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Short Communication

Comparative peptidomics analysis reveals the peptides involving tobacco seed ageing

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Abstract

Improvement of seed ageing tolerance is critical for germplasm conservation and field cultivation in tobacco (*Nicotiana tabacum* L.). However, whether peptides involved in the regulation of seed ageing remains unknown. In this study, a comparative peptidomics analysis conducted to reveal the regulators involving seed ageing in tobacco. Seed vigor was significantly reduced after 5 days artificial ageing treatment compared with that of the unaged seed. A total of 2812 and 4604 peptides identified in the 5 days aged seeds and unaged seeds, respectively; in which 542 peptides were differentially expressed between the aged and unaged seeds. Gene Ontology analysis revealed that the majority precursor proteins of the differentially expressed peptides involved in response to oxygen-containing compound, response to abscisic acid, and response to lipid. Further, the peptides derived from the precursor proteins, such as late embryogenesis abundant protein, peroxiredoxin, catalase, and globulin proteins, involved in seed ageing in tobacco. The identified peptides provide a foundation for the further exploration of seed ageing tolerance in tobacco.

Keywords: Peptidomics, Peptides, Seed ageing, Nicotiana tabacum L., Vigor index

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Introduction

Seed viability is often undermined due to seed ageing after storage (Li et al., 2022), and thus seed storability is an important agronomic trait in crops (Zhao et al., 2021). Tobacco (*Nicotiana tabacum* L.) has high seed lipid content (Zlatanov et al., 2007; Usta et al., 2011). Reduced seed storability is serious issue for germplasm conservation and field production in tobacco. The aged seeds of tobacco will cause low seed germination, poor seedling establishment, and enormous yield loss in fields. Therefore, it is critical to reveal the regulatory mechanisms of seed ageing for tobacco production. The excessive reactive oxygen species (ROS) is primary factor influencing seed ageing (Adetunji et al., 2021; Boucelha et al., 2021). Processes of protection, repair, and detoxification are important to maintain seed viability during storage (Rajjou and Debeaujon, 2008; Rajjou et al., 2012; Salvi et al.,

2022). However, molecular regulators of seed ageing remain unknown in tobacco.

Small peptides with less than 100 amino acids had been revealed involving stress tolerance (Matsubayashi, 2014; Christmann and Grill, 2018). For example, *Arabidopsis* Cysteine-rich secretory proteins (Antigen 5) and Pathogenesis-related 1 proteins (CAP)-derived peptide 1 (AtCAPE1) negatively regulate salt tolerance by reducing expression of salt-tolerance genes (Chien et al., 2015). C-terminal peptide fragment (AtPep3) plays important roles on salt tolerance in *Arabidopsis* (Nakaminami et al., 2018). In rice, drought and salt stress response-1 (OsDSSR1) peptide positively regulated drought tolerance by increasing activities of superoxide dismutase and ascorbate peroxidase (Cui et al., 2018). Drought tolerance 11 (OsDT11) peptide positively regulates drought tolerance thought the abscisic acid signaling pathway in rice (Li et al., 2017). However, whether peptides involved in seed ageing is largely unknown in tobacco.

Peptidomics employs techniques of genomics, modern proteomics, state-of-the-art analytical chemistry and innovative computational biology, with a specialized set of tools (Hellinger et al., 2023). Peptidomics is useful method to identify the potential peptides in stress tolerance in plants (Ma et al., 2022). In this study, comparative peptidomics conducted in aged seeds to detect peptides involving seed ageing in tobacco. Results showed that majority differentially expressed peptides (DEPs) in aged seeds of tobacco were derived from the precursor proteins, such as late embryogenesis abundant protein, peroxiredoxin, catalase, and globulin proteins. Identified peptides provide foundation for the further exploration of seed ageing tolerance in tobacco.

Material and Methods

Plant material

Tobacco cultivar 'K326' is popularly cultivated in Yunan Provinces, and then it was used in this study. Seeds harvested and dried to 4.5% moisture content to storage at 20 °C (Niu et al., 2022).

Accelerated seed ageing treatment

Accelerated ageing (AA) treatment was conducted according to Niu et al. (2022). Briefly, seeds subjected to the treatment conditions of 45 °C with 100% relative humidity for 0 to 10 days. After that, seeds dried to 4.5% moisture content for seed germination. The unaged seeds used as the control. Three biological replications were conducted.

Seed germination

One hundred seeds per replicate imbibed on the top of filter paper in Petri dishes with distilled water at 25 °C and light/dark for 12 hours for 12 days. Seeds considered as germinated when the normal radicle protruded through seed coat and the cotyledon unfurled. Germination percentage tested after 6 and 12 days germination, respectively. Meanwhile, germination index (GI) was calculated according to Niu et al. (2022). $GI = \Sigma (Gt/t)$, where Gt is the number of the germinated seeds on day t. A total of 10 plants were randomly for the evaluation of root length. Vigor index (VI) was calculated with minor revision (Guo et al., 2019) as follows: $VI = GI \times$ root length. Three biological replications were conducted.

