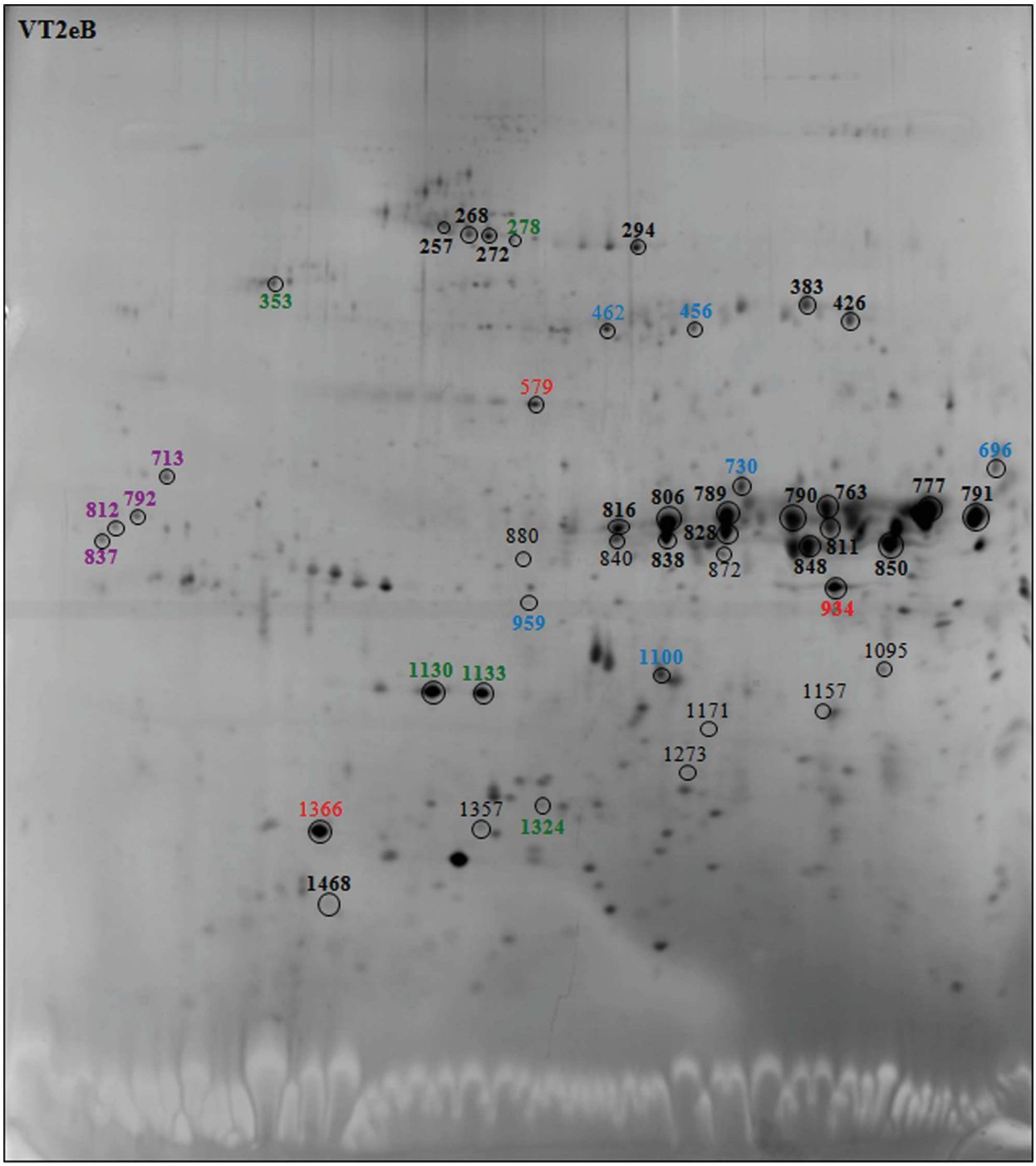


**Fig 8. 2D gel electrophoresis of VT2eB seeds.** Polypeptides changed in transgenic line withrespect to the WT were evidenced by circle. The number of spots correspond to polypeptides identified by MALDI TOF/TOF MS analysis showed in Table 2. Storage proteins were evidenced in black, Chaperone proteins in green, LEA proteins in violet, enzymes in blue, and other proteins in red. Spot numbers of the enhanced polypeptides compared to the WT are in bold.

