

The proteome of exudates from germinating *Lupinus albus* seeds is secreted through a selective dual-step process and contains proteins involved in plant defence

Alessio Scarafoni¹, Alessandro Ronchi^{1,*}, Bhakti Prinsi^{2,*}, Luca Espen², Gemma Assante², Giovanni Venturini² and Marcello Duranti¹

¹ Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Italy

² Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy, Università degli Studi di Milano, Italy

Keywords

germination; legume seed; plant defence; seed exudates; seed proteins

Correspondence

A. Scarafoni, Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, via G. Celoria 2, 20133 Milano, Italy
Fax: +39 02 5031 6801
Tel: +39 02 5031 6820
E-mail: alessio.scarafoni@unimi.it

*These authors contributed equally to this work

(Received 9 November 2012, revised 22 December 2012, accepted 15 January 2013)

doi:10.1111/febs.12140

The general knowledge of defence activity during the first steps of seed germination is still largely incomplete. The present study focused on the proteins released in the exudates of germinating white lupin seeds. During the first 24 h, a release of proteins was observed. Initially (i.e. during the first 12 h), the proteins found in exudates reflected the composition of the seed, indicating a passive extrusion of pre-formed proteins. Subsequently, when the rate of protein release was at its highest, the composition of the released proteome changed drastically. This transition occurred in a short time, indicating that more selective and regulated events, such as secretory processes, took place soon after the onset of germination. The present study considered: (a) the characterization of the proteome accumulated in the germinating medium collected after the appearance of the post-extrusion events; (b) the biosynthetic origin and the modalities that are the basis of protein release outside the seeds; and (c) an assessment of antifungal activity of these exudates. The most represented protein in the exudate was chitinase, which was synthesized *de novo*. The other proteins are involved in the cellular mechanisms responding to stress events, including biotic ones. This exudate was effectively able to inhibit fungal growth. The results of the present study indicate that seed exudation is a dual-step process that leads to the secretion of selected proteins and thus is not a result of passive leakage. The released proteome is involved in protecting the spermosphere environment and thus may act as first defence against pathogens.

Introduction

Germination starts when a quiescent dry seed begins to uptake water (imbibition) and is completed when the embryonic axes elongates. The uptake of water by a mature dry seed progresses through three rather distinct phases [1], where a rapid initial uptake (phase I) is followed by a plateau phase (phase II) and a further increase in water uptake occurs only after germination is completed (phase III). The influx of water into the

cells of dry seeds during phase I leads not only to crack the seed coat, but also to leakage of internal substances from the seed, a process known as seed exudation [2]. This is symptomatic of a transition of the tonoplast and plasmalemma phospholipid components from the gel state achieved during maturation drying to the normal hydrated state [3]. Upon imbibition, the quiescent dry seed resumes metabolic activity. The

Abbreviations

BSP, basic secretory protein; CBB, Coomassie Brilliant Blue; ConA, concanavalin A; ICP, inductively-coupled plasma; PB, protein body; SP, storage protein.

structures and enzymes necessary for this initial resumption of metabolic activity are generally present within the dry seed. At this time, the protein synthesis machineries remaining during the desiccation processes are used, including ribosomes and extant mRNAs [1,2].

The compounds exuded from seeds may have direct or indirect beneficial effects on germination itself, as well as on the general health of the seedling [4]. The substances released from seeds and roots during germination represent one of the major driving forces behind the growth of microorganisms and plant–microbe interactions in the spermosphere [5–8].

Several compounds exuded from germinating seeds have the capacity to inhibit pathogen growth, thus preventing seed infection. The best-described groups of defence compounds released from seeds are molecules with a low molecular mass, such as flavonoids, sterols, fatty acids and peptides [9,10]. Only few studies have reported that proteins and enzymes may contribute the defence activity of exudates [5,10,11]; thus, our general knowledge remains largely incomplete. The present study focused exclusively on this class of macromolecules.

In mature legume seeds, proteins comprise approximately 20% of the pea, fababean and common beans, and up to 37–45% of soybeans and lupins [12]. The majority of them are storage proteins (SP), which are globulins usually classified according to their sedimentation coefficients (S) as 7S (vicilin-like) and 11S (legumin-like). SPs are synthesized during seed development and are deposited inside the cells in specific membrane-bound organelles [13], called protein bodies (PBs). During germination, SPs are hydrolyzed up to free amino acids to support seedling growth. Although vicilins are defined as reserve proteins, studies suggest that they have an active role in defence processes [5,14–16]. A fragment of 45 amino acids generated during germination by proteolysis of *Macadamia integrifolia* vicilin was shown to inhibit several plant pathogenic fungi *in vitro* [17]. This polypeptide was found in both cotyledons and seed exudates.

No defensive bioactivities have been described for legumin-derived peptides.

The last class of SPs is represented by monomeric 2S albumins, which are proteins with a low molecular mass of approximately 10–12 kDa [16], and they may also play a physiological role in plant defence [18,19].

Further to these main protein families, seed storage tissues contain a number of other proteins. Most of them are involved in plant defence, including several kinds of hydrolytic enzymes, enzyme inhibitors, lectins, ribosome-inactivating proteins and lipid transfer proteins [20].

Although lupin is not considered as a model plant, some distinctive features make its seed an ideal system for investigating the potential role of proteins and peptides in the physiological processes that occur during germination. Lupin seeds contain one of the highest protein contents amongst the grain legumes, comprising up to 45% of the dry seed weight. All SP classes are present, in contrast to *Arabidopsis thaliana* seeds, which essentially contain 11S and 2S SPs [21], or *Medicago truncatula*, where mostly 11S and 7S SPs are accumulated [22]. In addition, lupin seeds contain significant amounts (5–6% of the total seed protein) of γ -conglutin, a distinctive tetrameric basic protein of approximately 200 kDa that has long been considered as an SP [23]. However, γ -conglutin is not degraded during germination (whereas SPs usually are) and it is secreted from the seed under conditions of stress and during germination (whereas SPs are not) [23,24]. γ -Conglutin has been detected in apoplasts of germinating lupin cotyledons [25], as well as in the germinating medium [24]. γ -Conglutin is likely to be involved in plant defence mechanisms against fungal attacks because it shares structural similarities to tomato xyloglucan-specific endo- β -1,4-glucanase inhibitor proteins and because its expression can be strongly elicited by chitosan during germination [26]. Another peculiarity of lupin SPs involves β -conglutin, the lupin seed vicilin-like protein. It is the most represented SP (approximately 45% of the total SP) and is characterized by a pronounced heterogeneity of its constituent polypeptides. This is a consequence of the copious post-translational proteolysis of the approximately 60-kDa precursor polypeptides [23,27,28]. As a result, more than 42 different vicilin-like polypeptides of varying molecular mass and pI have been detected in cotyledons of mature seeds [27]. The extent of this processing has no parallel in other plant species, including the pea and soybean, where endogenous cleavage of vicilins during seed development is known [29,30]. At least one breakdown peptide has been shown to possess antifungal properties [31].

The present study focused exclusively on the protein fraction of germinating seed exudates. In particular, we investigated the release of polypeptides during the first 24 h of white lupin (*Lupinus albus*) seed germination. The present study aimed to consider: (a) the characterization of the proteome secreted into the germinating medium; (b) the biosynthetic origin and the modalities that are the basis of protein release outside the seeds; and (c) the assessment of antifungal activity of these proteins. The results provide evidence that seed exudation is a dual-step process that leads to the secretion of selected proteins. The released proteome is

involved in protecting the spermosphere environment and thus may act as first defence against pathogens.

Results

The first seed exudation event and protein release

The timing of the early event of lupin seed material leakage was determined by monitoring the presence of K^+ and the proteins released and accumulated into the surrounding aqueous medium (Fig. 1). During the first 2–4 h, a consistent efflux of K^+ was observed (Fig. 1, dashed line), whereas, in the following period, the cation was rapidly recovered and, after 24 h, only very small amounts of K^+ were detected in the external medium. K^+ re-uptake by germinating seeds indicates the full resumption of seed metabolic activities [32,33]. More than 95% of the seeds were fully viable (i.e. able to originate seedlings when potted onto light expanded clay aggregates). A remarkable release of proteins started at approximately the fourth hour after imbibition, when K^+ efflux reached its maximum intensity. The highest amount of protein released in the medium (approximately $55 \mu\text{g}\cdot\text{mL}^{-1}$ under the adopted experimental conditions) was observed after approximately 24 h (Fig. 1, solid line).

The type and relative quantities of the polypeptides accumulated in the medium varied over time, as shown by SDS/PAGE (Fig. 2A). In this case, the germination media were removed every 12 h and replaced with sterile distilled water. After 12 h from the onset of germination, the size of the polypeptides ranged from approximately 10–70 kDa and no particular protein

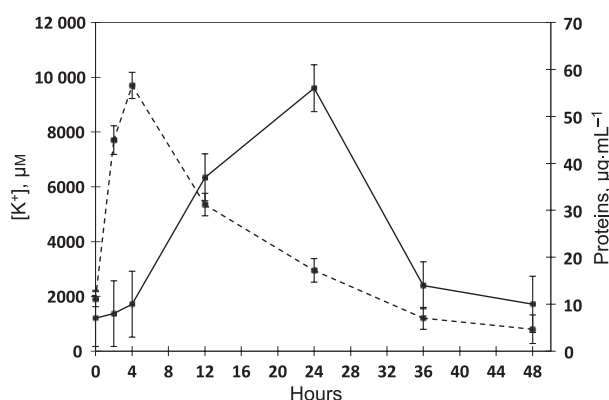


Fig. 1. K^+ ions (dash line) and proteins (solid line) released from the seeds and accumulated into the surrounding aqueous medium during germination. Quantification of ions was carried out by ICP-OES, whereas proteins were determined by the method of Bradford.

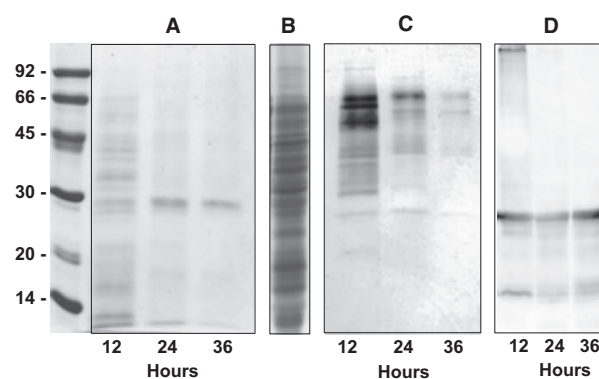


Fig. 2. Analysis of seed exudates and comparison with proteins extracted from mature dry seeds. Gels were stained with CBB or blotted onto nitrocellulose membranes. (A) SDS/PAGE of seed exudates collected 12, 24 and 36 h after the start of germination. (B) SDS/PAGE of cotyledonary proteins. (C) Western blotting of the exudates, as in (A), and detection with ConA. (D) Western blotting of the exudates, as in (A), and detection with anti- γ -conglutinin specific antibodies. Samples were reduced before the electrophoretic runs. Equal volumes of each sample (35 μL) have been loaded in (A), (C) and (D), whereas 15 μL of total seed protein extract was loaded in (B).

was predominant. The electrophoretic pattern of such an exudate protein fraction is essentially coincident to that obtained with the total proteins extracted from the cotyledons of mature dry seeds (Fig. 2B) [27,34].

A band of approximately 30 kDa started to be observable between 12 and 24 h, and its relative abundance also persisted from 24 to 36 h.

Figure 2C,D shows the identification of two main proteins of mature lupin seed by western blotting, namely β -conglutinin and γ -conglutinin. The presence of vicilins in the exudates was estimated by assessing the presence of carbohydrates covalently linked to the blotted polypeptides with concanavalin A (ConA) [35], whereas specific antibodies were used to detect the presence of γ -conglutinin [23,34]. The amount of vicilin decreased over time (Fig. 2C), whereas that of γ -conglutinin was found to be steady (Fig. 2D). As expected, ConA recognized the large subunit of γ -conglutinin, which is also glycosylated [24], as indicated by a 29-kDa faint band visible in Fig. 2C.

Proteomic map of exudate proteins and protein identification

The characterization of the proteome selectively released from the seed during the first exudation episode was one of the overriding aims of the present study. IEF/SDS/PAGE two-dimensional maps were prepared analysing the samples collected between 12

and 24 h after the start of germination, namely when the protein release showed its maximum. The map shown in Fig. 3A is a representative depiction of three independent experimental settings, each analyzed in quadruplicate.

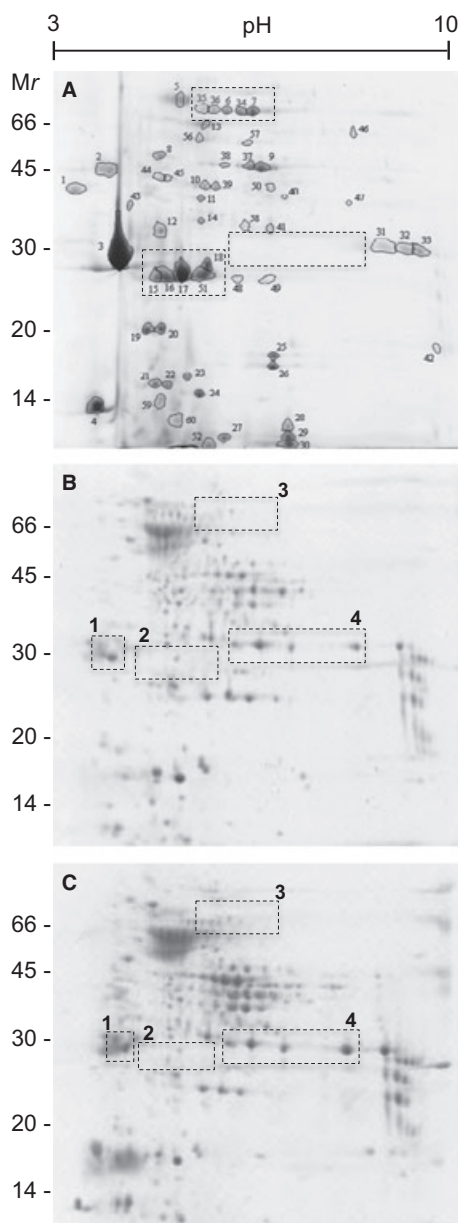


Fig. 3. IEF/SDS/PAGE two-dimensional analysis of the exudate proteome collected between 12 and 24 h after the start of germination (A) and of cotyledonary proteins at 24 h (B) and 12 h (C) after the start of germination. The gels were CBB-stained. Two hundred micrograms of total protein was loaded on each gel. (A) The spots analyzed by trypsin-digestion and MS are outlined and numbered. The full list of identified spots is provided in Table 1.