Polypeptide extraction

The polypeptide extraction conducted by Gene Denovo Biotechnology Co. (Guangzhou, China). Briefly, the aged (after 5 days artificial ageing) and unaged (control) seeds used for polypeptide extraction. The super natant was collected after the centrifugation at 4 ℃ for 15,000 rpm for 15 min. Polypeptide filtration was conducted using Ultrafiltration Spin Columns (Millipore, Billerica) to centrifuge at 4 ℃ for 8000 g for 30 min to remove high molecular weight protein. Enzymatic hydrolysis of 3 to 10 KD polypeptide was conducted as follows: concentrated by centrifugal concentrator, drained, and redissolved with 100 µL 100 mM TEAB; add 5 µL trypsin to each sample, and carry out enzymolysis at 37 ℃ overnight. The C18 Zip Tip (MonoSpin C18, GL) was used to desalinate samples. The eluted peptide was drained by vacuum concentrator and stored at 80 ℃ for mass spectrometry detection. Three biological replications were conducted.

Nano LC-MS/MS analysis

The nanoLC-MS/MS analysis conducted by Gene Denovo Biotechnology Co. (Guangzhou, China). Briefly, on-line nanospray LC-MS/MS on Thermo Scientific™ Orbitrap Fusion Lumos ™ coupled to an EASY-nano-LC 1200 system (Thermo Fisher Scientific, MA, USA) was used. 4 µL peptide was loaded (analytical column: Acclaim PepMap C18, 75 µm x 25 cm) and separated with a 120 min linear gradient, from 6% B (B: 0.1% formic acid in 80% ACN) to 36% B. The column flow rate was maintained at 400 nL/min with the column

temperature of 40 °C. The electrospray voltage of 2 kV versus the inlet of the mass spectrometer was used. The mass spectrometer was run under data dependent acquisition mode, and automatically switched between MS and MS/MS mode. Three biological replications were conducted.

Database search and analysis

Database search and analysis conducted by Gene Denovo Biotechnology Co. (Guangzhou, China). Briefly, raw data processed and analyzed by Spectronaut X (Biognosys AG, Switzerland) with default settings to generate an initial target list. Spectronaut was set up to search the database of tobacco along with contaminant database assuming trypsin as the digestion enzyme. Carbamidomethyl (C) was specified as the fixed modification. Oxidation (M) was specified as the variable modifications. The false discovery rate (FDR) Q value cutoff on precursor and protein level was applied 1%. Decoy generation was set to mutated which similar to scrambled but will only apply a random number of amino acid position swamps (min=2, max=length/2). All selected precursors passing the filters were used for quantification. The average top 3 filtered peptides which passed the 1% Q value cutoff used to calculate the major group quantities.

Differentially expressed peptide analysis and functional annotation

DEPs filtered with the selection criteria of fold change > 1.2 and *P* value < 0.05. For all the identified polypeptides, use the proteins to which they belong. Gene Ontology (EuKaryotic Orthologous Groups (KOG)/ Cluster of Orthologous Groups of Proteins (COG) database (Okuda et al., 2008) used to analyze the protein functions and classifications.

Statistical analysis

Significant differences among samples compared using Student's *t*-test or analysis of variance.

Results

Characteristics of tobacco seed vigor after artificial ageing treatment

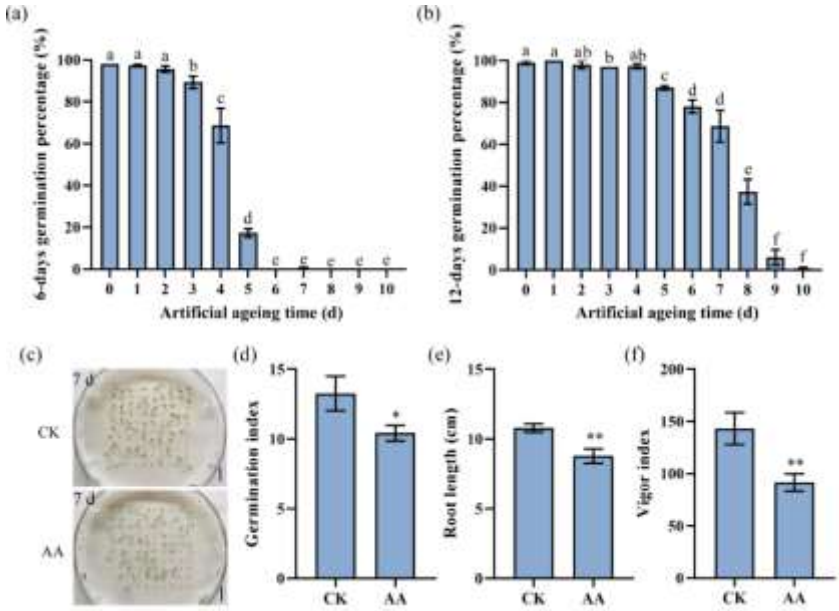
To reveal characteristics of tobacco seed ageing, the different durations (0 to 10 days) of AAtreatment were conducted. Generally, traits of seed vigor gradually decreased with the increased duration of AA treatment in tobacco. Germination percentages after 6 days (Figure 1a) and after 12 days (Figure 1b) of seeds after 5, 6, 7, 8, 9, and 10 days AA treatment significantly reduced in comparison with control treatment (CK, 0 d). The germination percentage after 6 days and 12 days was less than 50% and 90% in seeds after 5 days AA treatment, respectively. Further, significant reduction of seedling growth observed in seeds after 5 days AA treatment in comparison with CK (Figure 1c). Germination index, root length, and vigor index significantly decreased in seeds after 5 days AA treatment in comparison with CK (Figure 1de). These data suggest that seed ageing is occurred after 5 days AA treatment in tobacco.

