera VvPrx17	774	Berry, flower, mixed tissues	PeroxiBase database
V.vinifera VvPrx36	5119	Not determed	PeroxiBase database

Fig. 1 Physiological stages of styles used for RNA extraction: bud before the red-dot stage (a) and relative styles not exposed yet (b); bud at flowering time (c) and relative mature fully expanded styles (d) (Source: Arboriculture Department of University of Turin)

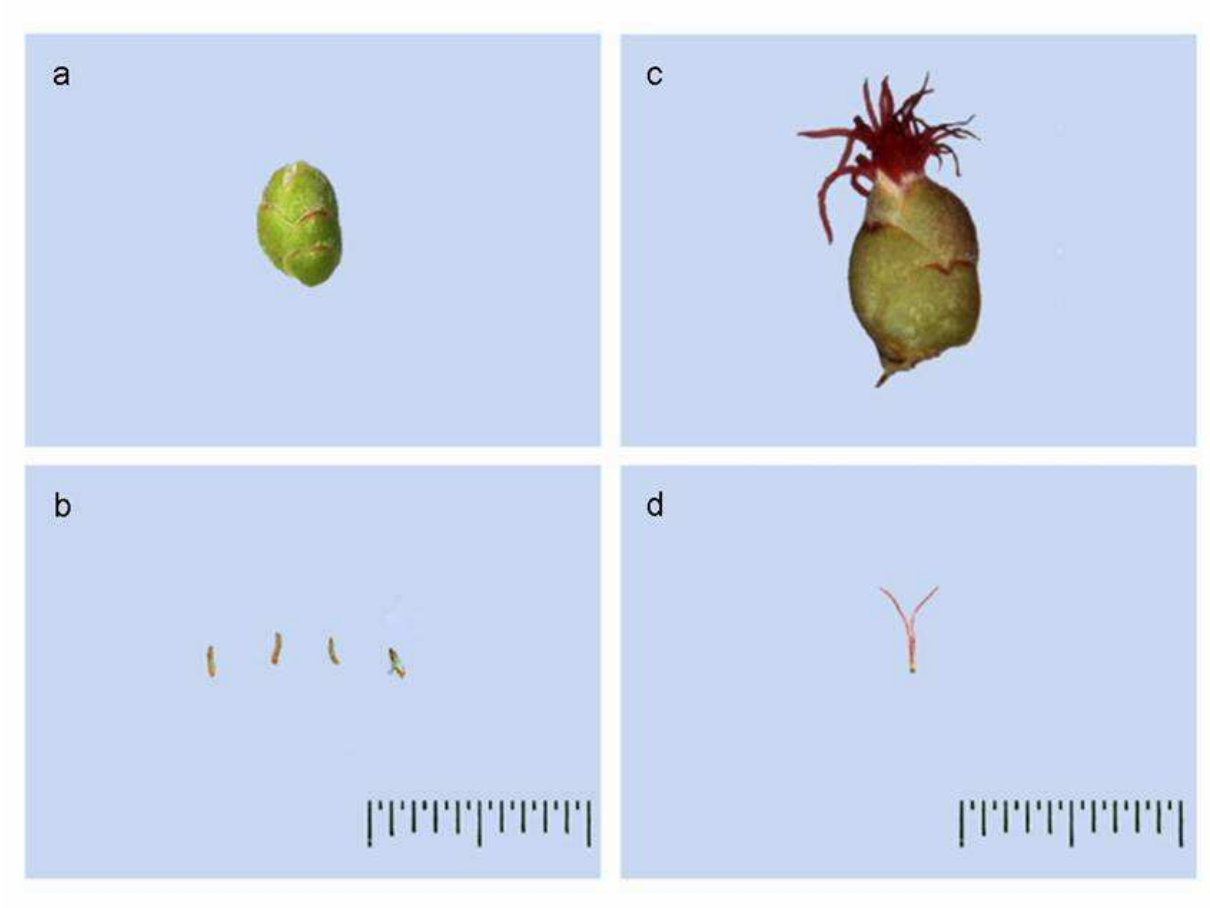

Fig. 2 cDNA sequence and translation of CavPrx. The 5' and 3' UTR are in lower case and the stop codon is represented by a bold asterisk. The eight cysteine residues conserved in class III peroxidases are indicated by ■. The putative signal peptide is underlined. The conserved peroxidase active site signature and the proximal heme-ligand signature are in bold type. Position of predicted alpha-helices, based on consensus with HRPC (Horseradish peroxidase C), are in grey (A: 38-52; B: 56-68; C: 102-115; D: 122-137; D': 156-162; E: 170-179; F: 184-190; F': 205-208; F'': 222-231; G: 256-262; H: 272-276; I: 283-291; J: 293-307). Intron positions are indicated by arrows. Residues involved in calcium binding are indicated by ○, salt bridge residues are indicated by Δ and other highly conserved residues important for integrity of peroxidase structure are indicated by □