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Table 1. 1D gel-resolved seed proteins identified by MALDI-TOF/TOF MS.

| Band N. | Protein description | Accession number | Organism | Mascot search results | | |
|---|---|---|---|---|---|---|
| | | | | N. of matched peptides | Sequence coverage (%) | Score |
| *Wild type* | | | | | | |
| 1 | PREDICTED: vicilin-like antimicrobial peptides 2–2 | gi\|697094707 | *N. tomentosiformis* | 14/37 | 26 | 119 |
| | PREDICTED: vicilin-like antimicrobial peptides 2–3 | gi\|698426490 | *N. sylvestris* | 13/37 | 24 | 106 |
| 2 | PREDICTED: vicilin-like antimicrobial peptides 2–3 | gi\|698557844 | *N. sylvestris* | 22/44 | 26 | 176 |
| | PREDICTED: vicilin-like antimicrobial peptides 2–3 isoform X2 | gi\|697173575 | *N. tomentosiformis* | 21/44 | 26 | 164 |
| 3 | PREDICTED: vicilin-like antimicrobial peptides 2–3 isoform X2 | gi\|697173575 | *N. tomentosiformis* | 24/70 | 29 | 139 |
| | PREDICTED: vicilin-like antimicrobial peptides 2–3 | gi\|698557844 | *N. sylvestris* | 23/70 | 25 C-fragment | 125 |
| 4 | PREDICTED: legumin B-like | gi\|698534493 | *N. sylvestris* | 18/102 | 33 | 107 |
| | PREDICTED: 11S globulin subunit beta-like | gi\|697189071 | *N. tomentosiformis* | 17/102 | 33 | 94 |
| | PREDICTED: centromere-associated protein E-like isoform X2 | gi\|721694598 | *B. distachyon* | 30/102 | 19 | 79 |
| 5 | PREDICTED: legumin A-like | gi\|697139891 | *N. tomentosiformis* | 18/81 | 39 | 122 |
| | PREDICTED: legumin A-like | gi\|698529732 | *N. sylvestris* | 17/81 | 38 | 111 |
| | PREDICTED: centromere-associated protein E-like isoform X2 | gi\|721694598 | *B. distachyon* | 26/81 | 19 | 94 |
| 6 | PREDICTED: legumin A-like | gi\|697139896 | *N. tomentosiformis* | 18/75 | 35 | 127 |
| | PREDICTED: 11S globulin seed storage protein 2-like | gi\|697139889 | *N. tomentosiformis* | 18/75 | 33 | 73 |
| 7 | PREDICTED: legumin B-like | gi\|698534493 | *N. sylvestris* | 18/91 | 31 C-fragment | 85 |
| 8 | PREDICTED: legumin B-like | gi\|698534493 | *N. sylvestris* | 15/73 | 23 | 93 |
| | PREDICTED: legumin A-like | gi\|698517368 | *N. sylvestris* | 14/73 | 24 C-fragment | 77 |
| 9 | PREDICTED: legumin B-like | gi\|698534493 | *N. sylvestris* | 17/95 | 34 | 92 |
| | PREDICTED: 11S globulin subunit beta-like | gi\|697189071 | *N. tomentosiformis* | 16/95 | 32 | 82 |
| 10 | PREDICTED: vicilin-like antimicrobial peptides 2–3 | gi\|698557844 | *N. sylvestris* | 22/89 | 24 | 103 |
| *F18* | | | | | | |
| 11 | PREDICTED: legumin A-like | gi\|698517368 | *N. sylvestris* | 12/39 | 26 | 103 |
| 12 | PREDICTED: vicilin-like antimicrobial peptides 2–3 | gi\|698426490 | *N. sylvestris* | 22/69 | 42 | 163 |
| 13 | PREDICTED: vicilin-like antimicrobial peptides 2–3 | gi\|698557844 | *N. sylvestris* | 31/64 | 34 | 223 |
| 14 | PREDICTED: 11S globulin seed storage protein 2-like | gi\|697139889 | *N. tomentosiformis* | 17/66 | 32 | 86 |
| | PREDICTED: legumin B-like | gi\|698534493 | *N. sylvestris* | 14/66 | 24 | 82 |
| | PREDICTED: 11S globulin subunit beta-like | gi\|697189071 | *N. tomentosiformis* | 14/66 | 24 | 82 |
| 15 | PREDICTED: legumin A-like | gi\|697139891 | *N. tomentosiformis* | 12/41 | 25 N-fragment | 103 |

(*Continued*)