Peptide profiles in developing seeds of tobacco

To reveal the mechanism of seed ageing in tobacco, seeds after 5 days AA treatment and CK used for peptidomics analysis. To investigate relationships among samples, principal component analysis (PCA) and correlation analysis performed for the peptide expression. PC1 and PC2 explained 72.2% and 14.3% of the peptide expression variation in all samples, respectively (Figure 2a). Pearson correlations between replicates of CK samples (CK-1, CK-2, and CK-3) and 5 days AA treatment samples (AA-1, AA-2, and AA-3) were larger than 0.9 (Figure 2b). These data suggest that peptide expression was consistent among samples. Peptidomics analysis identified 2812 and 4604 nonredundant peptides derived from seeds of CK and AA, respectively (Figure 2c). In which, 1241 peptides had fewer than 10 amino acids, and 2156 peptides had 10–15 amino acids and 1382 peptides with more than 15 amino acids (Figure 2d).

Characteristics of differentially expressed peptides

To reveal the mechanism of seed ageing in tobacco, theDEPs between 5 days AA seeds and CK analyzed. A total of 542 DEPs observed in AA seeds (Figure 3a). Of them, 125 and 417 peptides were up-regulated and down-regulated, respectively (Figure 3b). The potential functions of these DEPs according to their precursor proteins conducted using Gene Ontology (GO) analysis. It showed that these DEPs were mainly involved in the responses to oxygen-containing compound, abscisic acid, and lipid, and so on (Figure 3c). In which, the first one is response to oxygen-containing compound. These data indicate that the stress responses may play important roles on



seed ageing in tobacco.

Figure-1. Characteristics of seed vigor after artificial ageing treatment in tobacco. Germination percentage after (a) 6 days and after (b) 12 days in seeds after 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 days artificial ageing (AA) treatment. (c) Representative images of seed germination after 7 days in seeds after 5 days AA treatment. Scale bars represent 10 mm. Comparisons of (d) germination index, (e) root length, and (f) vigor index between seeds after 5 days AA treatment and control (CK). Data were presented as mean ± SD, $n = 3$ biological replicates. In (a), (b) different letters indicate significant differences ($P = 0.05$, one-way ANOVA). In (d), (e), (f) significant difference was determined by two-tailed Student's *t*-tests (*$P < 0.05$; **$P < 0.01$)

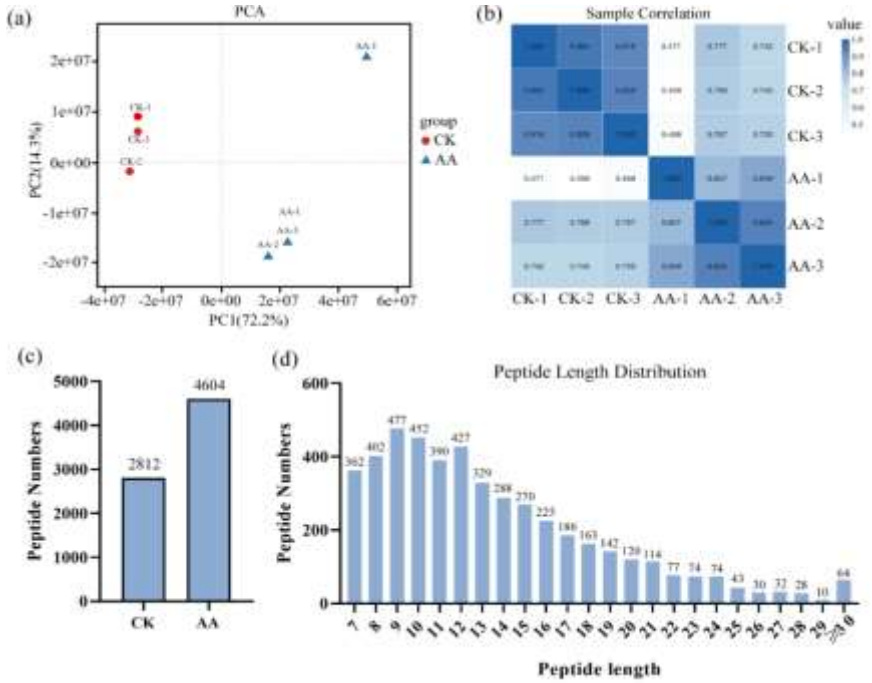


Figure-2. Identification of peptides in the aged seeds of tobacco. (a) Principal component analysis (PCA) and (b) Pearson correlation analysis among samples of aged (after 5 days AA treatment) and control (CK) seeds. (c) The number of identified peptides in aged (AA) and CK seeds. (d) Distribution of the identified peptide lengths.