A total of 60 spots were found present through all the gels. The intensity and volume of each detected spot is reported in Table S1. The detected spots were analyzed by MS. The sequence of all the peptides identified by MS/MS fragmentation and the associated statistical information obtained from database searches are reported in Table S2. Seventeen spots out of 60 (28%) remained unidentified. The identified spots are listed in Table 1. Most of the spots were assigned by matching of at least two peptides. Twenty-six spots (accounting for 60% of the identified polypeptides) showed > 5% sequence coverage, whereas 12 spots (28%) showed < 5% coverage. Table 1 also includes 11 spots for which identification was based on a single peptide assignment. Five of them (12% of the total assigned spots) showed > 5% sequence coverage. The identification of the last six spots was uncertain, with IDs tentatively being assigned according to the matching of only one peptide and < 5% sequence coverage.

The most abundant protein (spot number 3) accounted for approximately 33% of the total volume of spots (Table S1). MS analysis identified it as a chitinase belonging to class III [36]. The experimental molecular mass and pI coincided with the theoretical values. The molecular mass values were also in good accordance with that of the main band present at 24 and 36 h of incubation, as shown in Fig. 2A. The protein is not glycosylated, as assessed *in silico* via a PROSITE scan (<http://prosite.expasy.org>). Chitinases from class III comprised the GH18 family [37]. Members of GH18 can be devoid of chitinase activity and represent xylanase inhibitor proteins [38]. In the present study, the exudates collected between 12 and 24 h after the beginning of germination showed endochitinase activity but not xylanase inhibitory capacity (Table 2).

Ten spots (numbers 2, 5, 6, 7, 9, 34, 35, 36, 39 and 45) have been attributed to other types of glucanases. Altogether, they account for approximately 16% of the total number of spots and 11% of the total volume of spots (Table S1). Some of them appear to be present in more than one form, such as spots 6, 7, 34, 35 and 36, which have been all identified as β -xylosidase/ α -L-arabinosidase. This enzyme is a bifunctional exoglucanase that releases xylose and arabinose from cell walls, acting synergistically with endohydrolases, and is implicated in cell wall turnover in rapidly growing tissues [39]. The expression and synthesis of α -galactosidase (spot number 2) has been implicated in response to common conditions of stress, including defence against pathogens [40].

The so-called basic secretory proteins (BSPs; spots 15, 16, 17, 51) are also considered to be a part of the

Table 1. List of spots identified by LC-ESI-MS/MS. Statistical information about MS and alignment analysis is provided in Table S2.

ID	Accession number	Species	Protein description	EC	pI ^a /M _r ^a	pI ^b /M _r ^b	Coverage (%) ^c	n _{pep} ^d
2	P14749	<i>Cyamopsis tetragonoloba</i>	α -galactosidase	3.2.1.22	4.3/47.5	4.7/39.8 ^e	14 ^e	5
3	CAA76203	<i>Lupinus albus</i>	Class III chitinase	3.2.1.14	4.4/28.3	4.5/28.2 ^e	24 ^e	5
4	CAJ42100	<i>Lupinus albus</i>	Conglutin δ seed storage protein (large chain)	–	4.2/13.2	4.5/9.1 ^e	40 ^e	2
5	ACU18828	<i>Glycine max</i>	Polygalacturonase ^f	3.2.1.67	4.9/69.2	5.0/51.3	6	2
6	A5JTO3	<i>Medicago varia</i>	β -xylosidase/ α -L-arabinofuranosidase 2	3.2.1.37 3.2.1.55	5.3/65.4	8.5/79.8 ^e	3 ^e	2
7	A5JTO3	<i>Medicago varia</i>	β -xylosidase/ α -L-arabinofuranosidase 2	3.2.1.37 3.2.1.55	5.6/65.4	8.5/79.8 ^e	4 ^e	3
9	CAC44501	<i>Fragaria x ananassa</i>	β -galactosidase	3.2.1.23	5.8/48.6	7.2/91.2	6	4
	AAS97865	<i>Lupinus albus</i>	β -conglutina precursor	–		6.4/62.1	6	2
11	ACU22795	<i>Glycine max</i>	Putative NADP-dependent oxidoreductase ^f	1.3.1.74	5.1/41.0	5.9/37.9	6	2
12	ACU19183	<i>Glycine max</i>	Lactoylglutathione lyase ^f	4.4.1.5	4.7/34.0	5.4/31.6	9	2
	CAI84850	<i>Lupinus albus</i>	Vicilin-like protein	–		6.1/62.0 ^g	2 ^g	1
13	CAI84850	<i>Lupinus albus</i>	Vicilin-like protein	–	5.1/60.4	6.1/62.0 ^g	12 ^g	5
14	ACU23990	<i>Glycine max</i>	Annexin ^f	–	5.1/35.7	6.2/25.1 ^g	15 ^g	3
15	ACU14241	<i>Glycine max</i>	Plant basic secretory protein ^f	–	4.7/26.7	4.9/25.2	13	2
16	ACU14241	<i>Glycine max</i>	Plant basic secretory protein ^f	–	4.8/26.7	4.9/25.2	13	2
17	ACU14241	<i>Glycine max</i>	Plant basic secretory protein ^f	–	4.9/26.7	4.9/25.2	13	2
20	XP_002271261	<i>Vitis vinifera</i>	Phosphatidylethanolamine-binding protein ^f	–	4.8/20.1	5.3/17.8	9	1
21	BAB63949	<i>Lupinus albus</i>	Pathogenesis-related 10	–	4.7/14.9	4.9/16.9	34	4
22	CAC17729	<i>Lupinus albus</i>	Conglutin γ	–	4.8/14.9	6.5/45.9 ^e	4 ^e	1
24	Q02610	<i>Pisum sativum</i>	Superoxide dismutase [Cu-Zn]	1.15.1.1	5.1/14.0	5.6/15.2 ^e	9 ^e	1
25	CAC16394	<i>Lupinus albus</i>	Conglutin γ – small subunit	–	6.2/17.0	6.2/16.2 ^e	50 ^e	4
26	CAC16394	<i>Lupinus albus</i>	Conglutin γ – small subunit	–	6.2/16.4	6.2/16.2 ^e	50 ^e	4
28	ACY30464	<i>Ficus pumila</i>	Cystatin	–	6.4/11.8	9.7/12.8	10	1
29	CAN82794	<i>Vitis vinifera</i>	Cystatin ^f	–	6.4/11.2	6.6/13.0	23	3
30	ACY30464	<i>Ficus pumila</i>	Cystatin	–	7.7/10.7	9.7/12.8	10	1
31	CAC16394	<i>Lupinus albus</i>	Conglutin γ – large subunit	–	7.7/31.0	8.7/29.2	16	3
32	CAC16394	<i>Lupinus albus</i>	Conglutin γ – large subunit	–	8.0/31.0	8.7/29.2	16	3
33	CAC16394	<i>Lupinus albus</i>	Conglutin γ – large subunit	–	8.0/30.8	8.7/29.2	16	3
34	A5JTO3	<i>Medicago varia</i>	β -xylosidase/ α -L-arabinofuranosidase 2	3.2.1.37 3.2.1.55	5.4/65.4	8.5/79.8 ^e	3 ^e	2
35	A5JTO3	<i>Medicago varia</i>	β -xylosidase/ α -L-arabinofuranosidase 2	3.2.1.37 3.2.1.55	5.1/65.4	8.5/79.8 ^e	3 ^e	2
36	A5JTO3	<i>Medicago varia</i>	β -xylosidase/ α -L-arabinofuranosidase 2	3.2.1.37 3.2.1.55	5.2/65.4	8.5/79.8 ^e	3 ^e	2
37	AAS97865	<i>Lupinus albus</i>	β -conglutin precursor	–	5.5/48.4	6.4/62.1	4	2
	ABN08398	<i>Medicago truncatula</i>	D-galactoside/L-rhamnose binding SUEL	–		6.6/93.2	1	1
39	ACU23118	<i>Glycine max</i>	Glycosyl hydrolase family 31 ^f	–	5.2/43.3	5.9/43.6	3	1
40	AAS97865	<i>Lupinus albus</i>	β -conglutin precursor	–	6.4/40.9	6.4/62.1	11	4
41	AAN15136	<i>Lupinus angustifolius</i>	Putative TAG factor protein	–	6.1/34.3	6.3/32.2	5	1
	BAD20774	<i>Raphanus sativus</i>	β -galactosidase	3.2.1.23		8.7/92.6	2	1
42	ABB13526	<i>Lupinus albus</i>	BLAD	–	9.7/19.4	9.7/20.4 ^e	68 ^e	9
43	ACU19711	<i>Glycine max</i>	SGNH plant lipasi-like ^f	–	4.5/39.0	4.6/39.7	8	2
45	ACU23118	<i>Glycine max</i>	Glycosyl hydrolase family 31 ^f	–	4.8/45.4	5.9/43.6	3	1
46	XP_002302122	<i>Populus trichocarpa</i>	Xylanase inhibitor I like	–	7.3/57.5	8.8/46.4	2	1
50	ACU16628	<i>Glycine max</i>	Fructose-bisphosphate aldolase ^h	4.1.2.13	6.1/42.7	5.7/29.5 ^g	12	2
51	ACU14241	<i>Glycine max</i>	Plant basic secretory protein ^f	–	5.1/27.0	4.9/25.2	12	3

Table 1. (Continued).

ID	Accession number	Species	Protein description	EC	pI ^a /M _r ^a	pI ^b /M _r ^b	Coverage (%) ^c	n _{pep} ^d
56	BAB78700	<i>Nicotiana tabacum</i>	UDP-glucose pyrophosphorylase	2.7.7.9	5.1/56.2	7.1/41.0 ^g	7 ^g	2
58	ACJ85059	<i>Medicago truncatula</i>	Annexin ^f	–	5.4/34.7	7.8/36.2	3	1
59	AAS97865	<i>Lupinus albus</i>	β-conglutin precursor	–	4.7/13.5	6.4/62.1	2	1
60	CAC42084	<i>Pisum sativum</i>	Thioredoxin h	–	4.9/12.1	4.8/13.1	21	3

^a Experimental molecular mass (kDa) or isoelectric point. ^b Theoretical molecular mass (kDa) or isoelectric point. ^c Amino acid coverage (%).

^d Number of the unique peptides identified in the search. ^e Values referred to the mature form of the protein. ^f Annotation indicated by the authors of the present study. ^g Partial sequence. ^h Annotation obtained by the meaning of BLAST alignment against the protein NCBI-nr database.

Table 2. Chitinase and endo-xylanase activities of exudates collected between 12 and 24 h after the start of germination. ND, not detectable.

Enzyme activity	Substrate	U·mL ⁻¹
Chitinase	4-nitrophenyl <i>N</i> -acetyl-β-D-glucosaminide ^a	110.8 ± 6.6
Chitinase	β-D- <i>N,N,N'</i> -triacetylchitotriose ^b	99.2 ± 6.1
Endo-xylanase (AsN) ^{c,e}	Tamarind xyloglucan ^e	ND
Endo-xylanase (XEG) ^{d,e}	Tamarind xyloglucan ^e	ND

^a *N*-acetylglucosaminidase activity. ^b Endo-chitinase activity. ^c AnX: endo-xylanase from *A. niger* (belonging to the GH11 family). ^d XEG: xyloglucan-specific endo-β-1,4-glucanase from *A. aculeatus* (belonging to the GH12 family). ^e Assayed in accordance with Scarafoni *et al.* [26].

plant defence mechanism against pathogens [41]. Most likely, the spots identified as BSPs represent isoforms of the protein differing with respect to pI and, altogether, account for more than 12% of the gel total spot volume.

Annexins (spots 14 and 58) are proteins that are able to bind phospholipids of endomembranes and the plasma membrane and thus are assumed to be involved in transport and sorting during esocytotic and endocytotic events [42]. Annexins could also play a role in Nod factor signalling for the establishment of symbiosis with *Rhizobium* [43].

Only a few spots were identified as vicilin or vicilin-derived polypeptides (spot numbers 13, 40 and 42). The sum of the spot volumes of these polypeptides accounted for approximately 0.8% of the total spot volume detected on gels. Only spot 13 refers to the full-length precursor form of this protein, which has a molecular mass of approximately 62 000 [23,35].

Spots 25, 26, 31, 32 and 33 were identified as polypeptides of γ-conglutin subunits. All of these spots represent approximately 5.0% of the total spot volume of the seed exudate (Table S1). γ-Conglutin is a tetra-

meric protein, with each monomer consisting of two disulfide-linked polypeptides of 29 and 17 kDa [26]. As already noted, γ-conglutin is most likely involved in plant defence mechanisms [23].

δ-Conglutin (spot number 4), which is the fourth SP quantitatively present in mature lupin seed [23], represents approximately 6% of the total volume of spots of the exudate proteome (Table S1). This protein belongs to the 2S sulfur-rich albumin family and is structurally related to cereal bifunctional trypsin/α-amylase inhibitors [44]. It is formed by two disulfide-linked subunits of 4 and 9 kDa, which originate from the post-translational proteolytic processing of a single precursor polypeptide during seed development [23,45]. The molecular mass of spot 4 (approximately 13 kDa) is in good agreement with that of the precursor form.

The polypeptide of spot 21 is a member of the PR-10 class. Although the localization is usually cytosolic [46], experimental evidence indicates that PR-10 proteins are also located in the cell walls and intercellular spaces [47]. These proteins are involved in the general defence mechanisms of plants in response to pathogen infection and abiotic stress [46,47].

Spots 28, 29 and 30 are isoforms of cystatin and comprise up to approximately 7% of the calculated total spot volume in exudates.

Finally, a variety of proteins found in the exudates have been described (in other plant systems) with roles in both biotic and abiotic stress tolerance, namely NADPH oxidoreductase (spot number 11), lactoylglutathione lyase (spot number 12), superoxide dismutase [Cu-Zn] (spot number 24) and thioredoxin (spot number 60).

Selective protein release

After characterization of the proteome of the seed exudate (Fig. 3A), two-dimensional electrophoretic analysis of the proteins present inside the cotyledons during the time of germination was carried out aiming

to shed light on the polypeptide release mechanisms and dynamics. The map shown in Fig. 3B refers to the sample taken 24 h after the onset of germination, although cotyledons gathered after 12 h have been also analyzed (Fig. 3C). These maps are representative of three independent experimental settings, each of them analyzed in duplicate. The electrophoretic pattern is essentially identical to that previously obtained from dry seeds [27], indicating that no major proteolytic events resulting from the action of already existing or newly-synthesized proteases [48] took place at these stages of germination. A comparison between these protein profiles and that of the released protein (Fig. 3A) confirms that the polypeptides released outside the seed do not have a passive leakage origin. Indeed, differences in the relative abundance of several polypeptides are clearly evident, although any quantitative statistical determination has not been carried out. The presence of a small amount of chitinase was detected inside the seed not only after 24 h (box number 1), but also after 12 h of incubation. In both cases, the relative abundances of the protein are much lower than those found in exudates (Fig. 3A). In the same region of the gel are polypeptides identified as plant BSPs (spots 15, 16, 17 and 51) that are undetectable on the gels for cotyledonary proteins (box number 2). Box number 3 highlights a virtual absence inside cotyledons of the proteins identified as glucanases on the maps of the exudates (spots 6, 7, 34, 35 and 36). These spots were also undetectable on the sample from 12 h previously (Fig. 3C). Other relevant differences are related to vicilins, which were scarcely present in the germinating media (Fig. 3A and Tables 1 and S1). During germination, vicilin polypeptides are promptly hydrolyzed by a number of specific proteases, some of which are already present in the mature dry seeds, and others are synthesized *ex novo* [48,49]. The main breakdown products (box number 3) were not released in the exudates but, instead, accumulated inside cotyledons from the very beginning (Fig. 3B,C). These polypeptides are not present before germination, namely in dry mature seeds [27,34].