**Table 1.** (*Continued*)

| Band N. | Protein description | Accession number | Organism | Mascot search results | | |
|---|---|---|---|---|---|---|
| | | | | N. of matched peptides | Sequence coverage (%) | Score |
| 16 | PREDICTED: 11S globulin seed storage protein 2-like | gi\|697139898 | *N. tomentosiformis* | 14/60 | 68 | 98 |
| 17 | PREDICTED: 11S globulin seed storage protein 2-like | gi\|697139898 | *N. tomentosiformis* | 13/49 | 72 | 108 |
| 18 | PREDICTED: legumin B-like | gi\|697151558 | *N. tomentosiformis* | 15/54 | 22 C-fragment | 92 |
| *VT2eB* | | | | | | |
| 19 | PREDICTED: vicilin-like antimicrobial peptides 2–2 | gi\|697094707 | *N. tomentosiformis* | 10/25 | 25 | 88 |
| 20 | PREDICTED: vicilin-like antimicrobial peptides 2–3 | gi\|698426490 | *N. sylvestris* | 19/44 | 35 | 165 |
| 21 | PREDICTED: vicilin-like antimicrobial peptides 2–3 | gi\|698557844 | *N. sylvestris* | 30/62 | 32 C-fragment | 212 |
| 22 | PREDICTED: legumin B-like | gi\|698534493 | *N. sylvestris* | 15/73 | 25 | 98 |
| | PREDICTED: 11S globulin subunit beta-like | gi\|697189071 | *N. tomentosiformis* | 14/73 | 24 | 86 |
| 23 | PREDICTED: legumin A-like | gi\|697139896 | *N. tomentosiformis* | 12/42 | 27 N-fragment | 91 |
| | PREDICTED: centromere-associated protein E-like isoform X2 | gi\|721694598 | *B. distachyon* | 17/42 | 15 | 84 |
| 24 | PREDICTED: 11S globulin seed storage protein 2-like | gi\|697139898 | *N. tomentosiformis* | 13/45 | 64 | 98 |
| 25 | PREDICTED: 11S globulin seed storage protein 2-like | gi\|697139898 | *N. tomentosiformis* | 13/42 | 68 | 115 |
| 26 | PREDICTED: legumin B-like | gi\|698538015 | *N. sylvestris* | 14/41 | 25 C-fragment | 98 |

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suggested the presence of a different isoform of this protein. One of these isoforms disappeared from VT2eB seeds. The reason for the delay in root elongation for F18 and VT2eB (Fig 1B) could be due to the need for *de novo* production or the modification of proteins involved in cell cycle progression. Further experiments investigating seed maturation are needed to clarify this hypothesis.

Concerning the storage proteins, bands 8–10 comprise three classes of reserve proteins belonging to vicilin, legumin and globulin families and were only observed in the WT seeds. This suggests that the limited proteolysis leading to the destabilization of the tertiary structures and to the susceptibility of storage proteins to unlimited proteolysis occurred in the WT during seed maturation, whereas it was not observed in the transgenic lines in two independent experiments. In fact, the same proteins were identified in the corresponding bands 13–14 and 22–23, at a higher molecular weight, of the F18 and VT2eB mutants, respectively (Table 1 and Fig 5). The correct folding and packaging of storage proteins play a crucial role in regulating their resistance to proteolysis by specific enzymes [38]. The limited proteolysis of prolegumins in developing seeds as well as that of legumin and vicilin during the seedling development depends on the presence of accessible sites for proteolysis [55]. The limited cleavage destabilizes the tertiary structure of storage proteins and makes them susceptible to further unlimited proteolysis during seed germination and seedling [55]. In addition, the limited proteolysis of storage proteins in WT dry seeds could reflect differences in reserve protein folding and may facilitate the further accessibility of processing enzymes to mobilize the resources, leading to a faster seed germination.

Table 2. 2D gel resolved seed polypeptides identified by MALDI-TOF/TOF MS.