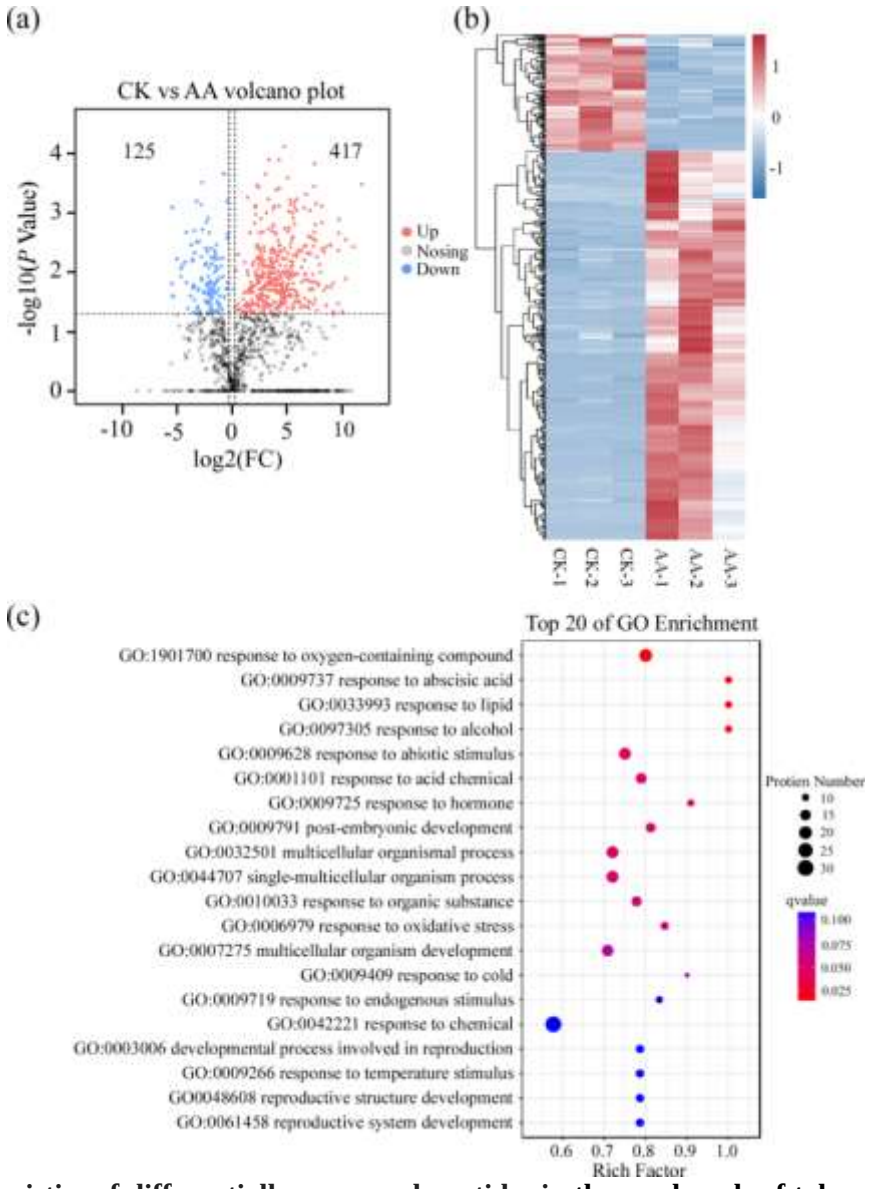


Figure-3. Characteristics of differentially expressed peptides in the aged seeds of tobacco. (a) Volcano plot of differentially expressed peptides. The horizontal axis represents the expressed fold change. The vertical axis represents the degree of statistical significance in differential expression. The higher -log10 (FDR) values represent greater differences. Black dots indicate no significant changes between aged (AA) and control (CK) seeds. The up-regulated peptides are represented by a red dot, down-regulated peptides by a blue dot. (b) Hierarchical clustering analysis of the differentially expressed peptides. Red, up regulation; Blue, down regulation. Values represent the log2 fold changes of peptide. (c) GO analysis of peptide precursor proteins. The horizontal axis represents the enrichment degree rich factor value, and the vertical axis represents the GO term information. The size of the circle represents the number of peptide precursor proteins in the pathway; the larger circle indicates the more number.

DEPs derived from stress-related precursor proteins involving seed ageing

To further reveal the factors involving tobacco seed ageing, the details of peptides derived from oxygen-containing compound proteins were analyzed. In which, most peptides derived from 11 kDa late embryogenesis abundant protein-like, 1-Cys peroxiredoxin-like, and catalase isozyme 1 (Table 1).

Table 1. Details of peptides derived from the precursor proteins involved in response to oxygen-containing compound in tobacco