Before undertaking any expression studies, we investigated the possibility that vacuole-deposited proteins might be directly secreted into the medium via a non-classical pathway, namely through secretion not triggered by a signal peptide. This eventuality was previously proposed for vacuolar enzymes such as chitinase, α -mannosidase and β -1,3-glucanases [50]. The amino acid sequences of the most significant or abundant proteins identified by MS were analyzed by using the neural network-based software SECRETOMEP, version 2.0 (<http://www.cbs.dtu.dk/services/Secretom->

[ep](http://www.cbs.dtu.dk/services/Secretom-)) [51]. Although the available training data sets are not specific for plant proteins, SECRETOMEP has been widely employed in the predictive characterization of *A. thaliana* and *M. truncatula* cell cultured secretomes [52,53], as well as other plant systems [53,54]. All of the submitted sequences of the signal peptide (if any) have been removed before prediction. The outputs are summarized in Table 3. The majority of searched proteins have scores higher than the threshold value, indicating that they might be secreted via a pathway where the proteins pass through the vacuolar compartment. It is worth noting that, although the calculated neural network score of the vicilin β -conglutinin was below the threshold value, its proteolytically derived peptide BLAD (spot 42) showed a score of 0.81. This suggests that polypeptides originating in the vacuolar compartment might be secreted via nonconventional mechanisms.

Expression profiles

The possible neosynthesis of the main proteins found in the germination medium was investigated by mean of the incorporation of radioactive amino acids and by quantitative RT-PCR. The results of the protein labelling experiments are shown in Fig. 4. Before 12 h has elapsed from the beginning of germination, a variety of labelled polypeptides were observable in the cotyledons (Fig. 4, lane 1), indicating that protein synthesis took place during imbibition. Instead, radioactive polypeptides appeared in exudate samples only after at least 12 h (Fig. 4, lanes 3 and 4). Here, a band of 30 kDa, not particularly visible inside cotyledons and with a molecular mass coincident with that of chitinase

Table 3. Predictions of nonclassical protein secretion by SECRETOMEP (<http://www.cbs.dtu.dk/services/SecretomEP/>). Neural network (NN) scores > 0.5 are considered to predict extracellular localization. γ -conglutin consists of two disulfide-linked polypeptides of 29 and 17 kDa [26].

Spot number	Protein name	UniprotKB ID	SECRETOMEP
			NN score
2	α -galactosidase	P14749	0.56
3	Chitinase III	O49876	0.73
4	δ -conglutin	Q333K7	0.68
13	β -conglutin	Q53HY0	0.44
14	Annexin	C6TM48	0.72
16	Basic secretory protein	C6SYB8	0.48
21	PR-10	Q93XI0	0.42
25 + 31	γ -conglutin	Q9FSH9	0.67
29	Cystatin	A5B2E1	0.64
42	BLAD	Q0RON3	0.81

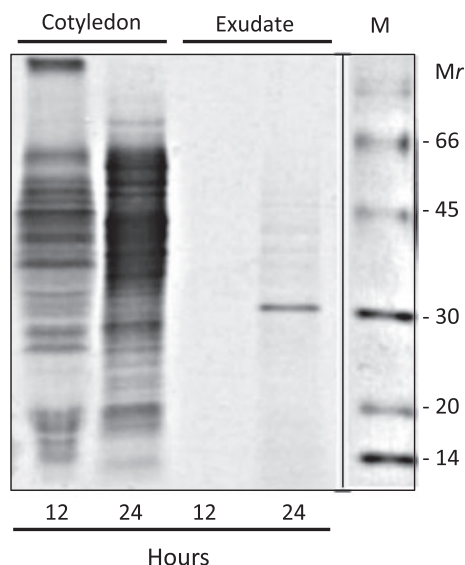


Fig. 4. Fluorography of SDS/PAGE of proteins labelled with ^{35}S -Met and ^{35}S -Cys, extracted from germinating cotyledons and collected from the exudates. Samples were analyzed 12 and 24 h after the start of germination.

III, did stand out from a number of other much less conspicuous bands.

To determine whether the *de novo* biosynthesis of the main released proteins might be a result of specific transcriptional events, total RNAs were extracted from germinating seed cotyledons and analyzed by PCR after the conversion of mRNAs to cDNAs (quantitative RT-PCR). The target gene was chitinase III, although other genes have been also selected, namely vicilin and the two γ -conglutins (C γ A32 and C γ E48) genes [55]. Vicilin, as a typical storage protein, was not expected to be expressed during germination. γ -Conglutin, as noted above, is expressed as a defence protein. The expression of the C γ A32 gene has been already analyzed previously [27] and, thus, the presence of the specific cDNA has been considered in the present study only as an experimental control. The C γ E48 gene was previously found to be expressed during late germination [55], although it has never been investigated in detail. The 18S gene was chosen as an internal control for data normalization. It has been established previously that the 18S gene displays a highly uniform expression within tissue samples during various phases of development and under different environmental conditions [56,57]. The results are shown in Fig. 5. Mature seeds contain conspicuous amounts of mRNAs [26] that constitute the pool of stored mRNAs formed at the late stage of seed maturation and are conserved in quiescent seeds to support germination [58]. The relative quantities of the four mRNAs are shown in

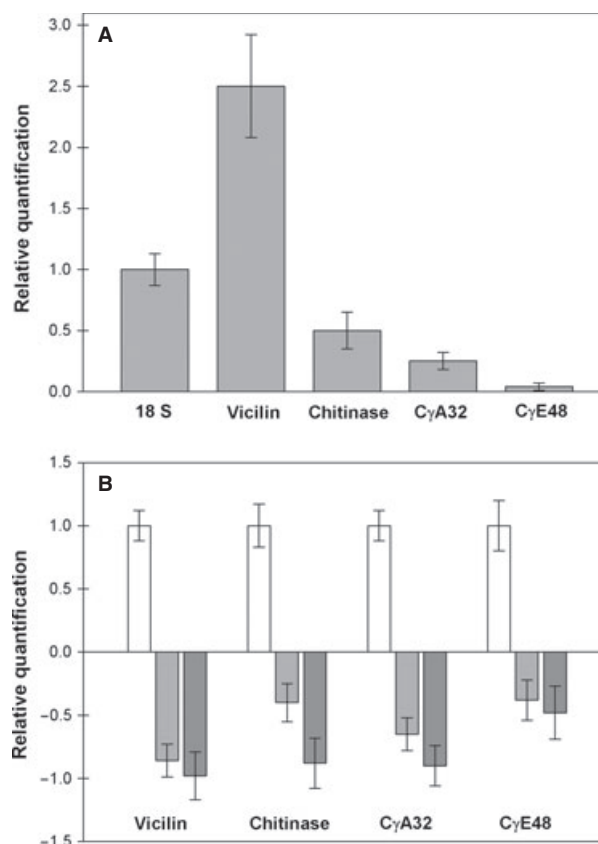


Fig. 5. Quantitative RT-PCR for relative quantification of vicilin (EMBL ID: AJ966470), chitinase III (EMBL ID: [Y16415](#)), γ -conglutins C γ A32 and C γ E48 (EMBL ID: AJ297490 and AJ297568, respectively) genes during germination. The housekeeping 18S rRNA (GenBank ID: CP002686.1) gene was used as normalizer. (A) Relative quantification of stored mRNAs in lupin seeds before the start of germination (t_0). (B) Relative quantification of expression during germination in water. After normalization, the control samples were set to one. White bars: control (t_0), light grey bars: 24 h, dark grey bars: 48 h. Standard errors represent biological repeats.

Fig. 5A. The analyses indicate that, with the amount of stored 18S mRNA set arbitrarily as '1', the quantities of vicilin, chitinase and C γ A32 mRNAs were approximately 2.5-, 0.5- and 0.25-fold that of stored 18S mRNA, respectively, whereas the amount of C γ E48 mRNA was one order of magnitude lower. The absolute quantification of such molecules is beyond scope of the present study.

The mRNAs coding for the four selected proteins extracted from overnight-vernalized seeds have been used as the control sample (t_0) and their amounts are set to a value of '1' in Fig. 5B. No *ex novo* transcription was evident within 48 h (Fig. 5B). mRNAs disappeared very rapidly during the time of germination and, thus, these selected transcripts are degraded

during germination. It is a general hydrolytic mechanism because all of the mRNA species considered in the present study underwent the same fate. The data shown in Fig. 5B suggest that the different kinds of mRNA disappeared at different rates, with the vicilin gene being faster and the C γ E48 gene being slower at decreasing the amounts. By contrast, the results (Figs 4 and 5B) indicate that the stored mRNAs may be translated to new polypeptides from the first moment of germination onward.

Antifungal activity of the exudates

Before testing their ability to inhibit fungi growth, exudates collected between 12 and 24 h after germination were subjected to gel filtration using a desalting resin, as described in the Experimental procedures. This chromatographic step allowed the removal of any organic compound with a molecular mass < 5000 Da from the protein fraction that might have the capacity to influence pathogen growth [9,10]. Aliquots of the resulting solution were added to fungal conidia suspensions, as described in the Experimental procedures. The pathogenic fungi *Fusarium verticillioides*, *Fusarium oxysporum*, *Botrytis cinerea*, *Alternaria solani*, *Aspergillus niger* and *Penicillium expansum* were selected as representatives of Eumycota containing chitin and β -1,3-glucans as structural cell wall components of hyphae and conidia [59–61]. Germination of the conidia and hyphal elongation of five out of six species were inhibited, although at different levels (Fig. 6). The only exception was *F. verticillioides*, which showed an increase of fungal biomass with respect to the control. Germination of *F. oxysporum* conidia was slightly inhibited (< 20% inhibition), whereas *A. niger* and *P. expansum*

were the most inhibited (approximately 80% inhibition).

Discussion

The present study describes the proteome accumulated in the seed exudates and the mechanisms at the basis of the selective protein release. An attempt to characterize the full set of proteins was impaired by the still largely insufficient information available in genomic, expressed sequence tags and protein databases for lupin sequences, as already reported and discussed previously [23,27]. After an initial burst of protein release during the first few hours, the exudates between 12 and 24 h are composed of a specific set of proteins selectively secreted from lupin seeds and connected with lytic processes (e.g. chitinase) and defence mechanisms against pathogenic fungi.

The influx of water into the cells of dry seeds not only causes structural perturbations to membranes leading to leakage of solutes into the surrounding imbibition solution [2,62], but also the outflow of cellular content, including starch grains and entire PBs [63], where the majority of the seed proteins are stored [13,45]. This generates an extrusion stream into the germinating medium [64]. It is expected that, shortly after rehydration, any spillage from the seed is drastically reduced or even stopped [62], including the stream of PBs [63,64]. In the present study, the proteins found in exudates collected during the first 12 h reflected the cotyledonary composition (Fig. 2). Afterwards, namely between 12 and 24 h after the beginning of germination, the protein composition of the exudate drastically differs from that of the seed. The number of polypeptides reduced and a band of 30 kDa prevailed (Fig. 2A). Proteins delivered by cell breakage would be expected to accumulate in the medium with similar kinetics. β -Conglutin and γ -conglutin have been used as molecular markers to monitor the flux of proteins from the seed because both are deposited in the same storage vacuoles during seed development [23]. The release modalities of the two proteins are clearly different. The leak of β -conglutin into the medium occurred only during the first 12 h. Subsequently, the presence of this protein outside the seed was scarcely detectable. The small amount of full-length β -conglutin found in the medium (spot 13) may derive from an early and transient translation of specific stored mRNA, which is particularly abundant in dry mature lupin seeds (Fig. 5). Alternatively, it may be part of a residual loss of cellular material after ruptures occurring during seed imbibition. By contrast, a continuous release of γ -conglutin was observed, although, in this case, no

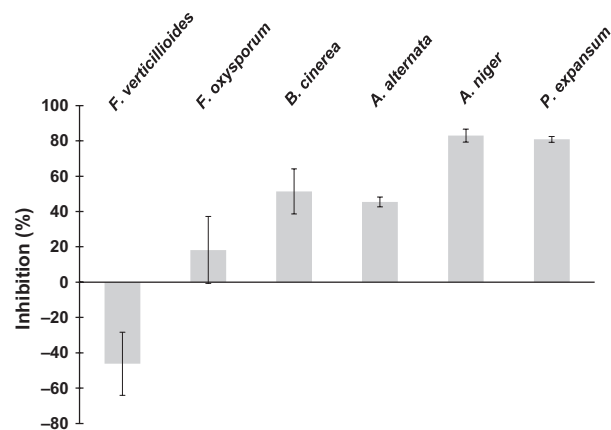


Fig. 6. Antifungal activity of seed exudate. Data are expressed as a percentage of growth inhibition of biomass of five fungi tested after 24 h of incubation of conidia suspension with the exudate.

newly-synthesized polypeptides were detected outside the seed. In conclusion, the extrusion stream of storage vacuoles contributes to protein release only at the very beginning of germination and this is not the sole mechanism responsible for the flux of polypeptides to seed exudates. It is not unlikely that the extrusion process itself could play an actual role in defence mechanisms, with it being a source of pre-formed bioactive molecules on which plants rely during germination [2,5,31,65]. More selective and regulated events, such as secretory processes, took place soon after the onset of germination. The proteomic analysis provides evidence to support this hypothesis. The virtual absence of proteins in the secretome that are normally abundant in the plant cytoplasm (e.g. ribosomal proteins, actin, tubulin and glycolytic enzymes) confirms that the appearance of these proteins is not a result of nonspecific cell lysis. A comparison of the electrophoretic maps of the released protein and those extracted from cotyledons (Fig. 3) also confirms that the polypeptides released outside the seed do not have a passive leakage origin. Indeed, differences in the relative abundance of several polypeptides are clearly evident. Chitinase, glucanases and BSPs largely predominate into the exudate, accounting for approximately 65% of the exudate proteins, whereas SP polypeptides represent only 10–12% of the total protein (half of which was δ -conglutin). Conversely, SPs comprise approximately 85% of the total seed proteins deposited inside cotyledons during seed development [23]. In addition, polypeptides ascribable to legumins have been identified in the exudates. Legumins are the second most highly represented SPs, accounting for approximately 35% of the total lupin seed proteins [23]. Unlike vicilins, no specific biological activities have been described for legumins or legumin-derived fragments.