| Spot N. | Protein description | Accession number | Organism | Mascot search results | | | Mean % V±SD x $10^{-4}$[a] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | N. of matched peptides | Sequence coverage (%) | Score | WT | VT2eB | F18 |
| *Storage proteins* | | | | | | | | | |
| 257 | PREDICTED: seed biotin-containing protein SBP65-like isoform X2 | gi\|1025096692 | *N. tabacum* | 7/14 | 20 | 80 | - | 3091 ±1484[$b] | 771 ±483[$] |
| 268 | PREDICTED: seed biotin-containing protein SBP65-like isoform X1 | gi\|1025096688 | *N. tabacum* | 11/19 | 29 | 129 | 140 ±122*[&] | 2300 ±781* | 1428 ±347[&] |
| 272 | PREDICTED: seed biotin-containing protein SBP65-like isoform X2 | gi\|1025096692 | *N. tabacum* | 17/27 | 39 | 168 | 153±133* | 4165 ±1220* | 2853 ±729[&] |
| 383 | PREDICTED: embryonic protein DC-8-like | gi\|1025354957 | *N. tabacum* | 9/13 | 25 | 109 | - | 3835±303 | 4155 ±114 |
| 426 | PREDICTED: embryonic protein DC-8-like | gi\|1025374019 | *N. tabacum* | 5/5 | 17 | 81 | - | 4201±656 | 3812 ±463 |
| 294 | PREDICTED: seed biotin-containing protein SBP65-like isoform X2 | gi\|698480227 | *N. sylvestris* | 18/13 | 31 | 172 | - | 5246 ±1718 | 4238 ±1551 |
| 763 | PREDICTED: 11S globulin subunit beta-like | gi\|697189073 | *N. tomentosiformis* | 16/32 | 30–N fragment | 173 | 6015 ±1507*[&] | 17341 ±3788* | 15940 ±2684[&] |
| 777 | PREDICTED: 11S globulin seed storage protein 2-like | gi\|1025029409 | *N. tabacum* | 18/33 | 46 | 228 | 21424 ±2836*[&] | 70003 ±14299* | 63336 ±1608[&] |
| 789 | PREDICTED: 11S globulin subunit beta-like | gi\|697189071 | *N. tomentosiformis* | 17/50 | 30 N-fragment | 171 | 3927 ±382*[&] | 18535 ±1247* | 23374 ±2004[&] |
| | PREDICTED: legumin B-like | gi\|698534493 | *N. sylvestris* | 14/50 | 29 N-fragment | 129 | | | |
| 790 | PREDICTED: 11S globulin subunit beta-like | gi\|1025251419 | *N. tabacum* | 17/36 | 32 N-fragment | 196 | 4564 ±244*[&] | 19335 ±5406* | 15106 ±369[&] |
| 791 | PREDICTED: legumin B-like PREDICTED: | gi\|698538015 | *N. sylvestris* | 16/30 | 26 N-fragment | 185 | 12033 ±746*[&] | 33821 ±6004* | 33199 ±8730[&] |
| | 11S globulin subunit beta-like | gi\|1025258047 | *N. tabacum* | 13/30 | 20 N-fragment | 137 | | | |
| 806 | PREDICTED: legumin B-like PREDICTED: | gi\|1025293602 | *N. tabacum* | 16/41 | 28 N-fragment | 171 | 5090 ±572*[&] | 34191 ±7100* | 30774 ±3758[&] |
| | 11S globulin subunit beta-like | gi\|697189071 | *N. tomentosiformis* | 13/41 | 23 N-fragment | 122 | | | |
| 811 | PREDICTED: 11S globulin subunit beta-like | gi\|1025251419 | *N. tabacum* | 18/63 | 33 N-fragment | 146 | 1993 ±747*[&] | 5722 ±753* | 5505 ±762[&] |
| | PREDICTED: legumin B-like | gi\|698538021 | *N. sylvestris* | 15/63 | 26 N-fragment | 103 | | | |
| 816 | PREDICTED: legumin B-like | gi\|697189071 | *N. tomentosiformis* | 18/41 | 30 N-fragment | 187 | 2716 ±427*[&] | 7565 ±564*[$] | 9658 ±1084[&$] |
| | PREDICTED: 11S globulin subunit beta-like | gi\|698534493 | *N. sylvestris* | 16/41 | 30 N-fragment | 156 | | | |
| 828 | PREDICTED: 11S globulin subunit beta-like | gi\|697189071 | *N. tomentosiformis* | 17/37 | 32 N-fragment | 178 | 1954 ±41*[&] | 9256 ±1498* | 9369 ±1131[&] |
| | PREDICTED: legumin B-like | gi\|698534493 | *N. sylvestris* | 14/37 | 30 N-fragment | 133 | | | |
| 838 | PREDICTED: legumin B-like | gi\|1025293602 | *N. tabacum* | 17/33 | 33 N-fragment | 194 | 2914 ±262*[&] | 9349 ±1096* | 8793 ±1982[&] |
| 840 | PREDICTED: legumin B-like | gi\|698534493 | *N. sylvestris* | 17/49 | 29 N-fragment | 158 | 1549 ±468[&] | 2447±221 | 3277 ±868[&] |
| | PREDICTED: 11S globulin subunit beta-like | gi\|697189071 | *N. tomentosiformis* | 14/49 | 28 N-fragment | 117 | | | |
| 848 | PREDICTED: legumin A-like | gi\|697139891 | *N. tomentosiformis* | 17/39 | 32 N-fragment | 155 | 9044 ±1245*[&] | 18285 ±2482* | 16861 ±1198[&] |

(*Continued*)