Protein_id	Peptide	log2(fold change)	*P* Value	Description
Nitab4.5_0000105g0110	M(+42.01)QAAKEKAANVA.A	5.53	0.0010	XP_009604364.1 PREDICTED: 11 kDa late embryogenesis abundant protein-like
Nitab4.5_0000105g0110	M(+42.01)QAAKEKAANVAASA.K	2.31	0.0152	XP_009604364.1 PREDICTED: 11 kDa late embryogenesis abundant protein-like
Nitab4.5_0000105g0110	M(+42.01)QAAKEKA.A	1.12	0.0225	XP_009604364.1 PREDICTED: 11 kDa late embryogenesis abundant protein-like
Nitab4.5_0000204g0010	M.PGLTIGDDLPNLE.V	11.88	0.0003	XP_016505780.1 PREDICTED: 1-Cys peroxiredoxin-like
Nitab4.5_0000204g0010	M.PGLTIGDDLPNLEVETTHGKM.K	-3.60	0.0065	XP_016505780.1 PREDICTED: 1-Cys peroxiredoxin-like
Nitab4.5_0000204g0010	M.PGLTIGDDLPNLEVETTHGKMKLH.D	-1.96	0.0141	XP_016505780.1 PREDICTED: 1-Cys peroxiredoxin-like
Nitab4.5_0000204g0010	Q.GYDTANLPSRKDYL.H	-3.45	0.0169	XP_016505780.1 PREDICTED: 1-Cys peroxiredoxin-like
Nitab4.5_0000204g0010	M.PGLTIGDDLPNLEVETTHGKM(+15.99).K	-5.41	0.0248	XP_016505780.1 PREDICTED: 1-Cys peroxiredoxin-like
Nitab4.5_0000204g0010	P.GEPVVISPSVSN.E	-3.10	0.0486	XP_016505780.1 PREDICTED: 1-Cys peroxiredoxin-like
Nitab4.5_0000261g0150	E.GVDIDESKFSTS	8.22	0.0014	XP_009606940.1 PREDICTED: protein SLE1
Nitab4.5_0000261g0150	M.A(+42.01)SGQQQQQRSELDARAR.Q	2.29	0.0330	XP_009606940.1 PREDICTED: protein SLE1
Nitab4.5_0000266g0030	T.FQGPPHGIQ.V	2.20	0.0340	PHT98340.1 Ribulose bisphosphate carboxylase large chain
Nitab4.5_0000563g0250	E.GVAEWQSSLH.S	-5.38	0.0257	XP_009595923.1 PREDICTED: nucleoside diphosphate kinase 1
Nitab4.5_0000723g0150	M.S(+42.01)HYDNQFSAG.Q	4.87	0.0078	XP_009624592.1 PREDICTED: dehydrin DHN1-like isoform X1
Nitab4.5_0000723g0150	M.S(+42.01)HYDNQFSAGQA.L	2.43	0.0114	XP_009624592.1 PREDICTED: dehydrin DHN1-like isoform X1
Nitab4.5_0000781g0010	A.NLPSGKDYLRFTNV	4.78	0.0104	XP_016490368.1 PREDICTED: 1-Cys peroxiredoxin-like
Nitab4.5_0000781g0010	Q.GYDTANLPSGKDYLRFTNV	0.58	0.0341	XP_016490368.1 PREDICTED: 1-Cys peroxiredoxin-like
Nitab4.5_0000797g0130	E.GIEIDESKYKTKS	7.60	0.0016	XP_009628792.1 PREDICTED: em protein H5
Nitab4.5_0000797g0130	M.A(+42.01)SQQEKSELNRRAKEGETVVPG GTGGKSLE.A	6.32	0.0044	XP_009628792.1 PREDICTED: em protein H5
Nitab4.5_0001618g0150	A.TFIDDHIRHHAQ.A	-3.16	0.0492	XP_009766444.1 PREDICTED: S-formylglutathione hydrolase
Nitab4.5_0003904g0050	E.IIPVPTK.I	-3.59	0.0059	XP_009619812.1 PREDICTED: 3-ketoacyl-CoA thiolase 2, peroxisomal
Nitab4.5_0003904g0050	T.KIVDPKTGDETP.V	-0.40	0.0148	XP_009619812.1 PREDICTED: 3-ketoacyl-CoA thiolase 2, peroxisomal
Nitab4.5_0003904g0050	F.YAGFPETVPVRT.V	-5.47	0.0154	XP_009619812.1 PREDICTED: 3-ketoacyl-CoA thiolase 2, peroxisomal
Nitab4.5_0003904g0050	E.IIPVPTKIVDPKTGDET.P	-1.21	0.0194	XP_009619812.1 PREDICTED: 3-ketoacyl-CoA thiolase 2, peroxisomal
Nitab4.5_0005796g0030	M.A(+42.01)DLRDEHGNPIQ.L	6.93	0.0048	XP_009765052.1 PREDICTED: late embryogenesis abundant protein
Nitab4.5_0006960g0010	M(+42.01)DPYKYRPS.S	-3.22	0.0006	XP_009794245.1 PREDICTED: catalase isozyme 1
Nitab4.5_0006960g0010	M(+42.01)DPYKYRPSS.A	-1.89	0.0348	XP_009794245.1 PREDICTED: catalase isozyme 1
Nitab4.5_0006960g0010	M(+42.01)DPYKYRP.S	-3.19	0.0406	XP_009794245.1 PREDICTED: catalase isozyme 1
Nitab4.5_0010922g0010	A.SFRKPVEPY	4.55	0.0044	XP_009764205.1 PREDICTED: enolase-like
Nitab4.5_0010922g0010	L.RIEEELGSDAVYAGASFRKPVEPY	-2.91	0.0454	XP_009764205.1 PREDICTED: enolase-like

Table 2. Details of peptides derived from the precursor proteins globulin involved in seed ageing tolerance in tobacco