As already noted, vicilins are commonly considered as reserve proteins, although their possible role in defence processes has been also proposed. Protein cleavage at germination is assumed to occur via a regulated mechanism involving the selective breakdown of specific peptide bonds which, before the native conformation is critically altered to allow the complete hydrolysis, results in the origin of large polypeptide fragments [48,49]. In recent studies, the activation of specific functions upon the limited cleavage of storage proteins has been reported [5,17]. This is the case of BLAD (spot number 42), a stable intermediary product of vicilin breakdown, which accumulates in cotyledons of *Lupinus* species during germination and shows defence and antibiotic properties [31]. Accordingly, the presence of cystatins in exudates is relevant. Cystatins form a family of cysteine protease inhibitors that are

widely distributed in plant seeds. They are involved in a large variety of physiological processes, ranging from the control of endogenous proteolysis to the inhibition of digestive proteases of herbivore arthropods, parasitic nematodes and microbial pathogens [66]. Cystatins are synthesized in developing seeds and promote the deposition of storage proteins by inhibiting endogenous intracellular proteases [67]. A cystatin/cys-protease balance maintained in dormant tissues acts to preserve the storage proteins over dormancy. The induction of protease-encoding genes and a concomitant repression of cystatin-encoding genes after imbibition leads to a decrease of the cystatin/cys-protease balance, which triggers storage protein hydrolysis and mobilization [68]. Release into the germinating medium of these protease inhibitors may effectively contribute to this regulatory mechanism. Cystatins have been predicted by SECRETOMEP software (Table 3) to be potentially secreted via nonclassical pathways.

The incorporation of radiolabelled amino acids indicated that protein synthesis took place during or immediately after imbibition, although radioactive polypeptides appeared in exudate samples only after at least 12 h (Fig. 4). Expression analysis (Fig. 5) revealed that *de novo* transcription of the major lupin seed proteins and chitinase III genes did not occur, even after a time period longer than those considered in the overall experimental setting of the present study. The presence of secreted chitinase III outside the seed can thus be considered as transient. The eventuality that small amounts of seed-stored chitinase [36] may be secreted along neosynthesized chitinase (Fig. 4) should be taken into account (Table 3).

Chitinase enzymatic activity was certainly responsible for the capacity of the exudate to inhibit conidia germination and hyphal elongation of the selected fungal species (Fig. 6). By contrast, the antifungal activity of *L. albus* chitinase III has been demonstrated previously *in vitro* [36]. The enzyme was stable for at least 24 h after secretion because activity was fully retained after incubation of the collected exudate at 20 °C for 24 h (Table S3). The potential persistence of chitinase activity outside the seed might suggest specific functionalities. Apart from the role as an inhibitor of the growth of fungi via hydrolysis of cell wall chitin [69], a possible function of chitinases is to generate signal molecules. These molecules not only elicit plant resistance to external environmental factors, but also are involved in plant growth and development events, such as host specificity of *Rhizobia*, nodule development and the process of cold acclimatization [70,71].

In conclusion, the present study unveils a well-defined process that takes place soon after imbibition

of germinating seeds via the selective secretion of proteins involved in defence mechanisms against fungal pathogens.

Experimental procedures

Lupinus albus L. seeds of the sweet Multitalia variety were kindly provided by A. Conocchiari (Agroservice SpA, S. Severino Marche, Italy). All reagent grade chemicals were obtained from Sigma-Aldrich (Milan, Italy) unless otherwise indicated. Media for fungal growth were obtained from Difco (Becton Dickinson & Company, Spark, MD, USA).

Protein concentrations were determined by the dye binding method of Bradford [72] using BSA as the standard.

Seed germination and sampling

Lupin seeds were surface sterilized with 0.5% sodium hypochlorite, washed extensively with distilled water and kept overnight at 4 °C to vernalize. The seeds have been then manually dehulled, taking care not to damage the embryos. Five seeds were incubated under mild shaking in distilled water (1 mL per seed) at 20 °C in a 50-mL Falcon tube capped with a sterile cotton plug and kept slightly slanted. Alternatively, the germination media were removed every 12 h and replaced with sterile distilled water (pre-warmed at 20 °C). In both cases, each experimental session or experimental point was made by 15 different tubes, the contents of which were pooled at the end of the incubation time. The samples were immediately centrifuged at 6000 *g* for 10 min to remove any insoluble material and frozen at −80 °C until analyzed.

Protein sample processing for electrophoresis

For preparation of the proteins contained in the germination media, collected samples were lyophilized and resuspended in 5 mL of distilled water. Proteins were precipitated by adding trichloroacetic acid (final concentration: 10% v/v), followed by extensive washing with cold acetone. Pellets were air-dried and dissolved in 500 µL of 10 mM NaOH for protein quantification, or of a solution consisting of 7 M urea, 2 M thiourea, 2% Chaps and 65 mM dithiothreitol, if the proteins were further subjected to electrophoresis.

Protein sample processing for biological activity assays

The proteins contained in the pooled germination media were lyophilized and resuspended in 5 mL of sterile distilled water. The solutions were then loaded onto PD-10 desalting disposable columns (GE Healthcare, Milan, Italy) in accordance with the manufacturer's instructions. The

columns were previously equilibrated in 50 mM sodium citrate buffer (pH 6.5). The fraction containing the high molecular mass molecules was collected and immediately concentrated to 20 mg·mL^{−1} using an Amicon Ultra-4 centrifugal filter device (Millipore, Bedford, MA, USA) with a cut-off of 10 kDa.

Potassium content determination

Before reading potassium ion content in germination media, all the used glassware was washed with nitric acid (20% v/v) and then rinsed with distilled water. For each experimental point, the medium of three independent flasks was pooled, diluted 1000 times with distilled water, and analyzed by inductively-coupled plasma (ICP) techniques (Liberty AX sequential ICP-OES; Varian, Palo Alto, CA, USA) using calibration solutions in the range 0.05–10 µg·mL^{−1}. Internal standards were added for all samples.

SDS/PAGE and western blotting

SDS/PAGE was performed according to Laemmli [73]. Protein samples were denatured in the presence of β-mercaptoethanol by heating at 100 °C for 10 min in the SDS/PAGE sample buffer and then loaded onto SDS gels. The run was carried out at 16 mA, constant for each gel. After electrophoresis, the protein bands were visualized using Coomassie Brilliant Blue (CBB). Molecular mass markers were: β-phosphorilase (92 kDa), BSA (66 kDa), egg albumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and lysozyme (14 kDa).

Electrotransfer onto nitrocellulose membranes was performed for 1 h at 50 mA with a semi-dry apparatus (GE Healthcare) using 39 mM glycine, 48 mM Tris-HCl (pH 9), 1.3 mM SDS and 12% methanol as transfer buffer. Membranes were blocked for 2 h with casein (2% w/v) in NaCl/P_i (15 mM NaH₂PO₄ and 150 mM NaCl, pH 7.2). The membranes were equilibrated in NaCl/P_i containing 0.05% Tween 20 and incubated with anti-γ-conglutinin antibodies, diluted 1 : 1500 in the same buffer, or ConA (20 µg·mL^{−1}). To visualize the bands in the first case, horseradish peroxidase conjugate with goat anti-rabbit antibodies (dilution 1 : 3000; Bio-Rad, Hercules, CA, USA) and H₂O₂ with 4-chloronaphthol as substrate were used. Following reaction with ConA, the positive bands were visualized by the peroxidase reaction according to Hawkes [74].

Two-dimensional IEF/SDS/PAGE and image processing

IEF was performed on 13-cm, pH 3–10 linear IPG strips (GE Healthcare). The strips were rehydrated overnight with a solution of 7 M urea, 2 M thiourea, 2% Chaps, 65 mM dithiothreitol, 2% IPG buffer pH 3–10 (GE Healthcare).

Approximately 250 µg of proteins were applied with the cup loading system positioned near the anode. Strips were focused at 40 000 Vh, with a maximum of 6500 V, at 20 °C using an Ettan IPGphor II electrophoresis unit (GE Healthcare). Prior to the second dimension, strips were incubated in equilibration buffer (375 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol) with 65 mM dithiothreitol for 15 min, and then with 243 mM iodoacetamide in the same buffer without dithiothreitol for 10 min. The second dimension was performed on 15 × 15 × 0.1 cm 12% acrylamide gels, containing 0.2% bis-acrylamide. The running buffer consisted of 2.5 mM Tris-base, 192 mM glycine and 0.01% SDS. The electrophoresis conditions for each gel were 2 mA for 20 min, 4 mA for 20 min, 12 mA for 40 min and 22 mA until the end of the run. The gels were stained overnight with 0.25% CBB R-250 in ethanol/acetic acid/water (20 : 10 : 70) and destained with the same solution without the dye, until a colourless background was achieved. The gels were digitalized in an Epson Expression 1680 Pro Scanner (Epson Corp., Long Beach, CA, USA) and analyzed with IMAGEMASTER 2D PLATINUM software (GE Healthcare). Master gels were generated for each sample by matching all the available gels with the same software, with the spot detection parameters: smooth 4, saliency 1.7 and minimal area 24. When needed, manual matching complemented computer-assisted matching. To determine the quantitative presence of the spots in the proteomic maps, the relative spot volumes were evaluated using the same software. Data are given as the mean of the values calculated for each gel analyzed.

LC-ESI-MS/MS analysis

Spots excised from gels stained with CBB were digested as described by Prinsi *et al.* [75]. The LC-ESI-MS/MS experiments were conducted using a Finnigan Surveyor (MS pump Plus) HPLC system directly connected to the ESI source of a LCQ DECA XP MAX ion trap mass spectrometer (ThermoFisher Scientific Inc., Waltham, USA). Chromatography separations were obtained on a BioBasic C18 column (inner diameter 180 µm, length 150 mm length, particle size 5 µm; ThermoFisher Scientific Inc.), using a linear gradient from 5% to 80% solvent B [solvent A: 0.1% (v/v) formic acid; solvent B: acetonitrile containing 0.1% (v/v) formic acid] with a flow of 3 µL·min⁻¹. ESI was performed in positive ionization mode with spray voltage and capillary temperature set at 3 kV and at 220 °C, respectively. Data were collected in the full-scan and data dependent MS/MS mode with collision energy of 35% and a dynamic exclusion window of 3 min. Spectra were searched by TURBOSEQUENCE incorporated in BIOWORKS BROWSER, version 3.2 (ThermoFisher Scientific Inc.) against the *Fabaceae* protein subset (66061 entries), *Fabaceae* expressed sequence tag subset (3040898 entries) and against the protein NCBI-nr database (11160352 entries), all available from the National Center for Biotechnology Information

(<http://www.ncbi.nlm.nih.gov/>). The searches were carried out by assuming parent ion and fragment ion mass tolerances of ± 2 and ± 1 Da, respectively, one possible missed cleavage per peptide, fixed carboxyamidomethylation of cysteine and variable methionine oxidation. Positive hits were filtered on the basis of peptides scores [$xcorr \geq 1.5$ (+1 charge state), ≥ 2.0 (+2 charge state), ≥ 2.5 (≥ 3 charge state), $\Delta C_n \geq 0.1$, peptide probability $< 1 \times 10^{-3}$ and $Sf \geq 0.70$]. If needed, identified peptides were used in protein similarity search performed by alignment analysis against the NCBI-nr database using the FASTS algorithm [76]. Physical properties of the characterized proteins were predicted and calculated using *in silico* tools (<http://expasy.org/>).

Antifungal activity assay

The biological activity of the seed exudates prepared as described before was assessed in 96-well microplates (Sero-wel; Bibby Sterilin Ltd, Stone, UK) in three replicas in accordance with the quantitative method proposed by Raposo *et al.* [77]. Isolates of *F. verticillioidea* (Sacc.) Nirenberg, *F. oxysporum* Schlechtendahl emend. Snyder & Hansen, *B. cinerea* Pers., *A. solani* Sorauer, *A. niger* Tiegh. and *P. expansum* Link, stored in potato dextrose agar slants kept at 4 °C, were grown on the same medium in Petri dishes for 7 days at 24 °C. Conidial suspensions from each fungus were prepared by transferring a loop of mass spores differentiated on the mycelium surface in a 1.5-mL tube containing sterile potato dextrose broth at double concentration and adjusted at the final count of 2×10^4 conidia·mL⁻¹. The exudate was tested at the final concentration of 1 mg per well. Wells were filled with 50 µL of conidial suspension and 50 µL of seed exudate. Each fungus was tested in 30 wells, whose half represented the untreated control (sterile distilled H₂O without exudate). A_{492} for each well was measured using a Sunrise Absorbance Reader (Tecan Group Ltd, Männedorf, Switzerland) immediately before (t_0) and after incubation for 24 h (t_{24}) at 24 °C in the dark. The growth of each strain was quantified in both treated and untreated wells by comparing the absorbance values at t_0 and at the end of the incubation period. Growth inhibition ($I\%$) was calculated as percentage according to: $[(AC_{t_{24}} - AC_{t_0}) - (AT_{t_{24}} - AT_{t_0})] \times 100 / (AC_{t_{24}} - AC_{t_0})$, where AC_{t_0} and AT_{t_0} are the initial absorbance of control and protein-treated samples, whereas $AC_{t_{24}}$ and $AT_{t_{24}}$ are the final ones, respectively.

Protein labelling during seed germination

Lupin seeds were surface sterilized with 0.5% sodium hypochlorite and washed with sterile water. The seeds were placed in capped glass vials with 450 µL per seed of labelling solution, prepared by diluting 500 µCi per seed of radioactive [³⁵S]Met/Cys (Redivue Pro-Mix; Amersham,

Little Chalfont, UK) in sterile water, and incubated overnight at 4 °C with mild shaking. Seeds were extensively washed with water and then transferred into a 50-mL Falcon tube (five seeds per tube) and incubated with water (1 mL per seed) at 20 °C with mild shaking. The germination media were removed every 12 h for analysis and replaced with fresh distilled water (pre-warmed at 20 °C). Proteins were extracted from the cotyledons according to Magni *et al.* [27], whereas those contained in germination media were prepared as described previously. Samples were submitted to SDS/PAGE. Gels were fluorographed at −80 °C for 1 week, after treatment with dimethylsulfoxide/2,5-diphenylloxazole and drying.

RNA extraction and transformation into cDNA

Total RNAs were extracted from 100 mg of cotyledonary tissue using the RNeasy Plant Mini Kit (Qiagen, Milan, Italy) in accordance with the manufacturer's instructions. In addition, a digestion step with DNase (DNase I-RNase free; Qiagen) was performed at 37 °C for 15 min to remove any possible genomic DNA. RNA was quantified with a biophotometer (Eppendorf, Milan, Italy) at 260 nm using a Tray-Cell (Hellma, Müllheim, Germany). Typically, the yield of total RNA was in the range 20–32 µg per 100 mg of tissue. The samples were stored at −80 °C until use. Five hundred nanograms of total RNA were transformed into cDNA by reverse transcription reaction using the cDNA synthesis kit (iScript cDNA Synthesis kit; Bio-Rad) with random primers in a total volume of 20 µL. The reaction conditions adopted were 20 min at 40 °C, 20 min at 42 °C, 20 min at 45 °C and 5 min at 85 °C for blocking the reaction. The cDNA samples obtained were diluted 1 : 100 with sterile water before use as templates in quantitative PCR.