Table 2. (*Continued*)

| Spot N. | Protein description | Accession number | Organism | Mascot search results | | | Mean % V±SD x $10^{-4a}$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | N. of matched peptides | Sequence coverage (%) | Score | WT | VT2eB | F18 |
| **850** | PREDICTED: legumin A-like | gi\|697139896 | *N. tomentosiformis* | 20/33 | 40 N-fragment | 226 | 8934 ±635*[&] | 18326 ±712* | 23113 ±6615[&] |
| **872** | PREDICTED: legumin A-like | gi\|697139891 | *N. tomentosiformis* | 11/22 | 24 N-fragment | 124 | 799±124[&] | 1210±162 | 2081 ±683[&] |
| **880** | PREDICTED: 11S globulin subunit beta-like | gi\|697189073 | *N. tomentosiformis* | 7/10 | 13 Central fragment | 81 | 6154 ±1607*[&] | 402±158* | 443±87[&] |
| **1157** | PREDICTED: legumin A-like | gi\|697139896 | *N. tomentosiformis* | 7/9 | 19 | 102 | 2116 ±838*[&] | 422±126* | 136 ±235[&] |
| **1171** | PREDICTED: legumin A-like | gi\|697139891 | *N. tomentosiformis* | 5/5 | 11 Central fragment | 83 | 1856 ±212*[&] | 391±161* | 428 ±176[&] |
| **1095** | PREDICTED: vicilin-like antimicrobial peptides 2–3 | gi\| 1025308817 | *N. tabacum* | 8/8 | 8 Central fragment | 108 | 16436 ±512*[&] | 1139 ±333* | 989 ±462[&] |
| **1273** | PREDICTED: legumin A-like | gi\|698517368 | *N. sylvestris* | 8/11 | 20 Central fragment | 115 | 4248 ±330*[&] | 796±437* | 423±80[&] |
| **1357** | PREDICTED: 11S globulin subunit beta-like | gi\|697189071 | *N. tomentosiformis* | 11/16 | 17 Central fragment | 139 | 2462 ±442*[&] | 740±176* | 1055 ±185[&] |
| **1468** | PREDICTED: 11S globulin subunit beta-like | gi\|697189073 | *N. tomentosiformis* | 7/11 | 13 Central fragment | 79 | - | 667±447 | 1089 ±241 |
| ***Chaperone proteins*** | | | | | | | | | |
| **278** | PREDICTED: heat shock 70 kDa protein, mitochondrial | gi\| 1025062529 | *N. tabacum* | 10/13 | 17 | 110 | 207±196[+] | 1006 ±278[+] | 790±484 |
| **1130** | PREDICTED: 17.1 kDa class II heat shock protein-like | gi\| 1025386162 | *N. tabacum* | 9/14 | 64 | 157 | 5317 ±328*[&] | 13584 ±3145* | 13999 ±4152[&] |
| **1133** | PREDICTED: 17.1 kDa class II heat shock protein-like | gi\|698539535 | *N. sylvestris* | 8/14 | 61 | 116 | 2933 ±181*[&] | 7652 ±1605* | 8485 ±2818[&] |
| **1324** | 16.9 kDa class I heat shock protein 1-like | gi\| 1025247079 | *N. tabacum* | 7/17 | 37 | 103 | | 614±167 | 815±308 |
| **353** | PREDICTED: protein disulfide-isomerase-like | gi\|698574414 | *N. sylvestris* | 7/8 | 15 | 104 | 266±275* | 1333 ±197* | 845±299 |
| ***LEA proteins*** | | | | | | | | | |
| **713** | PREDICTED: late embryogenesis abundant protein D-34-like | gi\| 1025097779 | *N. tabacum* | 9/15 | 37 | 124 | 191±51*[&] | 1365 ±418* | 1023 ±132[&] |
| **792** | PREDICTED: late embryogenesis abundant protein D-34-like | gi\| 1025079598 | *N. tabacum* | 8/16 | 43 | 106 | 142±124* | 1260 ±523* | 883±78 |
| **812** | PREDICTED: late embryogenesis abundant protein D-34-like | gi\| 1025097783 | *N. tabacum* | 5/12 | 40 | 73 | 149±69*[&] | 780±285* | 661±84[&] |
| **837** | PREDICTED: late embryogenesis abundant protein D-34-like | gi\| 1025073492 | *N. tabacum* | 9/12 | 47 | 102 | 173±41*[&] | 898±280* | 855 ±200[&] |
| ***Enzymes*** | | | | | | | | | |
| **0,7** | PREDICTED: enolase-like | gi\|697116359 | *N. tomentosiformis* | 14/16 | 39 | 223 | 290±97*[&] | 2092 ±424* | 2427 ±480[&] |
| **462** | PREDICTED: enolase-like | gi\|697116359 | *N. tomentosiformis* | 6/6 | 18 | 101 | 484±208[£] | 1647 ±1484 | 1940 ±484[£] |
| **959** | PREDICTED: aspartic proteinase | gi\|698433659 | *N. sylvestris* | 7/10 | 14 central fragment | 93 | | 415±105 | 458±75 |
| **696** | PREDICTED: glucose and ribitol dehydrogenase homolog 1 | gi\|698551643 | *N. sylvestris* | 5/5 | 16 | 90 | | 3197±600 | 3275 ±890 |