Protein_id	Peptide	log2(fold change)	*P* Value	Description
Nitab4.5_0000044g0350	A.IVAPHWNMNAH.S	3.57	0.009854871	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	E.ELRLTLPEESEQEERREQQQREGGRW.P	8.42	0.004485347	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	E.SEQEERREQQQREGGRW.P	3.95	0.032063299	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	F.AIVKRAGNEGLEYIAF.K	2.80	0.024693519	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	F.DTEILSEAF.N	4.47	0.03057303	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	F.FLAGNPQ.R	2.49	0.024402908	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	F.FLAGNPQRGLQ.Q	6.60	0.006199966	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	F.FLAGNPQRGLQQQ.F	7.99	0.007421698	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	F.FLAGNPQRGLQQQF.V	7.48	0.01437893	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	F.KTNDNAM(+15.99)VNPLAGRLSAL.R	7.46	0.034065327	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	F.KTNDNAMVNPLAGRLSAL.R	6.11	0.004198995	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	F.LAGNPQRGLQQ.Q	2.08	0.001478513	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	F.LAGNPQRGLQQQF.V	5.24	0.023472556	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	K.RAGNEGLEYIAF.K	3.54	0.044750441	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.ALPAGVTFW.M	3.57	0.004600271	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.DTSNHANQLDLTFRSF.F	11.10	0.003719116	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.IVPQNFAIVK.R	4.86	0.047516264	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.KLRENIGHPSRSDVYN.P	-2.20	0.022899842	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.LDTSNHANQL.D	3.62	0.035969392	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.LDTSNHANQLDLTFRSF.F	5.24	0.012913905	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.QLSAERGTLYRNA.I	1.28	0.038434823	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.RAMPEEVLM.N	4.04	0.000406303	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.RAMPEEVLMN.S	5.75	0.001531199	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.RAMPEEVLMNSY.Q	9.03	0.003541628	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.RAMPEEVLMNSYQIS.R	3.62	0.010267537	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.RLTLPEESEQEERREQQQREGGRWP.L	7.41	0.019027537	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.SAERGTLYRNA.I	3.60	0.016049151	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.SLPILNFL.Q	4.45	0.020600061	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.SLPILNFLQL.S	8.98	0.003418218	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	L.TLPEESEQEERREQQQREGGRW.P	5.69	0.000649841	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	N.AIVAPHWNM.N	2.42	0.028560352	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	N.AQEPTRRFESEAG.V	3.06	0.015749566	XP_009603582.1 PREDICTED: 11S globulin

				subunit beta-like
Nitab4.5_0000044g0350	N.DNAMVNPLAGRLSAL.R	7.53	0.002111477	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	N.FLQLSAERGTLYRNA.I	4.55	0.032221399	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	N.IGHPSRSDVYNPRGGRVSTVNSL.S	1.82	0.029067803	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	N.PRGGRVSTVNSL.S	2.33	0.013510788	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	N.S(+42.01)YQ(+.98)ISRQEARSLKYN.R	4.62	0.020334075	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	N.SYQISRQEARSLKYN.R	4.92	0.019725622	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	N.VWQRLQQQ.Q	5.53	0.002511654	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	N.VWQRLQQQQQHRAL.R	2.48	0.012534492	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	Q.FVGRQQETT.M	5.76	0.030858928	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	Q.LSAERGTLYRNA.I	7.83	0.014998963	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	Q.NFAIVKRAGNEGLE.Y	4.89	0.032083271	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	Q.NFAIVKRAGNEGLEYIAF.K	4.01	0.005066811	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	R.AMPEEVLMNSY.Q	10.08	0.046007541	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	R.AMPEEVLMNSYQISRQEARSLKYN.R	-1.98	0.040104034	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	R.SDVYNPRGGRVSTVN.S	1.89	0.007622902	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	S.AERGTLY.R	-3.03	0.019056136	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	S.ALRAMPEEVLMNSYQISRQEARSLKYN.R	-2.01	0.014988708	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	S.DVYNPRGGRVSTVNSL.S	2.84	0.002044224	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	S.EQEKTSKGGNIFNGFDTEILSE.A	8.67	0.017331629	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	S.LLDTSNHANQL.D	4.42	0.037648218	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	S.RQEARSLKYNRGRVD.C	3.34	0.026345024	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	T.NDNAM(+15.99)VNPLAGRLSAL.R	6.20	0.032867094	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	T.NDNAMVNPLAGRLSAL.R	5.58	0.011432039	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	Y.NDAEEPIVTVSLL.D	4.99	0.017445916	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	Y.Q(+42.01)(+.98)ISRQEARSL.K	9.77	0.001282809	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	Y.Q(+42.01)(+.98)ISRQEARSLKYN.R	4.94	0.009893117	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	Y.QISRQEARSL.K	9.70	0.004259262	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	Y.QISRQEARSLKYN.R	5.32	0.000592294	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	Y.RNAIVAPHWNMNAH.S	2.87	0.008019712	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000044g0350	Y.TNTPMLM.Y	-1.43	0.003411681	XP_009603582.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	A.GNPQRGVQQQMLG.R	4.31	0.004381964	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	D.ELNVFGPGAKSGRQQY.A	2.86	0.011734928	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	E.SEEQQQQQWQQGRRGGSWP.L	2.59	0.006261552	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like