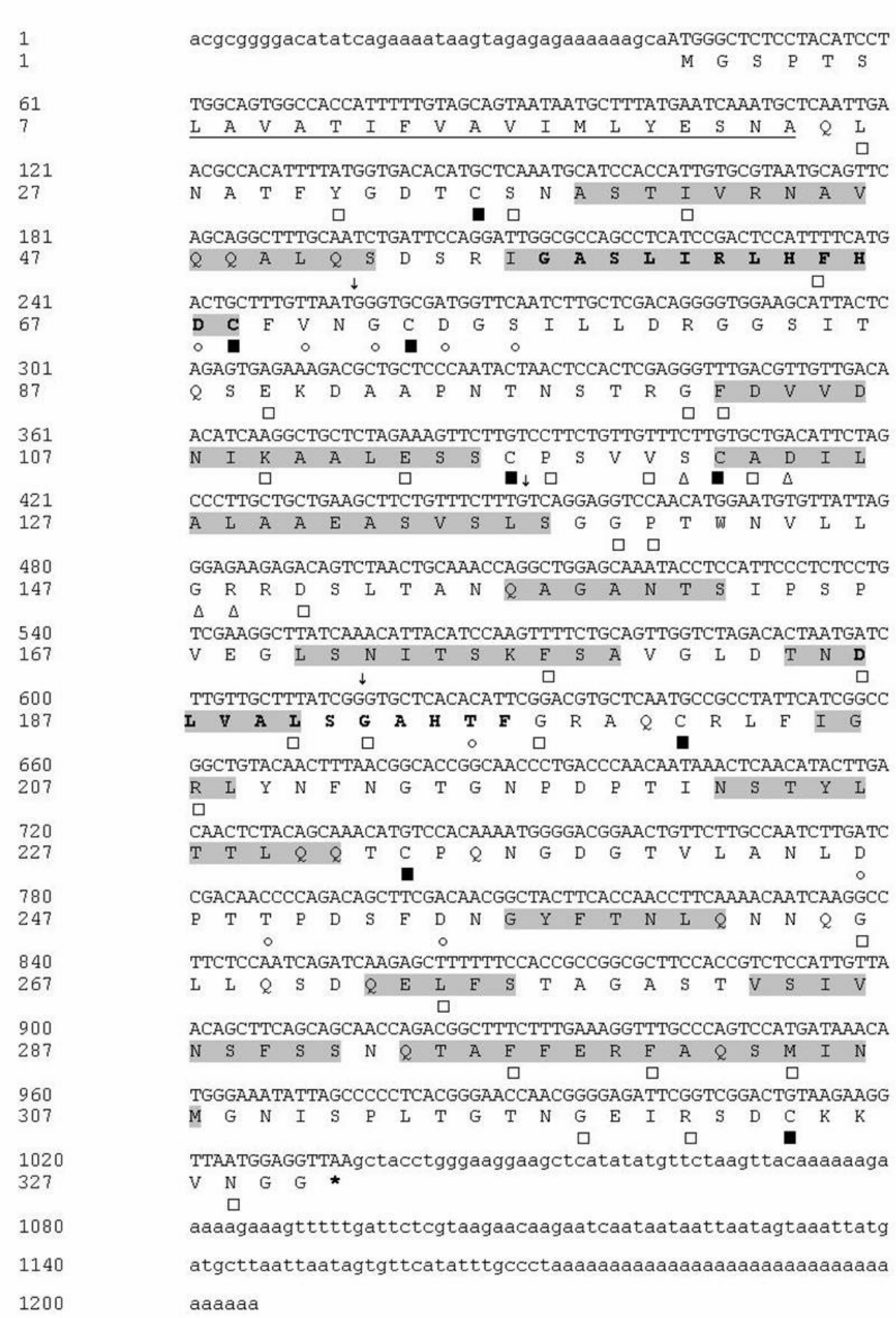

Fig. 3 mRNA abundance of CavPrx in catkins (1:stage I, 2:stage II, 3:stage III), leaves (4) and styles of TGdL hazelnut cultivar (5:immature styles, 6:mature styles not pollinated, 7:mature styles pollinated with compatible pollen, 8:mature styles pollinated with incompatible pollen). Relative expression of peroxidase gene in catkins, leaves and mature styles is calculated by comparison with the reference sample (immature styles) using a ΔCt method, after normalization with housekeeping genes *VvUbiquitin1* and *VvACT1*. Data represent the average of three technical replicates (±SD)

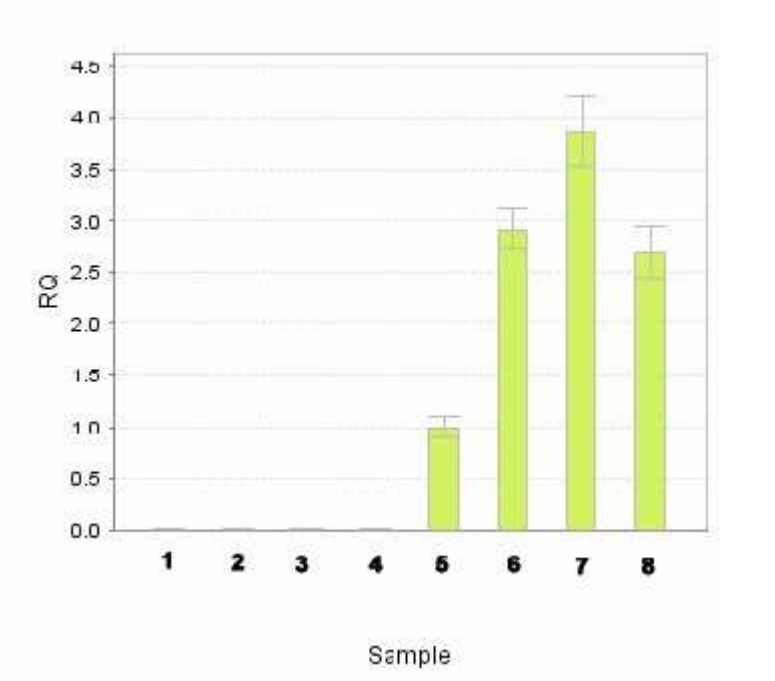

Fig. 4 Phylogenetic relationship between CavPrx peroxidase and class III peroxidases with the highest sequence similarity. The tree is constructed in MEGA version 3.1 and is based on sequence alignments of predicted protein sequences. Node values indicate bootstrap support (2000 replicates). The main groups and subgroups are indicated by letters and numbers. The expression pattern is represented by filled quadrants indicating: floral, root, vegetative tissues (leaf or stem) and fruit expression. The hypothetical role is represented by filled circles: stress-induced, lignification and other function (nodulation, senescence). For accession numbers and references of the sequences used to establish the phylogenetic tree, see Table 1