(*Continued*)

**Table 2.** (*Continued*)

| Spot N. | Protein description | Accession number | Organism | Mascot search results | | | Mean % V±SD x $10^{-4}$[a] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | N. of matched peptides | Sequence coverage (%) | Score | WT | VT2eB | F18 |
| **730** | PREDICTED: glucose and ribitol dehydrogenase homolog 1 | gi\|698563269 | *N. sylvestris* | 9/10 | 28 | 153 | 1319 ±304*& | 4750 ±619* | 4526 ±779& |
| **1100** | PREDICTED: methionyl-tRNA formyltransferase-like isoform X3 | gi\| 1025077239 | *N. tabacum* | 5/6 | 15 | 84 | 1664±99+ | 3515 ±390+ | 5265 ±2801 |
| | | | ***Others*** | | | | | | |
| **0,7** | PREDICTED: actin-97 | gi\|698564562 | *N. sylvestris* | 18/29 | 54 | 206 | 1180 ±280*& | 2738 ±193* | 2602 ±751& |
| **934** | PREDICTED: uncharacterized protein LOC104224147 | gi\|698568389 | *N. sylvestris* | 18/42 | 75 | 190 | 3888 ±543*& | 8829 ±824* | 10434 ±2910& |
| **1366** | PREDICTED: MLP-like protein 423 | gi\|697098884 | *N. tomentosiformis* | 7/11 | 13 central fragment | 79 | 6624 ±381*& | 8694 ±3214* | 6701 ±4613& |

[a] Each value represents the mean±SD of individually computed %V in spot maps from wild-type (WT), VT2eB-N and F18-N. tabacum dry seeds.

[b] Pair-wise comparison was performed using a two-tailed Student's t-test ($p \leq 0.05$) and the Tukey's post hoc test ($p \leq 0.05$). Only protein spots showing both statistical reliability and at least 2 fold change in expression are listed as significant differences: WT vsVT2eB-N(*),WT vs F18-N(&),VT2eB-NvsF18-N($). Significant differences according Student's t-test between WT and Vt2eB-N, and WT and F18-N are visualized by (+) and (£), respectively

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Mass spectrometry analysis of 2D gel resolved polypeptides, performed on dry seeds, confirmed the data obtained by protein identification from 1D gel electrophoresis. In fact, most polypeptides excised from 2D gels, which differed in the transgenic seeds with respect to the WT, were reserve proteins belonging to the vicilin, globulin and legumin protein families (Table 2). A 2D gel image analysis showed that a number of spots identified as storage proteins were significantly more abundant in F18 dry seeds, compared with the WT (compare Figs 6 and 7, black bold spots). The number of enhanced reserve proteins also significantly increased in the VT2eB seeds, with respect to the WT (compare Figs 6 and 8, black bold spots). These data confirm that the delay in the germination time of VT2eB and F18 seeds could be correlated to the amount of storage proteins and/or to their folding/assembly state [38].

Although previous studies have suggested that there is no relationship between total protein content and germination rate, the authors do not exclude the possible relationship between germination and specific classes of seed proteins [47].

Our data support a correlation between the increase/folding state of reserve proteins both with delayed germination and with the persistent seed profile. The higher storage protein content for buried seeds could be to support the higher need for nutrients of embryos that need to grow for a longer time until the seedling reaches the soil surface and initiates photosynthesis. In addition, buried seeds showed a round shape and a delayed germination [46, 47] like the transgenic line seeds.

2D gel analysis also confirmed that the proteolysis of storage proteins (legumin, globulin, vicilin) occurred in WT dry seeds, since polypeptides which are shown in black in Figs 7 and 8 with a low molecular weight, were significantly higher in WT seeds with respect to the transgenic lines. These differences suggested that the proteolysis of storage proteins during seed development was poor in the VT2eB and F18 seeds, explaining the retarded germination of mutant seeds.