Nitab4.5_0000436g0260	F.FLAGNPQRGVQQQ.M	4.03	0.013526495	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	F.FLAGNPQRGVQQQM.L	2.67	0.001232723	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	F.LAGNPQRGVQQQM.L	6.75	0.003561065	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	F.LRPEESEEQQQQQWQQGRRGGSWP.L	9.04	0.017684466	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	G.EQERGGNILSGFDAQLL.S	4.09	0.016094761	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	G.LTLPVLNF.L	4.80	0.020910016	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	K.SLKYNRDELNVFGPGAKSGRQQ.Y	2.18	0.000741247	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	K.SLKYNRDELNVFGPGAKSGRQQY.A	2.42	0.000364757	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	K.YNRDELNVFGPGAKSGRQQ.Y	-2.17	0.023600729	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	K.YNRDELNVFGPGAKSGRQQY.A	-2.20	0.001212669	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	L.AGNPQRGVQQQM.L	1.48	0.037415104	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	L.DLTFRKF.F	1.88	0.018167242	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	L.IDTSNQANQLDLTFRKFF.L	4.65	0.019256248	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	L.IDTSNQANQLDLTFRKFFLA.G	0.97	0.046279465	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	L.IDTSNQANQLDLTFRKFFLAGNPQRGVQQQ.M	-0.48	0.019257982	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	L.KYNRDELNVFGPGAKSGRQQY.A	3.97	0.041061526	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	L.KYNRDELNVFGPGAKSGRQQYA	3.75	0.004567453	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	L.SDAFNVDPEIIRRL.Q	7.77	0.015068786	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	L.SGFDAQLL.S	5.11	0.024968897	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	L.TLPVLNF.L	7.41	0.003591541	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	L.TLPVLNFLQ.L	6.71	0.010620893	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	N.RDELNVFGPGAKSGRQQ.Y	4.14	0.038373658	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	N.VDPEIIRR.L	3.38	0.000124523	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	N.VFGPGAKSGRQQY.A	6.60	0.000815942	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	Q.VWQRLQQQ.Q	5.53	0.002511654	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	Q.VWQRLQQQQQHRAL.R	2.48	0.012534492	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	R.FLRPEESEEQQQQQWQQGRRGGSWP.L	4.19	0.040511142	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	R.GEQERGGNIL.S	5.57	0.035399417	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	R.GEQERGGNILSGFDAQLL.S	4.40	0.007555133	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	S.LIDTSNQANQLDLTFRKFFLAGNPQRGVQ.Q	3.82	0.014122825	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	S.RQEAKSLKYNRDELNVFGPGAKSGRQQ.Y	3.23	0.007950311	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	T.LPVLNFL.Q	4.46	0.022590923	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	Y.NRDELNVFGPGAKSGRQQY.A	-2.49	0.0073843	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0000436g0260	Y.QISRQEAKSLKYNRDELNVFGPGAKSGRQQY.A	8.20	0.000760393	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like

Nitab4.5_0000436g0260	Y.RNAIVAPHWNMNAH.A	2.87	0.008019712	XP_016490392.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	A.AVRNVIQPQ.G	6.00	0.010568769	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	A.GNPNPRGQSGSRYEEE.I	4.62	0.023023793	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	A.IRAMPEEVLMN.S	8.44	0.019745617	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	A.IRAMPEEVLMNSYQIS.R	5.93	0.031148973	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	F.FLAGNPNPRGQSG.S	5.87	0.025740495	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	F.FLAGNPNPRGQSGSRY.E	5.73	0.000776514	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	F.FLAGNPNPRGQSGSRYE.E	6.63	0.037145272	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	F.FLAGNPNPRGQSGSRYEE.E	4.70	0.01518857	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	F.LAGNPNPRGQSGS.R	9.25	0.015267494	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	F.NVDPELVRN.L	6.38	0.014530441	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	I.RAMPEEVLM.N	4.04	0.000406303	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	I.RAMPEEVLMN.S	5.75	0.001531199	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	I.RAMPEEVLMNSY.Q	9.03	0.003541628	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	I.RAMPEEVLMNSYQIS.R	3.62	0.010267537	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	K.YNREEATVFAGRRSGGYSTRA.F	-1.28	0.045380624	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	L.AGRLSAI.R	3.59	0.019887339	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	L.KYNREEATVFAGRRSGGY.S	3.08	0.000218309	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	L.KYNREEATVFAGRRSGGYS.T	3.11	0.019103333	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	L.KYNREEATVFAGRRSGGYST.R	2.61	0.011376069	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	L.KYNREEATVFAGRRSGGYSTRA.F	2.97	0.004337539	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	L.LLPHYNNAPQLL.Y	4.25	0.009284594	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	L.NSHKLPIL.N	6.77	0.000480179	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	L.NSHKLPILNWL.Q	3.44	0.013126517	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	L.SAERGVLY.Q	3.26	0.015089134	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	L.SAIRAMPEEVLMN.S	3.26	0.026245405	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	L.SAIRAMPEEVLMNSY.Q	5.24	0.012095225	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	M.RLGENLGRPSRAD.V	-1.83	0.026315081	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	N.AVMAPYWNM.N	2.82	0.018275064	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	N.AVMAPYWNMNAHSIL.Y	6.89	0.004685172	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	N.LGRPSRADVYNPRGGRISTL.N	4.18	0.013047174	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	N.REEATVFAGRRSGGYSTR.A	-1.78	0.016025784	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	N.REEATVFAGRRSGGYSTRA.F	-1.71	0.021414759	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	Q.GLLLPHYNNAPQLLY.I	2.30	0.025705735	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like

Nitab4.5_0004662g0050	Q.NFAVVKRAGNQGLDYIAF.K	3.77	0.000844334	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	S.AIRAMPEEVLMN.S	4.93	0.005936842	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	S.AIRAMPEEVLMNSY.Q	7.00	0.002488012	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	S.AIRAMPEEVLMNSYQIS.R	1.58	0.04503679	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	T.NDQAMTSPLAGRLSA.I	4.46	0.012362749	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like
Nitab4.5_0004662g0050	W.FYNNGQERLVTVA.L	5.26	0.010330232	XP_016489177.1 PREDICTED: 11S globulin subunit beta-like

Interestingly, the expressions of all peptides derived 11 kDa late embryogenesis abundant protein-like significantly increased in seeds after 5 days AA treatment compared with those of CK, while the expressions of majority peptides derived 1-Cys peroxiredoxin-like and catalase isozyme 1 significantly decreased. The up- and down-regulated peptides may participate in stress responses involving tobacco seed ageing.