Quantitative RT-PCR and data analysis

Primer pair selection was carried out using PRIMER3 (<http://frodo.wi.mit.edu/>). The sequences to be amplified were pitched on: CγA32 and CγE48 genes (EMBL ID: AJ297490 and AJ297568, respectively), encoding for two isoforms of seed glycoprotein γ-conglutin [54]; chitinase class III gene (EMBL ID: [Y16415](#)); and vicilin gene (EMBL ID: AJ966470) 18S rRNA (GenBank ID: CP002686.1). Intron-specific sequences have been not considered. The primers used are listed in Doc. S1. Specificity of the primers was checked by BLAST searches through the EMBL nucleotide database. Except for the expected results, no other significant similarities were found. Potential primer–dimer formation was checked using the OLIGO ANALYSIS AND PLOTTING TOOL (<http://www.operon.com/tools/oligo-analysis-tool.aspx?>).

Reactions were carried out in triplicate using an iCycler thermocycler equipped with a MyiQ detection system (Bio-Rad), in 96-well reaction plates sealed with optical tapes

(Bio-Rad). Reactions were set up in a final volume of 20 µL using iQ SYBR Green Supermix (Bio-Rad, Milan, Italy) with 2 µL of diluted cDNA samples. Each primer was added to a final concentration of 0.25 µM. The cycling conditions were: one cycle at 95 °C for 3 min; 40 cycles of 95 °C for 15 s (denaturation), 60 °C for 30 s (annealing and extension). The thermal profile used was the same for all target genes. The fluorescence signal was captured at the end of each cycle (excitation wavelength of 490 nm). Melting curves were obtained by progressive heating at 0.3 °C every 15 s starting from 70 °C to check the specificity of the analysis. Each amplicon was then been checked for correct identity by sequencing. For each primer pair, reaction efficiencies were determined from the standard dilution series of lupin genomic DNA [26] spanning five orders of magnitude and were found to range between 98% and 102% (Doc. S1). Data were collected and processed, including baseline subtraction and threshold definition, with iq5 software (Bio-Rad). No-template control and not retro-transcribed samples (10 ng of RNA) were also included in the experimental set to detect spurious signals arising from the amplification of any DNA contamination or primer dimers formed during the amplification reaction. Raw data were analyzed with iq5 software with the parameters: baseline from the second to the tenth cycle, with the threshold calculated by the software for every reaction. Prior to acceptance of data for quantitative work, the amplicons in all samples were checked for specificity by analysis of the dissociation curve. Differences in gene expression were calculated by the comparative $\Delta\Delta C_t$ method [78] with a dedicated Excel (Microsoft Corp., Redmond, WA, USA) macro created by Bio-Rad. cDNA from seeds after overnight vernalization (t_0) were used as control samples for quantification of those corresponding to test samples. All quantifications were normalized to the housekeeping 18S.

Enzyme activities and inhibitory assays

The chitinase activity of the exudates was assessed with the Chitinase Assay Kit (Sigma-Aldrich, catalogue number CS0980) using 4-nitrophenyl *N*-acetyl-β-D-glucosaminide and 4-nitrophenyl β-D-*N,N',N''*-triacetylchitotriose as substrates, in accordance with the manufacture's instructions. Endo-xylanase activities and inhibitory assays were carried out using xyloglucan-specific endo-β-1,4-glucanase from *Aspergillus aculeatus* (XEG) and *A. niger* β-xylanase (AnX) according to Scarafoni *et al.* [26].

Acknowledgements

We thank Dr R. Bollini and Dr F. Sparvoli (IBBA-CNR, Milan, Italy) for sharing facilities and for assistance with the radiochemical incorporation experiments.

References

- Bewley JD (1997) Seed germination and dormancy. *Plant Cell* **9**, 1055–1066.
- Nelson EB (2004) Microbial dynamics and interactions in the spermosphere. *Annu Rev Phytopathol* **42**, 271–309.
- Crowe JH & Crowe LM (1992) Membrane integrity in anhydrobiotic organisms: toward a mechanism for stabilizing dry seeds. In *Water and Life* (Somero NG, Osmond CB, Bolis CL, eds), pp. 87–103. Springer-Verlag, Berlin.
- Barbour WM, Hattermann DR & Stacey G (1991) Chemotaxis of *Bradyrhizobium japonicum* to soybean exudates. *Appl Environ Microbiol* **57**, 2635–2639.
- Rose TL, da Silva Conceicao A, Xavier-Filho J, Okorokov LA, Fernandes KVS, Marty F, Marty-Mazars D, Carvalho AO & Gomes VM (2006) Defense proteins from *Vigna unguiculata* seed exudates: characterization and inhibitory activity against *Fusarium oxysporum*. *Plant Soil* **286**, 181–191.
- Nobrega FM, Santos IS, Da Cunha M, Carvalho AO & Gomes VM (2005) Antimicrobial proteins from cowpea root exudates: inhibitory activity against *Fusarium oxysporum* and purification of a chitinase-like protein. *Plant Soil* **272**, 223–232.
- Peters NK & Long SR (1988) Alfalfa root exudates and compounds which promote or inhibit induction of *Rhizobium meliloti* nodulation genes. *Plant Physiol* **88**, 396–400.
- Mel'nikova NN & Omel'chuk SV (2009) Effect of legume seed exudates on the formation of *Rhizobium*–legume symbiosis. *Appl Biochem Microbiol* **45**, 297–302.
- Casey CE, O'Sullivan OB, O'Gara F & Glennon JD (1998) Ion chromatographic analysis of nutrients in seed exudate for microbial colonization. *J Chromatogr* **804**, 311–318.
- Terras FRG, Eggermont K, Kovaleva V, Raikhel NV, Osborn RW, Kester A, Rees SB, Torrekem S, Van Leuven F, Vanderleyden J *et al.* (1995) Small cysteine-rich antifungal proteins from radish: their role in host defense. *Plant Cell* **7**, 573–588.
- Krishnan HB, Karr DB & Emerich DW (1999) Purification of an autophosphorylating protein from imbibing soybean (*Glycine max* L.) seed exudate and its identification as a nucleoside diphosphate kinase. *J Plant Physiol* **154**, 584–590.
- Guéguen J & Cerletti P (1994) Proteins of some legume seeds: soybean, pea, fababean and lupin. In *New and Developing Sources of Food Proteins* (Hudson BJB, ed.), pp. 145–193. Chapman & Hall, New York.
- Derbyshire E, Wright DJ & Boulter D (1976) Legumin and vicilin, storage protein of legumes seeds. *Phytochemistry* **15**, 3–24.
- Gomes VM, Mosqueda M-I, Blanco-Labra A, Sales MP, Fernandes KVS, Cordeiro RA & Xavier-Filho J (1997) Vicilin storage proteins from *Vigna unguiculata* seeds inhibit fungal growth. *J Agric Food Chem* **45**, 4110–4115.
- Wang X, Bunkers GJ, Walters MR & Thoma RS (2001) Purification and characterization of three antifungal proteins from cheeseweed (*Malva parviflora*). *Biochem Biophys Res Commun* **282**, 1224–1228.
- Wang X, Thoma RS, Carroll JA & Duffin KL (2002) Temporal generation of multiple antifungal proteins in primed seeds. *Biochem Biophys Res Commun* **292**, 236–242.
- Marcus JP, Green JL, Goulter KC & Manners JM (1999) A family of antimicrobial peptides is produced by processing of a 7S globulin protein in *Macadamia integrifolia* kernels. *Plant J* **19**, 699–710.
- Terras FRG, Goderis IJ, Van Leuven F, Vanderleyden J, Cammue BPA & Broekaert WF (1992) *In vitro* antifungal activity of a radish (*Raphanus sativus* L.) seed protein homologous to nonspecific lipid transfer protein. *Plant Physiol* **100**, 1055–1058.
- Agizzio AP, Carvalho AO, Ribeiro Sde F, Machado OL, Alves EW, Okorokov LA, Samarão SS, Block C Jr, Prates MV & Gomes VM (2003) A 2S albumin-homologous protein from passion fruit seeds inhibits the fungal growth and acidification of the medium by *Fusarium oxysporum*. *Arch Biochem Biophys* **416**, 188–195.
- Domoney C (1999) Inhibitors of legume seeds. In *Seed Proteins* (Shewry PR & Casey R, eds), pp. 635–655. Kluwer Academic Publishers, Amsterdam.
- North H, Baud S, Debeaujon I, Dubos C, Dubreucq B, Grappin P, Jullien M, Lepiniec L, Marion-Poll A, Miquel M *et al.* (2010) *Arabidopsis* seed secrets unravelled after a decade of genetic and omics-driven research. *Plant J* **61**, 971–981.
- Gallardo K, Le Signor C, Vandekerckhove J, Thompson RD & Burstin J (2003) Proteomics of *Medicago truncatula* seed development establishes the time frame of diverse metabolic processes related to reserve accumulation. *Plant Physiol* **133**, 664–682.
- Duranti M, Consonni A, Magni C, Sessa F & Scarafoni A (2008) The major proteins of lupin seed: characterisation and molecular properties for use as functional and nutraceutical ingredients. *Trends Food Sci Technol* **19**, 624–633.
- Duranti M, Faoro F & Harris N (1994) The unusual extracellular localization of conglutin γ in germinating *Lupinus albus* seeds rules out its role as a storage protein. *J Plant Physiol* **143**, 711–716.
- Duranti M, Scarafoni A, Gius C, Negri A & Faoro F (1994) Heat-induced synthesis and tunicamycin-sensitive secretion of the putative storage glycoprotein conglutin

- gamma from mature lupin seeds. *Eur J Biochem* **222**, 387–93.
- 26 Scarafoni A, Ronchi A & Duranti M (2010) γ -Conglutin, the *Lupinus albus* XEGIP-like protein, whose expression is elicited by chitosan, lacks of the typical inhibitory activity against GH12 endo-glucanases. *Phytochemistry* **71**, 142–148.
 - 27 Magni C, Scarafoni A, Herndl A, Sessa F, Prinsi B, Espen L & Duranti M (2007) Combined 2D electrophoretic approaches for the study of white lupin mature seed storage proteome. *Phytochemistry* **68**, 997–1007.
 - 28 Foley RC, Gao LL, Spriggs A, Soo LYC, Goggin DE, Smith PMC, Atkins CA & Singh KB (2011) Identification and characterisation of seed storage protein transcripts from *Lupinus angustifolius*. *BMC Plant Biol* **11**, 59.
 - 29 Shutov AD, Kakhovskaya IA, Bastrygina AS, Bulmaga VP, Horstmann C & Müntz K (1996) Limited proteolysis of beta-conglycinin and glycinin, the 7S and 11S storage globulins from soybean [*Glycine max* (L.) Merr.]. Structural and evolutionary implications. *Eur J Biochem* **241**, 221–228.
 - 30 Gatehouse JA, Lycett GW, Delauney AJ, Croy RRD & Boulter D (1983) Sequence specificity of the post-translational proteolytic cleavage of vicilin, a seed storage protein of pea (*Pisum sativum* L.). *Biochem J* **212**, 427–432.
 - 31 Monteiro S, Freitas R, Rajasekhar BT, Teixeira AR & Ferreira RB (2010) The unique biosynthetic route from *Lupinus* beta-conglutin gene to blad. *PLoS One* **5**, 11.
 - 32 Cocucci S & Cocucci M (1977) Effect of ABA, GA3 and FC on the development of potassium uptake in germinating radish seeds. *Plant Sci Lett* **10**, 85–95.
 - 33 Pardi D, Morgutti S & Cocucci S (1980) K^+ uptake in the early phases of germination of the photoblastic and thermosensitive seed of *Phacelia tanacetifolia*. *Physiol Plant* **48**, 379–384.
 - 34 Magni C, Herndl A, Sironi E, Scarafoni A, Ballabio C, Restani P, Bernardini R, Novembre E, Vierucci A & Duranti M (2005) One- and two-dimensional electrophoretic identification of IgE-binding polypeptides of *Lupinus albus* and other legume seeds. *J Agric Food Chem* **53**, 4567–4571.
 - 35 Duranti M, Sessa F & Carpen A (1992) Identification, purification and properties of the precursor of conglutin β , the 7S storage globulin of *Lupinus albus* L. seeds. *J Exp Bot* **43**, 1373–1378.
 - 36 Regalado AP, Pinheiro C, Vidal S, Chaves I, Ricardo CPP & Rodrigues-Pousada C (2000) The *Lupinus albus* class-III chitinase gene, IF3, is constitutively expressed in vegetative organs and developing seeds. *Planta* **210**, 543–550.
 - 37 Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V & Henrissat B (2009) The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res* **37**, 233–238.
 - 38 Durand A, Hughes R, Roussel A, Flatman R, Henrissat B & Juge N (2005) Emergence of a subfamily of xylanase inhibitors within glycoside hydrolase family 18. *FEBS J* **272**, 1745–1755.
 - 39 Xiong JS, Balland-Vanney M, Xie ZP, Schultze M, Kondorosi A, Kondorosi E & Staehelin C (2007) Molecular cloning of a bifunctional beta-xylosidase/alpha-L-arabinosidase from alfalfa roots: heterologous expression in *Medicago truncatula* and substrate specificity of the purified enzyme. *J Exp Bot* **58**, 2799–2810.
 - 40 Evers D, Ghislain M, Hoffmann L, Hausman JF & Dommes J (2006) A late blight resistant potato plant overexpresses a gene coding for α -galactosidase upon infection by *Phytophthora infestans*. *Biol Plant* **50**, 265–271.
 - 41 Basu U, Francis JL, Whittall RM, Stephens JL, Wang Y, Zaiane OR, Goebel R, Muench DG, Good AG & Taylor GJ (2006) Extracellular proteomes of *Arabidopsis thaliana* and *Brassica napus* roots: analysis and comparison by MudPIT and LC-MS/MS. *Plant Soil* **286**, 357–376.
 - 42 Gerke V, Creutz CE & Moss SE (2005) Annexins: linking Ca^{2+} signaling to membrane dynamics. *Nat Rev Mol Cell Biol* **6**, 449–461.
 - 43 Mortimer JC, Laohavisit A, MacPherson N, Webb A, Brownlee C, Battey NH & Davies JM (2008) Annexins: multifunctional components of growth and adaptation. *J Exp Bot* **59**, 533–544.
 - 44 Gourinath S, Alam N, Srinivasan A, Betzel C & Singh TP (2000) Structure of the bifunctional inhibitor of trypsin and alpha-amylase from ragi seeds at 2.2 Å resolution. *Acta Crystallogr D Biol Crystallogr* **56**, 287–293.
 - 45 Müntz K (1998) Deposition of storage proteins. *Plant Mol Biol* **38**, 77–99.
 - 46 Chadha P & Das RH (2006) A pathogenesis related protein, AhPR10 from peanut: an insight of its mode of antifungal activity. *Planta* **225**, 213–222.
 - 47 Pinto MP & Ricardo CPP (1995) *Lupinus albus* L. pathogenesis-related proteins that show similarity to PR-10 proteins. *Plant Physiol* **109**, 1345–1351.
 - 48 Shutov AD & Vaintraub IA (1987) Degradation of storage proteins in germinating seeds. *Phytochemistry* **26**, 1557–1566.
 - 49 Müntz K & Shutov AD (2002) Legumains and their function in plants. *Trends Plant Sci* **8**, 340–344.
 - 50 Kunze I, Kunze G, Bröker M, Manteuffel R, Meins F Jr & Müntz K (1998) Evidence for secretion of vacuolar alpha-mannosidase, class I chitinase, and class I beta-1,3-glucanase in suspension cultures of tobacco cells. *Planta* **205**, 92–99.