Interestingly, in addition to the storage proteins, five spots were identified as chaperone proteins with a different molecular mass and belonging to Hsp70, small Hsp proteins (sHsp)

and protein disulfide isomerase families (Table 2; Figs 7 and 8, green spots). Two were significantly enhanced only in VT2eB dry seeds, compared with the WT (Table 2; Fig 8, green spots in bold), while sHsp were also significantly enhanced in F18 (Table 2; Fig 7, green spots in bold). One spot identified as 16.9 kDa class I heat shock protein 1-like was detected only in mutant seeds (Table 2; Figs 7 and 8, green spot 1324). A high range of proteins were found to have chaperone activity. These included many proteins that were identified as heat shock proteins (Hsp), while others were identified as protein disulfide isomerases [56, 57]. Hsps and chaperones are considered as the major class of stress responsive proteins, involved in decreasing cellular damage following abiotic stresses [58, 59]. Hsps/chaperones can be localized in the cytoplasm or in membranous organelles where they assist protein folding and transport in control and in stress conditions [60]. They are involved in a wide range of stress responses such as cold, heat, drought and oxidative stress.

Thirteen sHsps were identified in the *Arabidopsis* genome and classified into six classes, depending on the subcellular localization and sequence homology [61], highlighting the high capacity of plants to deal with stress adaptation [62]. In addition, the endoplasmic reticulum Hsp70 (Bip) also regulates the protein trafficking to the Golgi apparatus before the further sorting to the PM or the vacuole. Bip also assists and facilitates protein folding and assembly [63] and may have a crucial role in assisting and regulating the appropriate folding of storage proteins during seed development. In *Arabidopsis*, Hsp70 was observed at high levels in after-ripening non dormant seeds and is required during dormancy release to maintain the correct folding of other proteins [50].

Among the chaperone proteins, a protein disulfide isomerase spot was also significantly enhanced in VT2eB, compared to the WT (Table 2). This protein showed an active thioredoxin-like domain and an ER resident signal and was involved in introducing disulfide bonds to nascent polypeptides in the ER lumen [64]. In seeds, the protein disulfide isomerase was found to play a different role related to protein folding (Kim et al, 2012; Kimura et al., 2015), regulation of cysteine protease activity [65], chaperone activity [66, 67], promotion of specific localization of Cys-rich prolaminin in the core of PBs [68] and regulation of the proportion of various seed proteins, including storage proteins [69]. In wheat, disulfide isomerase protein play an important role in assisting the folding of newly synthetized proteins during germination and in forming disulfide bonds in seed storage proteins [70].

Both Hsp70 and protein disulfide isomerase control protein folding thereby stabilizing their structure. The increase in these chaperon proteins may go hand in hand with the increase in storage proteins observed in tobacco VT2eB seeds with respect to WT. It is possible that the increase in these proteins in transgenic seeds was insufficient for the correct folding of storage proteins and thus for the correct mobilization of storage material for embryo development. In F18 seeds, the increase in storage proteins was not accompanied by the enhancement of these chaperone proteins (Hsp70, protein disulfide isomerase), thus explaining the higher germination delay observed in this transgenic line (Fig 1).

Interestingly, in wheat seeds, proteomic and mRNA analyses showed that the repression of disulfide isomerase in after-ripening compared to dry seeds, promotes proteolysis and in turn seed dormancy release and germination [71]. It is possible that the increase in this protein in transgenic lines contributes to germination delay.

In VT2eB seeds, the number of stress-related proteins such as late embryogenesis abundant (LEA) proteins and sHSPs (Table 2; Fig 8, violet/green bold spots), was also significantly higher than in WT seeds. These proteins also increased significantly in F18 seeds although to a lesser extent with respect to VT2eB (Table 2; Fig 7, violet/green bold spots). LEA genes are expressed during the later stage of seed maturation and are involved in the acquisition of desiccation tolerance [72]. It has also been proposed that LEA proteins, which are localized in the nuclei,

may have enzymatic or chaperone activity in nucleus proteins that unwind or repair DNA, regulate transcription, and might be associated with chromatin or cytoskeleton [73].

The LEA-like proteins, which increased in VT2eB and F18, belong to Group 5 which includes atypical LEA proteins with a significantly higher proportion of hydrophobic residues [74–76]. Group 5 LEA proteins are also expressed in seeds during the late maturation stage of development [77]. Unlike other groups of LEA proteins, which show high hydrophilic residues and play a role in protein protection from desiccation, very few studies have characterized group 5 LEA functions in abiotic stress tolerance. This LEA group could be involved in membrane protection [78]. This protective effect has also been observed in tobacco seeds overexpressing a novel atypical group 5 LEA gene from *A*. *diogoi* (AdLEA). This protein plays a role in abiotic stress tolerance, most specifically in water limiting conditions by increasing $O_2$-scavenging and up-regulating various stress-related genes [79].