DEPs derived from globulin proteins involving seed ageing

Larger number of DEPs derived from 11S globulin subunit beta-like proteins (Table 2). More than one peptide derived from same precursor protein, and many peptides came from the same 11S globulin subunit beta-like proteins. Especially, more than 35 peptides derived from three types of 11S globulin subunit beta-like proteins (Nitab4.5_0000044g0350, Nitab4.5_0000436g0260, and Nitab4.5_0004662g0050). Interestingly, the expressions of majority peptides significantly increased in 5 days AA seeds. It suggests that up-regulated peptides derived from 11S globulin subunit beta-like proteins may participate in seed ageing tolerance in tobacco.

Discussion

Seed ageing is usually occurred under high temperatures and humid conditions in crops (Chen et al., 2022; Niu et al., 2022). In this study, seed germination and seedling establishment significantly decreased after 5 days AA treatment under high temperatures and humid conditions in tobacco. Several genes have been used as biomarkers, such as *NtPIMT* and *NtOGG1*, for seed ageing tolerance in tobacco (Niu et al., 2022). It is well known that proteins are associated with seed vigor (Wang et al., 2015) and peptides play critical roles in stress responses (Matsubayashi, 2014; Christmann and Grill, 2018). In this study, a comparative peptidomics analysis conducted to identify the peptides that related with seed ageing in tobacco. The identified DEPs derived from the late embryogenesis abundant protein-like, 1-Cys peroxiredoxin-like, catalase isozyme 1, and globulin precursor proteins. Theses DEPs may play important roles on the regulation of seed ageing in tobacco.

The analysis of precursor proteins can elucidate the potential functions of peptides. In this study, the functions of precursor proteins mainly related to stress tolerance. Lipid peroxidation, protein oxidation, DNA and RNA damage, and repair system damage are involved in seed ageing (Salvi et al., 2022). It is well known that the excessive ROS is related with seed ageing in crops (Adetunji et al., 2021; Boucelha et al., 2021). Late embryogenesis abundant proteins as the protective molecules can improve stress tolerance through replacing water, sequestering ions, and removing ROS and so on (Wang et al., 2015). In this study, several peptides derived late embryogenesis abundant proteins in aged seeds in tobacco. Moreover, the peptides derived from peroxiredoxin and catalase proteins significantly decreased in the aged seeds, which play important roles on the regulation of ROS level (Li et al., 2022). Further, many DEPs derived from the precursor protein globulin in the aged seeds. It had been reported that the globulin is involved in rice seed vigor by regulating hydrogen peroxide (H_2O_2) levels (Peng et al., 2022ab). Moreover, seed storage proteins may be a primary target for oxidative stress and protect other proteins for oxidative damage (Davies, 2005; Nguyen et al., 2015; Yin et al., 2017). Thus, the potential functions of peptides derived from the late embryogenesis abundant proteins, peroxiredoxin, catalase, and globulin proteins may promote ageing tolerance by influencing ROS level in tobacco.

Plant peptides can be mainly divided into the precursor-derived and nonprecursor-derived peptides

(Tavormina et al., 2015). Proteolytic processing from precursor proteins is the main source of peptides in plants (Brito et al., 2018; Chen et al., 2020). Similarly, we observed that the most peptides were derived from the precursor proteins in tobacco in this study. Peptides can also be directly translated from the nonprecursor-derived group of small open reading frames (sORF) (Tavormina et al., 2015). However, these peptides need to be further investigated in the future. It had been reported that peptides play important roles on signaling and cell-cell communication during seed development (Qu et al., 2015). Storage proteins are hydrolyzed into small peptides during seed germination (Salmenkallio and Sopanen, 1989). It would be interesting to assess whether the peptides act as signals involved in seed ageing during seed storage or germination in tobacco.

Conclusion

Peptidomics analysis used to identify the peptides that may regulate tobacco seed ageing in this study. Many candidate peptides derived from stress response-related precursor proteins, such as late embryogenesis abundant protein, peroxiredoxin, catalase, and globulin proteins, may involve in the response of seed ageing in tobacco. These findings could make a significant contribution to understand the biological functions of peptides on seed ageing tolerance in tobacco. However, the roles of identified peptides, such as the peptides derived from the late embryogenesis abundant proteins, peroxiredoxin, catalase, and globulin proteins, on seed ageing tolerance need to be further investigated in tobacco. In this study, the natural seed ageing under the humidity and temperature of tobacco storage conditions was not considered for peptidomics analysis. Therefore, whether identified peptides involving in tobacco seed ageing tolerance during natural storage also needs to be further investigated.

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Contribution of Authors

Wang G: Performed experiments, wrote manuscript and approved final version for submission.
Zheng Y & Suo W: Performed experiments, read and approved final manuscript for submission.
Zhou D, Xu J & He Y: Analyzed data, read and approved final manuscript for submission.
Wang Z: Conceptualized study and wrote manuscript
Zhang L: Conceptualized study, read and approved final manuscript for submission.

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