- 51 Bendtsen JD, Jensen J, Blom N, von Heijne G & Brunak S (2004) Feature based prediction of non-classical and leaderless protein secretion. *Protein Eng Des Sel* **17**, 349–356.
- 52 Cheng FY, Blackburn K, Lin YM, Goshe MB & Williamson JD (2009) Absolute protein quantification by LC/MS(E) for global analysis of salicylic acid-induced plant protein secretion responses. *J Proteome Res* **8**, 82–93.
- 53 Agrawal GK, Jwa NS, Lebrun MH, Job D & Rakwal R (2010) Plant secretome: unlocking secrets of the secreted proteins. *Proteomics* **10**, 799–827.
- 54 Zhou L, Bokhari SA, Dong CJ & Liu JY (2011) Comparative proteomics analysis of the root apoplasts of rice seedlings in response to hydrogen peroxide. *PLoS One* **6**, e16723.
- 55 Scarafoni A, Di Cataldo A, Vassilevskaia TD, Bekman EP, Rodrigues-Pousada C, Cecilian F & Duranti M (2001) Cloning, sequencing and expression in the seeds and radicles of two *Lupinus albus* conglutin γ genes. *Biochim Biophys Acta* **1519**, 147–151.
- 56 Brunner AM, Yakovlev IA & Strauss SH (2004) Validating internal controls for quantitative plant gene expression studies. *BMC Plant Biol* **4**, 14.
- 57 Jain M, Nijhawan A, Tyagi AK & Khurana JP (2006) Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. *Biochem Biophys Res Commun* **345**, 646–651.
- 58 Rajjou L, Gallardo K, Debeaujon I, Vandekerckhove J, Job C & Job D (2004) The effect of alpha-amanitin on the *Arabidopsis* proteome highlights the distinct roles of stored and neosynthesized mRNAs during germination. *Plant Physiol* **134**, 1598–1613.
- 59 Larson TM, Kendra DF, Busman M & Brown DW (2011) *Fusarium verticillioides* chitin synthases CHS5 and CHS7 are required for normal growth and pathogenicity. *Curr Genet* **57**, 177–189.
- 60 Arbelet D, Malfatti P, Simond-Côte E, Fontaine T, Desquilbet L, Expert D, Kunz C & Soulié MC (2010) Disruption of the Bchs3a chitin synthase gene in *Botrytis cinerea* is responsible for altered adhesion and overstimulation of host plant immunity. *Mol Plant Microbe Interact* **23**, 1324–1334.
- 61 Munoz A, Lopez-Garcia B & Marcos JF (2007) Studies on the mode of action of the antifungal hexapeptide PAF26. *Antimicrob Agents Chemother* **50**, 3847–3855.
- 62 Simon EW & Raja Harun RM (1972) Leakage during seed imbibition. *J Exp Bot* **23**, 1076–1085.
- 63 Spaeth SC (1989) Extrusion of protoplasm and protein bodies through pores in cell walls of pea, bean and faba bean cotyledons during imbibition. *Crop Sci* **29**, 452–458.
- 64 Spaeth SC (1987) Pressure-driven extrusion of intracellular substances from bean and pea cotyledons during imbibition. *Plant Physiol* **85**, 217–223.
- 65 Yokotani-Tomita K, Goto N, Kosemura S, Yamamura S & Hasegawa K (1998) Growth-promoting allelopathic substance exuded from germinating *Arabidopsis thaliana* seeds. *Phytochemistry* **47**, 1–2.
- 66 Benchabane M, Schlüter U, Vorster J, Goulet M-C & Michaud D (2010) Plant cystatins. *Biochimie* **92**, 1657–1666.
- 67 Hwang JE, Hong JK, Je JH, Lee KO, Kim DY, Lee SY & Lim CO (2009) Regulation of seed germination and seedling growth by an *Arabidopsis* phytocystatin isoform, AtCYS6. *Plant Cell Rep* **28**, 1623–1632.
- 68 Müntz K (2001) Stored proteinases and the initiation of storage protein mobilization in seeds during germination and seedling growth. *J Exp Bot* **52**, 1741–1752.
- 69 Schlumbaum A, Mauch F & Vogeli U (1986) Plant chitinases are potent inhibitors of fungal growth. *Nature* **324**, 355–356.
- 70 Kasprzewska A (2003) Plant chitinases regulation and function. *Cell Mol Biol Lett* **8**, 809–824.
- 71 Hon W-C, Griffith M, Mlynarz A, Kwok YC & Yang DSC (1995) Antifreeze proteins in winter rye are similar to pathogenesis-related proteins. *Plant Physiol* **109**, 879–889.
- 72 Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- 73 Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- 74 Hawkes R (1982) Identification of concanavalin A-binding proteins after sodium dodecyl sulphate-gel electrophoresis and protein blotting. *Anal Biochem* **123**, 143–146.
- 75 Prinsi B, Negri AS, Pesaresi P, Cocucci M & Espen L (2009) Evaluation of protein pattern changes in roots and leaves of *Zea mays* plants in response to nitrate availability by two-dimensional gel electrophoresis analysis. *BMC Plant Biol* **9**, 113.
- 76 Mackey AJ, Haystead TAJ & Pearson WR (2002) Getting more from less: algorithms for rapid protein identification with multiple short peptide sequences. *Mol Cell Proteomics* **1**, 139–147.
- 77 Raposo R, Colgan R, Delcan J & Melgarejo P (1995) Application of an automated quantitative method to determine fungicide resistance in *Botrytis cinerea*. *Plant Dis* **79**, 294–296.
- 78 Pfaffl MW (2001) A new mathematical model for relative quantification in realtime RT-PCR. *Nucleic Acids Res* **29**, 1–6.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Table S1. Spot reports obtained from the analysis of the two-dimensional maps of seed exudates.

Table S2. List of the identified proteins by LC-ESI-MS/MS and bioinformatics analyses.

Table S3. Chitinase activities of exudates following incubation at 20 °C for 24 h.

Doc. S1. Quantitative PCR set-up and the results of the assessment of experimental conditions.

Table S4. Nucleotide sequences and specifications of primers probes used in quantitative RT-PCR for amplification of analyzed transcripts.

Table S5. Coefficients of the regression lines for each primer pairs according to the formula $y = mx + q$.

Fig. S1. Post-amplification melting curves of the amplicons obtained using the selected primer pairs in RT-PCR.

Fig. S2. Standard curves of dilution series of lupin genomic DNA amplified with the selected primer pairs.



WILEY
Blackwell

the **FEBS**
Journal

www.febsjournal.org

The proteome of exudates from germinating *Lupinus albus* seeds is secreted through a selective dual-step process and contains proteins involved in plant defence

Alessio Scarafoni, Alessandro Ronchi, Bhakti Prinsi, Luca Espen, Gemma Assante, Giovanni Venturini and Marcello Duranti

DOI: 10.1111/febs.12140

Table S1. Spot reports obtained from the analysis of the 2D maps of seed exudates.

Spot number	pI	MW	Intensity	Area	Vol.	%Total Intensity	%Total Vol.
1	4.02	43100	48.00	18.13	316.33	1.42	1.21
2	4.30	47500	53.00	28.90	778.44	1.57	2.99
3	4.39	28300	147.00	85.81	8745.72	4.35	33.54
4	4.20	13200	134.25	24.39	1507.87	3.97	5.78
5	4.91	69200	53.25	12.00	280.26	1.58	1.07
6	5.29	65400	56.25	6.90	164.92	1.66	0.63
7	5.59	65400	97.50	9.94	415.16	2.88	1.59
8	4.74	51600	55.50	8.45	163.06	1.64	0.63
9	5.85	48600	96.75	11.68	481.05	2.86	1.84
10	5.11	44300	42.75	7.42	109.47	1.26	0.42
11	5.08	41000	54.00	3.81	75.68	1.60	0.29
12	4.75	34000	64.00	16.77	270.59	1.89	1.04
13	5.12	60400	42.75	6.45	104.55	1.26	0.42
14	5.08	35700	55.50	3.42	65.90	1.64	0.25
15	4.71	26700	84.00	12.84	452.45	2.49	1.74
16	4.79	26700	102.75	20.32	959.79	3.04	3.68
17	4.92	26700	138.00	33.55	2513.61	4.08	9.64
18	5.14	28300	108.00	10.84	557.47	3.20	2.14
19	4.62	20100	99.00	11.48	434.13	2.93	1.66
20	4.75	20100	100.50	10.77	395.19	2.97	1.52
21	4.70	14900	78.00	9.35	227.03	2.31	0.87
22	4.80	14900	56.25	6.52	140.11	1.66	0.54
23	4.96	15500	60.75	5.16	124.42	1.80	0.48
24	5.07	14000	108.75	7.29	355.89	3.22	1.36
25	6.21	17100	92.25	6.52	235.15	2.73	0.90
26	6.19	16400	111.00	6.58	303.61	3.28	1.16
27	5.28	11100	75.75	9.16	274.19	2.24	1.05
28	6.41	11800	51.75	10.84	230.03	1.53	0.88
29	6.42	11200	91.50	10.90	444.55	2.71	1.70
30	6.43	10700	99.00	16.06	636.04	2.93	2.44
31	7.72	31000	23.00	31.16	313.82	0.68	1.20
32	8.00	31000	33.75	18.06	254.42	1.00	0.98
33	8.00	30800	48.00	15.87	267.74	1.42	1.03
34	5.41	65400	64.50	8.32	256.35	1.91	0.98
35	5.10	65400	24.00	11.87	108.65	0.71	0.42
36	5.19	65400	36.75	8.84	129.50	1.09	0.50
37	5.49	48400	66.00	7.74	192.65	1.95	0.74
38	5.27	48600	30.75	4.39	52.48	0.91	0.20
39	5.21	43300	48.00	6.06	101.68	1.42	0.39
40	6.35	40900	22.00	2.39	23.41	0.65	0.12
41	6.11	34300	23.00	6.13	60.83	0.68	0.18
42	9.66	19400	15.75	6.71	36.23	0.47	0.24
43	4.48	39000	33.00	4.77	57.35	0.98	0.22
44	4.74	45500	30.00	8.39	82.04	0.89	0.31
45	4.80	45400	25.50	4.65	48.26	0.75	0.19
46	7.29	57500	21.75	5.42	34.81	0.64	0.13
47	7.24	39500	10.00	2.39	11.87	0.30	0.05
48	5.39	26200	16.00	7.16	56.64	0.47	0.22

49	5.98	26100	21.00	10.71	56.29	0.62	0.22
50	6.14	42700	23.00	6.26	48.10	0.68	0.18
51	5.09	27000	105.00	24.26	1222.91	3.11	4.69
52	5.15	10700	53.25	13.23	293.92	1.58	1.13
56	5.06	56200	27.00	5.61	44.00	0.80	0.17
57	5.48	54800	27.00	4.84	47.10	0.80	0.18
58	5.44	34700	21.00	8.19	44.38	0.62	0.17
59	4.74	13500	39.00	14.13	211.11	1.15	0.81
60	4.88	12100	34.00	17.94	254.75	1.01	0.98

Table S2. List of the identified proteins by LC-ESI-MS/MS and bioinformatics analyses. The worksheet shows the sequence of all the peptides identified by MS/MS fragmentation and the associated statistical information obtained from database searches conducted by BioworksBrowser using TurboSEQUEST® software. For each identified protein, statistical information related to direct protein database search or to alignment analysis of the identified peptides by FASTS software are reported. Spot ID: spot identifier number. Protein A.N.: protein NCBI accession number (version). DB: database downloaded from NCBI: NR= protein non-redundant database (6/9/2010, 11160352 entries); NRF: subset of Fabaceae family protein (6/9/2010, 66061 entries); EST: subset of Fabaceae family EST (6/9/2010, 3040898 entries). n. pep.: number of the unique peptides used to identify the protein. aa cov. (%): sequence coverage %. Sf (pro): protein SEQUEST Sf score. FASTS (E) value: FASTS expectation (E) value of the entry resulting from the alignment of the peptides against NCBI non-redundant database. Hom. Protein A.N.: homologous protein NCBI accession number (version). EST A. N.: EST NCBI accession number (version). Peptide: sequence of the identified peptide; the symbol M* indicates oxidized methionine. MH+: molecular mass of the peptide; z: charge state of the peptide. Sf (pep): SEQUEST Sf score of the peptide. Xcorr: SEQUEST cross-correlation value. ΔCn: delta correlation value Sp: SEQUEST preliminary score. (a): mature form. (b): partial sequence.

Spot ID	Protein A.N.	Species	DB	n. pep.	aa cov. (%)	Sf (pro)	FASTS (E) value	Hom. Protein A.N.	EST A. N.	Peptide	MH+	z	Sf (pep)	Xcorr	ΔCn	Sp
2	P14749.1	Cyamopsis tetragonoloba	NRF	5	14,0 (a)	4,66	0	P14749.1		K.ALADYVHSK.G K.TFASWGVGDYLK.Y K.TFASWGVGDYLK.Y R.AHFSIWALAKA K.APLLIIGDIR.A R.TTGDIEDNWDMSR.A	1004,12 1287,44 1287,44 1144,35 1128,37 1728,78	2 2 2 2 2 2	0,90 0,97 0,96 0,91 0,95 0,92	2,779 4,084 3,732 2,832 3,472 4,304	0,111 0,562 0,523 0,487 0,435 0,327	1084,9 1455,1 1126,1 727,1 1261,0 1283,8
3	CAA76203.1	Lupinus albus	NRF	5	24,4 (a)	5,61	0	CAA76203.1		R.ALNGFSSQK.K R.DGLNADIK.G R.PFGDAVLGDIGDIEAGGAQHYDELAR.A R.PFGDAVLGDIGDIEAGGAQHYDELAR.A K.YGGVMLWNR.R K.YGGVM*LNWR.R -ALNGFSSQK.- -FNDIQTGYSDAIK.- -PFGDAVLGDIGDIEAGGAQHYDELAR.- -PFGDAVLGDIGDIEAGGAQHYDELAR.-	952,05 845,92 2893,07 2893,07 1096,29 1112,29 952,05 1472,58 2893,07 2893,07	2 2 3 3 2 2 2 2 3 3	0,94 0,84 0,98 0,98 0,94 0,96 0,83 0,95 0,97 0,98	2,743 2,598 5,313 6,132 3,860 3,780 3,379 4,258 5,313 6,132	0,653 0,476 0,581 0,668 0,147 0,191 0,292 0,297 0,418 0,481	596,4 456,3 2081,0 1874,2 1013,6 1490,2 657,8 1324,3 2081,0 1874,2
4	CAJ42100.1	Lupinus albus	NRF	2	40,3 (a)	1,93	n.d.	CAJ42100.1		R.ALQIYENQSEQCQGR.Q R.QEEQLLEQELENLPR.T R.QEEQLLEQELENLPR.T	1953,08 1869,02 1869,02	2 2 3	0,95 0,97 0,95	4,123 3,429 2,996	0,518 0,443 0,338	1077,2 2315,0 2257,3
5	ACU18828.1	Glycine max	NRF NR	2	5,6	1,81	4,30E-21	ACU18828.1 NP_001056466.1		K.AFQNAIFYLNSFADK.G K.SGWDEYGFAGR.P	1749,95 1358,44	2 2	0,89 0,93	3,022 3,789	0,462 0,276	744,3 1779,6
6	A5JQTQ.1	Medicago varia	NRF	2	3,0 (a)	1,93	9,80E-14	CBD33472.1 CBC23849.1		K.NPALAGYGFCDK.S K.IGNLVNSAVDVSR.L	1313,46 1344,50	2 2	0,97 0,96	4,298 3,869	0,597 0,494	1512,0 1097,3
7	A5JQTQ.1	Medicago varia	NRF	3	4,0 (a)	2,80	1,20E-20	A5JQTQ.1 CBD33472.1		K.DVCTPENQELAR.E K.HYTAYVDNWK.G K.NPALAGYGFCDK.S	1432,54 1412,49 1313,46	2 2 2	0,86 0,96 0,98	2,681 4,138 4,588	0,407 0,228 0,697	544,2 1437,8 1758,5
9 (1)	CAC44501.1	Fragaria x ananassa	NRF	4	5,8	5,26	0	CAW93195.1		R.STPQM*WPDLIQK.A R.STPQMWPDLIQK.A R.STPQM*WPDLIQK.A R.GGTFQNYM*YHGGTNFGR.T R.GGTFQNYMYHGGTNFGR.T R.GGTFQNYMYHGGTNFGR.T R.VLISGSIHYPR.S	1460,68 1444,68 1460,68 2087,22 2071,22 2071,22 1242,45	2 2 2 3 3 2 2	0,84 0,89 0,91 0,78 0,95 0,91 0,96	2,750 2,638 2,771 3,173 4,070 3,397 3,086	0,474 0,690 0,678 0,496 0,554 0,616 0,545	369,3 370,2 377,3 490,8 1436,5 713,0 1488,5
9 (2)	AAS97865.1	Lupinus albus	NR NRF	2	6,0	1,83	n.d.	ACP18875.1 AAS97865.1		R.PVEDLAFSVAR.F R.IPAGSTSYLNPDDNQK.L R.IVEFQSKPNTLILPK.H	1204,36 1833,98 1728,07	2 2 3	0,88 0,98 0,85	3,240 4,875 2,729	0,371 0,822 0,733	1120,2 1198,9 359,5
11	ACU22795.1	Glycine max	NRF EST	2	5,5	1,87	7,20E-11	ACU22795.1	CA410655.1	K.LPGSNDVLLK.N -TLDVLLNLK.- -TLDVLLNLK.-	1184,37 1100,33 1100,33	2 2 2	0,94 0,74 0,93	3,351 2,525 3,850	0,429 0,148 0,199	966,3 910,2 1351,0
12 (1)	ACU19183.1	Glycine max	EST	2	9,3	1,87	4,90E-20		GT627873.1 BG881552.1	-DPDGYTFALIQR.- -GNAYAQIGIGTDDVYK.-	1396,53 1685,82	2 2	0,97 0,90	5,219 4,171	0,336 0,185	1532,8 1176,3
12 (2)	CAI84850.2	Lupinus albus	NRF	1	2,4 (b)	0,97	n.d.	CAI84850.2		R.LLGFGINAYENQR.N	1495,66	2	0,97	3,436	0,548	1849,8
13	CAI84850.2	Lupinus albus	NRF	5	11,9 (b)	4,75	n.d.	CAI84850.2		K.DQGSYFSGFSR.N R.IPAGSTSYLNPDDNQK.L R.LLGFGINAYENQR.N R.NFLAGSEDNVIR.Q R.NTLEATFNTRY	1322,36 1833,98 1495,66 1335,45 1167,25	2 2 2 2 2	0,91 0,95 0,98 0,95 0,96	2,674 4,287 4,360 4,064 3,523	0,489 0,542 0,560 0,472 0,510	886,4 923,6 2097,6 1618,7 1265,6
14	ACU23990.1	Glycine max	NRF	3	14,5 (b)	2,82	2,00E-25	ACU23990.1		K.LILPLVSSYR.Y R.YEGDEVNLTAK.T -AQINATLNHYR.-	1161,42 1352,47 1301,44	2 2 2	0,95 0,95 0,92	2,936 3,913 3,427	0,420 0,423 0,337	1097,2 1012,9 1205,1
15	ACU14241.1	Glycine max	NRF	2	12,9	1,92	n.d.	ACU14241.1		K.TVDQLWQDYK.L K.TVDQLWQDYK.L K.WDQGYDVTAFHFLDYCDLSK.S K.WDQGYDVTAFHFLDYCDLSK.S	1296,41 1296,41 2334,51 2334,51	2 2 3 2	0,96 0,95 0,97 0,93	4,286 3,337 4,705 3,600	0,446 0,437 0,654 0,587	1236,7 1177,5 1365,0 779,7
16	ACU14241.1	Glycine max	NRF	2	12,9	1,92	n.d.	ACU14241.1		K.TVDQLWQDYK.L K.TVDQLWQDYK.L K.TVDQLWQDYK.L K.WDQGYDVTAFHFLDYCDLSK.S K.WDQGYDVTAFHFLDYCDLSK.S	1296,41 1296,41 1296,41 2334,51 2334,51	2 2 2 3 2	0,96 0,95 0,96 0,95 0,96	3,940 3,574 4,266 4,286 3,834	0,401 0,460 0,453 0,523 0,619	1380,9 1082,0 1201,7 1191,9 1286,6
17	ACU14241.1	Glycine max	NRF	2	12,9	1,93	n.d.	ACU14241.1		K.TVDQLWQDYK.L	1296,41	2	0,96	3,663	0,399	1550,9

									K.WDQGYDVTAFHLDYCDSLK.S	2334,51	2	0,97	4,179	0,593	1559,6
20	XP_002271261.1	Vitis vinifera	EST	1	8,8	0,93	2,30E-08	GR405059.1	-.NISPPLEWYNLPQGTK.- -.NISPPLEWYNLPQGTK.-	1858,09 1858,09	2 2	0,79 0,93	3,315 4,412	0,400 0,430	387,7 619,2
21	BAB63949.1	Lupinus albus	NRF	4	34,2	3,79	0	BAB63949.1	K.AVEAIQSVETVEGNGGPGTIK.K K.LTLIEGGETK.Y K.LVEGVNGGSIGK.V K.AIESYLSAHPDYN	2057,25 1061,21 1130,28 1480,56	2 2 2 2	0,94 0,91 0,97 0,97	4,057 2,731 4,535 4,229	0,236 0,426 0,454 0,647	1431,2 861,9 1446,2 1477,8
22	CAC17729.2	Lupinus albus	NRF	1	4.1 (a)	0,98	n.d.	CAC17729.2	K.EAQVESVGPFGLCYDSR.K	1915,07	2	0,98	5,214	0,644	1312,7
24	Q02610.2	Pisum sativum	NRF	1	8.6 (a)	0,97	n.d.	Q02610.2	R.AVVVHADPDDLKG.G	1336,48	2	0,97	4,147	0,528	1571,4
25	CAC16394.1	Lupinus albus	NRF	4	50,0 (a)	4,7	n.d.	CAC16394.1	R.AGIALGTHQLEENLVVFDLAR.S K.AVGPFGLCYDTK.K R.ISGENLM*VQAQDGVSVCLGFVDGGVHTR.A R.ISGENLMVQAQDGVSVCLGFVDGGVHTR.A K.KISGGVPSVDLIMDK.S	2267,57 1328,52 2864,16 2848,17 1559,85	2 2 3 3 2	0,97 0,80 0,97 0,98 0,97	4,045 2,095 5,653 7,678 4,716	0,633 0,413 0,575 0,584 0,512	1752,1 733,6 1314,5 2240,4 1282,1
26	CAC16394.1	Lupinus albus	NRF	4	50,0 (a)	4,81	n.d.	CAC16394.1	R.AGIALGTHQLEENLVVFDLAR.S K.AVGPFGLCYDTK.K K.AVGPFGLCYDTK.K R.ISGENLMVQAQDGVSVCLGFVDGGVHTR.A R.ISGENLM*VQAQDGVSVCLGFVDGGVHTR.A R.ISGENLM*VQAQDGVSVCLGFVDGGVHTR.A R.ISGENLMVQAQDGVSVCLGFVDGGVHTR.A K.KISGGVPSVDLIMDK.S	2267,57 1328,52 1328,52 2848,17 2864,16 2864,16 2848,17 1559,85	3 2 2 3 3 3 3 2	0,96 0,94 0,94 0,98 0,97 0,96 0,85 0,95	4,612 3,400 3,330 7,697 5,471 4,697 3,136 4,312	0,546 0,601 0,495 0,515 0,587 0,583 0,462 0,474	1285,7 839,6 960,9 2160,7 1443,6 1508,9 797,8 967,7
28	ACY30464.1	Ficus pumila	EST	1	9,6	0,96	6,20E-06	FG092921.1	-.EIADFAVTEHNMK.-	1374,48	2	0,96	4,477	0,352	1445,7
29	CAN82794.1	Vitis vinifera	EST	3	22,9	2,75	4,50E-16	FG092381.1	-.GESQVVSQTNRY.- -.LVLVASDGSSTAK.- FG092921.1 -.EIADFAVTEHNMK.-	1297,36 1161,33 1374,48	2 2 2	0,89 0,90 0,96	3,277 3,594 4,448	0,308 0,239 0,414	1322,9 958,7 1324,7
30	ACY30464.1	Ficus pumila	EST	1	9,6	0,92	6,20E-06	FG092921.1	-.EIADFAVTEHNMK.-	1374,48	2	0,92	3,803	0,395	938,6
31	CAC16394.1	Lupinus albus	NRF	3	15,7 (a)	2,82	n.d.	CAC16394.1	K.PNLLVLPQQDASTK.L K.QGEYFIQVSAIR.V K.QGEYFIQVSAIR.V R.TPLM*QVPVLLDLNGK.H R.TPLM*QVPVLLDLNGK.H	1637,90 1411,59 1411,59 1655,00 1655,00	2 2 2 2 3	0,95 0,96 0,92 0,97 0,91	3,291 4,193 3,282 4,929 3,767	0,569 0,510 0,460 0,606 0,426	1139,2 1018,9 720,5 956,2 966,0
32	CAC16394.1	Lupinus albus	NRF	3	16,1 (a)	2,89	n.d.	CAC16394.1	K.IPOQLFSCAPFTLTQK.G K.PNLLVLPQQDASTK.L K.PNLLVLPQQDASTK.L K.QGEYFIQVSAIR.V	1899,24 1637,90 1637,90 1411,59	2 2 2 2	0,98 0,86 0,97 0,94	5,636 2,860 4,030 3,313	0,549 0,377 0,627 0,517	1717,0 806,6 1326,2 811,2
33	CAC16394.1	Lupinus albus	NRF	3	16,1 (a)	2,87	n.d.	CAC16394.1	K.IPOQLFSCAPFTLTQK.G K.IPOQLFSCAPFTLTQK.G K.PNLLVLPQQDASTK.L K.PNLLVLPQQDASTK.L K.QGEYFIQVSAIR.V K.QGEYFIQVSAIR.V	1899,24 1899,24 1637,90 1637,90 1411,59 1411,59	2 2 2 2 2 2	0,98 0,96 0,93 0,97 0,95 0,97	4,745 3,924 4,126 4,260 3,502 4,083	0,509 0,604 0,373 0,615 0,545 0,569	2085,5 1231,1 860,1 1203,2 839,6 1182,7
34	A5JTQ3.1	Medicago varia	NRF	2	2,7 (a)	1,95	3,80E-13	CBD33472.1 A5JTQ2.1	K.NPALAGYGFCDK.S K.HYTAYDVNDWK.G	1313,46 1412,49	2 2	0,98 0,97	4,512 3,824	0,691 0,581	1814,7 1341,4
35	A5JTQ3.1	Medicago varia	NRF	2	3,0 (a)	1,93	9,80E-14	CBD33473.1 CAW96521.1	K.NPALAGYGFCDK.S K.IGNLVNSAVDVSRL	1313,46 1344,50	2 2	0,97 0,96	3,729 4,266	0,664 0,551	1320,2 1135,7
36	A5JTQ3.1	Medicago varia	NRF	2	3,0 (a)	1,94	9,80E-14	CBD33473.1 CAW96521.1	K.NPALAGYGFCDK.S K.IGNLVNSAVDVSRL	1313,46 1344,50	2 2	0,98 0,97	4,426 4,265	0,682 0,517	1656,5 1268,2
37 (1)	AAS97865.1	Lupinus albus	NRF	2	4,3	1,86	n.d.	AAS97865.1	K.DQGSYFSGFSR.N R.QAYNLEYGDALR.I	1322,36 1413,52	2 2	0,97 0,89	3,615 2,780	0,555 0,383	1581,2 768,6
37 (2)	ABN08398.1	Medicago truncatula	NRF	1	1,4	0,79	n.d.	ABN08398.1	R.STPEMWPDLIQK.A	1445,67	2	0,79	3,242	0,003	576,1
39	ACU23118.1	Glycine max	NRF	1	2,6	1,83	n.d.	ACU23118.1	K.SPLMYGGDVR.R K.SPLM*YGGDVR.R	1095,25 1111,25	2 2	0,94 0,89	3,315 2,343	0,475 0,304	1109,7 932,3
40	AAS97865.1	Lupinus albus	NRF	4	11,3	3,5	n.d.	AAS97865.1	K.DQGSYFSGFSR.N R.IPAGSTSYILNPDDNQK.L K.LAIPINNPGEFYDFYPSSTK.D R.QAYNLEYGDALR.I	1322,36 1833,98 2308,57 1413,52	2 2 2 2	0,75 0,98 0,83 0,95	2,369 5,952 2,707 3,705	0,354 0,685 0,559 0,420	564,4 1369,3 423,3 945,1
41 (1)	AAN15136.1	Lupinus angustifolius	NRF	1	5,1	0,86	n.d.	AAN15136.1	K.EGSSIINTTSVNAYK.G	1584,71	2	0,86	2,368	0,846	474,3
41 (2)	BAD20774.2	Raphanus sativus	EST	1	2,1	0,93	2,60E-11	BQ148037.1	-.TNFDAPSGSNPVAIDFTGMGK.-	2127,32	2	0,93	4,193	0,411	933,2
42	ABB13526.1	Lupinus albus	NRF	9	67,6 (a)	8,38	n.d.	ABB13526.1	R.ATTIVNPD.R K.DQGSYFSGFSR.N K.DQGSYFSGFSR.N K.DQGSYFSGFSR.N K.HSDADYVLVVLNGR.A K.HSDADYVLVVLNGR.A R.IPAGSTSYILNPDDNQK.L R.IPAGSTSYILNPDDNQK.L R.IVEFQSKPNTLILPK.H K.LAIPINNPGEFYDFYPSSTK.D R.NPYHFSSQR.F R.NTLEATFNTRY R.TNRLENLQNYR.I	1100,25 1322,36 1322,36 1322,36 1558,72 1558,72 1833,98 1833,98 1728,07 2308,57 1136,20 1167,25 1421,54	2 2 2 2 1 2 2 2 3 2 2 2 2	0,89 0,98 0,97 0,97 0,94 0,98 0,98 0,96 0,81 0,97 0,91 0,97 0,94	2,346 4,294 3,931 4,294 3,422 6,110 5,807 4,629 3,572 5,823 2,922 3,550 3,513	0,282 0,579 0,559 0,562 0,468 0,568 0,591 0,410 0,612 0,229 0,525 0,379	1032,2 1736,0 1426,4 948,3 2342,9 1264,4 1202,2 424,0 534,5 997,1 1472,5 1143,0
43	ACU19711.1	Glycine max	NRF	2	7,9	1,81	n.d.	ACU19711.1	R.ADYLPYGIDFPGGSPGR.F K.GVNYASAAAGIR.E	1782,93 1150,27	2 2	0,86 0,95	3,550 2,927	0,484 0,257	398,5 1886,7
45	ACU23118.1	Glycine max	NRF	1	2,6	0,95	n.d.	ACU23118.1	K.SPLMYGGDVR.R	1095,25	2	0,95	2,802	0,568	1133,1
46	XP_002302122.1	Populus trichocarpa	EST	1	2,0	0,96	1,70E-01	GO0419851.1	-.LGFSSLLYGR.-	1113,29	2	0,96	3,632	0,374	1843,7

50	ACU16628.1	<i>Glycine max</i>	NRF	2	12,5 (b)	1,79	n.d.	ACU16628.1	K.GILAADESTGTIGK.R K.IGPNPESELAIHENAYGLAR.Y	1333,47 2152,35	2 3	0,97 0,82	3,874 3,193	0,509 0,487	1756,9 510,4
51	ACU14241.1	<i>Glycine max</i>	NRF	3	12,0	2,74	5,90E-19	ACU14241.1	K.TVDQLWQDYK.L K.TVDQLWQDYK.L	1296,41 1296,41	2 2	0,97 0,96	3,947 4,145	0,468 0,382	1394,6 1076,4
								ACU14269.1	R.FLDYCDSLK.S	1161,31	2	0,87	2,305	0,290	694,3
			EST					FG089881.1	-SGFVAQLNNK.-	1078,20	2	0,90	3,485	0,278	947,7
56	BAB78700.1	<i>Nicotiana tabacum</i>	NRF	2	7,4 (b)	1,69	2,40E-24	AAL33919.1	K.LEIPDGAVIANK.E	1240,43	2	0,90	2,940	0,386	612,9
			EST					GW583419.1	-YTNSNVEHTFNQSQYPR.-	2199,32	3	0,79	3,509	0,480	390,3
58	ACJ85059.1	<i>Medicago truncatula</i>	EST	1	2,9	0,87	4,00E-02	DT454433.1	-LLDEASDDFQK.-	1281,35	2	0,87	2,821	0,175	1145,5
59	AAS97865.1	<i>Lupinus albus</i>	NRF	1	2,1	0,97	n.d.	AAS97865.1	K.YGNFYEITPDR.N	1375,47	2,00	0,97	3,445	0,869	1314,37
60	CAC42084.1	<i>Pisum sativum</i>	NRF	3	21,2	2,59	7,10E-16	CAC42084.1	R.FIAPILAEIAK.K K.KEEELQTIK.H	1186,47 1173,38	2 2	0,93 0,89	2,974 3,236	0,407 0,312	1103,3 678,9
			EST					GR465771.1	-TPEVTLK.-	935,10	2	0,77	2,539	0,108	677,4

Table S3. Chitinase activities of exudates following incubation at 20 °C for 24 hours.

Enzyme activity	Substrate	U/mL
Chitinase	4-nitrophenyl N-acetyl- β -D-glucosaminide ^a	96.7 $\pm$ 8.4
Chitinase	β -D-N,N',N''-triacetylchitotriose ^b	91.4 $\pm$ 7.5

^aN-acetylglucosaminidase activity

^bendo-chitinase activity

Doc. 1. Quantitative PCR set-up and the results of the assessment of experimental conditions

The final PCR conditions were determined by testing several template DNA quantities, primers concentrations, duration of each PCR step and annealing temperatures. The post-amplification melting curve (Fig. S1) showed a sole, symmetric and sharp curve for each primers pair, indicating that only one product was accumulated. Primer-dimers products were virtually absent. The T_m s of the amplicons are reported in Table S4. The identity of the PCR product has been checked by nucleic acid sequencing, which confirmed the amplification of the expected region on the template DNA (data not shown).

The selected primers were also tested to determine their efficiency reaction capacity (Fig. S2). During gene expression analysis it is indeed very important to consider also the efficiency of the used primers for relative quantification [78]. This was accomplished by the comparative delta-delta Ct method, with a dedicated Microsoft Excell macro created by Bio-Rad. For each primer pairs, reaction efficiencies were determined from the standard dilution series of lupin genomic DNA.

The slope of the standard curve measures the efficiency of the amplification process, according to the equation: $\eta = [10^{(-1/\text{slope})}] - 1$, where η is the efficiency [78]. It follows that, for $\eta = 1$ (namely 100% efficiency), the standard curve reaches a slope of -3.32 and that η is not dependent from the dilution factor of the standard. For a dilution series it will be: $Ct_2 - Ct_1 = \log(N_1/N_2)/\log(1 + \eta)$, where Ct is the threshold cycle and N_1/N_2 is the dilution factor.

Standard curves have been produced showing linear correlation coefficients (R^2) ranging between 0.996 and 0.999 (over more than 15 runs). Reaction efficiencies, determined from the standard dilution series spanning 5 orders of magnitude, ranged between 94.5% and 110% (Tab. S5). By and large, the results indicated that the designed primers and the protocol of amplification are suitable for quantification purposes.

Table S4: Nucleotide sequences and specifications of primers probes used in RT-qPCR for amplification of analysed transcripts.

Primer	Sequence (5'-3')	T _m (°C)	Position	T _m of the amplicon (°C)	Product size (bp)
18S					
Forward	ATGATAACTCGACGGATCGC	60,07	NA	87.5	nd ¹
Reverse	CTTGGATGTGGTAGCCGTTT	59,99	NA		
CγA32					
Forward	ATGGTGTACACCCCATTAACC	58,42	1206	81	136
Reverse	GGTATGAAGATGATGATGATGATG	58,28	1341		
CγE48					
Forward	GCTATGATTACAACTACAAATCCT	53,79	925	82.5	202
Reverse	TCCAAACATCATCATGGC	56,06	1126		
Vicilin					
Forward	TCCTCAAATCTTCGCTTGCT	60,10	949	83.5	201
Reverse	TTGTTGAGGCAGAGCATTTG	59,99	1190		
Chitinase					
Forward	CTGGTGGAGCCCAACACTAT	59,99	489	85	181
Reverse	TGGCATTGAGGGTTGTTGTA	59,96	669		

¹ not determined

Table S5: coefficients of the regression lines for each primer pairs according to the formula $y=mx+q$. Values are the mean of 15 assays.

	m	q	r²	Efficiency
18S	-3.432	20.647	0.996	96%
CγA32	-3.403	26.969	0.998	97.6%
CγE48	-3.599	22.487	0.999	94.5%
Vicilin	-3.014	29.930	0.995	110%
Chitinase	-3.252	24.445	0.999	95.9%

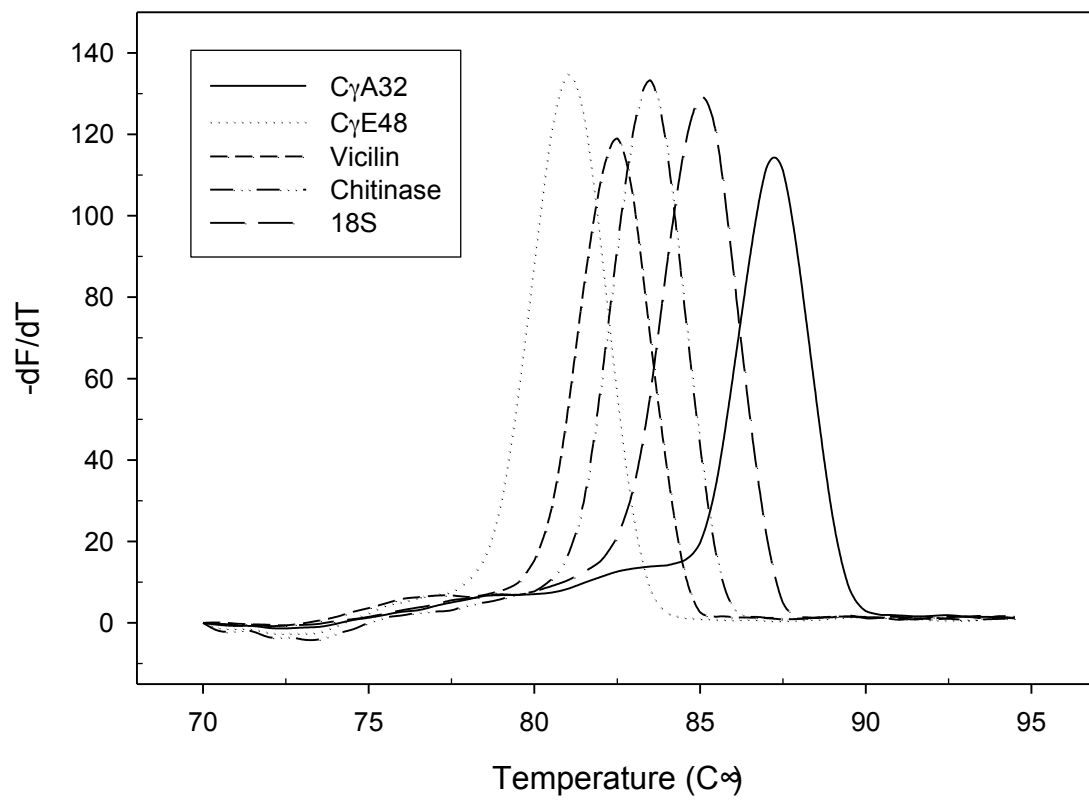


Figure S1. Post-amplification melting curves of the amplicons obtained using the selected primer pairs in real-time PCR.

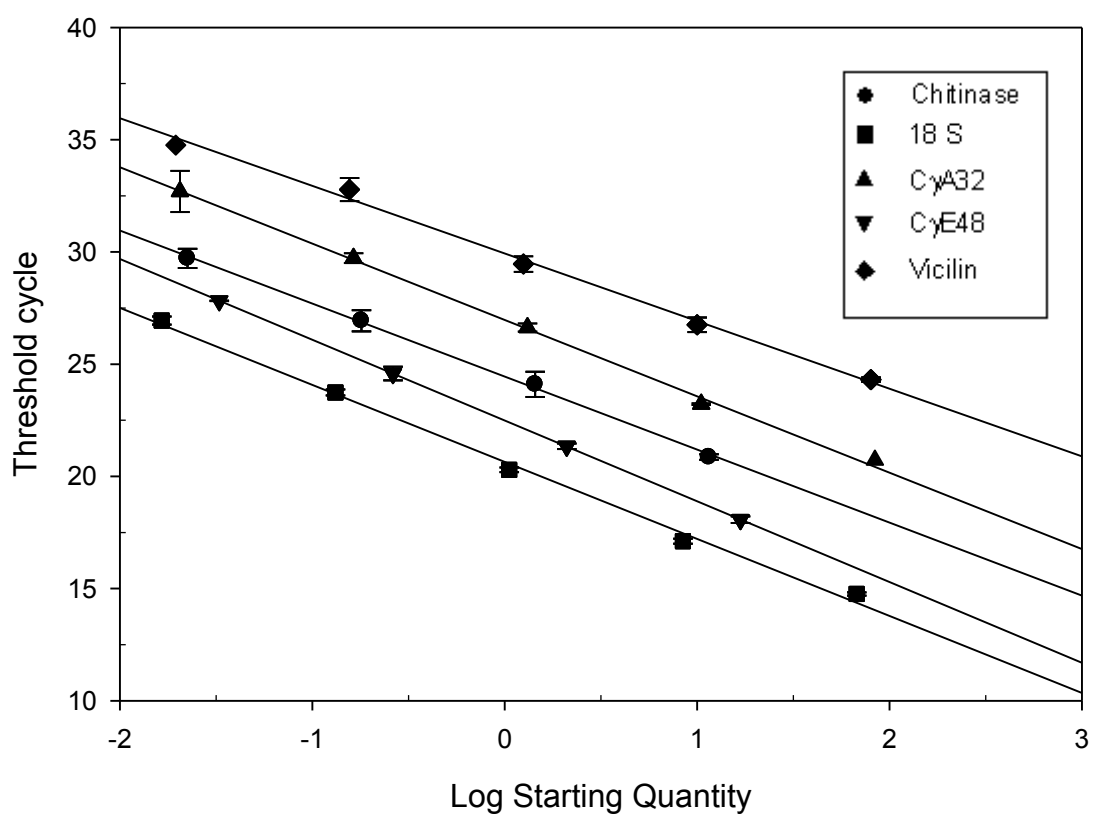


Figure S2. Standard curves of dilution series of lupin genomic DNA amplified with the selected primer pairs. The slope of each curve represents the efficiency of the amplification reaction.