The abundance of these proteins in transgenic lines could be due to the necessity to protect membranes and storage lipids from desiccation and to defend them from ROS activity during after-ripening and early germination. This characteristic could be related with persistent trait of buried seeds, which remain longer in the soil and are more subjected to oxidative damages.

In F18, the increase in these proteins was lower than VT2eB and, in particular, spot 792, identified as a LEA protein, was not significantly different from WT. This difference could further explain the higher delay in F18 seed germination compared to WT and VT2eB seeds.

Therefore, in F18 seeds, the increase in storage proteins was not accompanied by a parallel increase in chaperone proteins (Table 2), thus favoring protein oxidative stress and aggregate formation during dehydration, thereby resulting in an inability to use seed storage material for germination.

Interestingly, LEA proteins are also considered important for the persistence of buried seeds in a natural environment since they facilitate intracellular 'glass formation' in dehydrated cells [80, 81] inducing low metabolic activity and facilitating the persistence of dry seeds in the soil. The higher increase in LEA proteins represents an additional trait that, together with the shape change (Fig 2F), correlate the behavior of the transgenic lines with that of persistent buried seeds. This data suggests that the relative number of storage proteins and of proteins regulating their folding/accumulation state represent common mechanisms to control seed germination and that the destiny of seeds is already determined during maturation.

In addition to LEA proteins, sHsps also have an overall protective effect during seed drying. In both transgenic lines, the number of two sHSPs belonging to Class I (16.9 kDa class I heat shock protein 1-like) and II (17.1 kDa class II heat shock protein-like), increased in dry seeds, as detected by 2D gel analyses (Table 2; Fig 8, green spot 1130, 1133, 1324). sHsps might act as molecular chaperones during seed dehydration and during the first few days of rehydration. In seeds, class I and class II sHsps are developmentally regulated: they accumulate during seed maturation, before the acquisition of desiccation tolerance [82], and disappear in parallel to storage protein degradation [83]. These proteins stabilize protein conformation and help in protein folding, oligomer formation, intracellular transportation, and marking for degradation [78, 84, 85]. As observed for LEA proteins, sHsps may be required for desiccation tolerance [72, 86], and it has been observed that, in *Synechocystis*, Hsp17 could play an important role in membrane quality control and in the maintenance of membrane integrity [87]. In transgenic seeds, a 17.1 kDa class II heat shock-like protein significantly increased with respect to WT in dry seeds (Table 2). This protein has not yet been characterized and may participate in the protection of proteins or the membrane during seed desiccation.

Interestingly, spot 1324 identified as 16.9 kDa class I heat shock protein 1-like was only detected in mutant seeds and not in WT. In rice, Oshsp16.9 gene is expressed during stress responses and transgenic plants have shown tolerance to salt, cold, heat and dehydration

stresses [88, 89]. As observed with other stress response proteins which increase in transgenic lines in parallel with storage proteins, it is possible that these proteins play a role in keeping proteins in a folding-competent state during seed desiccation and in preventing them from irreversible aggregation until ATP-dependent chaperones (such as Hsp70 and Hsp60 GroE) restore the refolding of denatured proteins to native physiological conditions [90]. In this way, storage proteins become accessible to degradation during germination. In summary, the delay in transgenic seed germination was probably due to the increasing number of storage proteins which was associated with the higher persistence of seeds in the natural environment. In the VT2eB line, the increase in storage material was accompanied with an increase in chaperone and stress related proteins. However, the increase in chaperone proteins in parallel with storage proteins appeared insufficient for the correct germination of transgenic seeds. In the F18 line, the increase in storage proteins was only partially accompanied by an increase in chaperone proteins so that the storage proteins did not fold correctly for proteolysis, further delaying early germination events.

Notably, sHsp, which increase in F18 seeds (Table 2), are not able by themselves to determine the protein folding but they bind and stabilize proteins to prevent their possible non-native aggregation, facilitating subsequent refolding by other chaperones such as Hsp70 [91, 92]. Therefore, Hsp70s, which in turn did not increase significantly in F18 seeds (Table 2), interconnect with other chaperones to form the chaperone cell network and are also involved in responding to environmental stimuli [93]. Therefore, the higher delay in the germination of F18 seeds with respect to WT and VT2eB may also be due to the loss of cooperation between sHsp and Hsp70 in the protein folding activity.

In addition to storage material and chaperone proteins, F18 and VT2eB showed significant alteration in the enzymes involved in amino acid, lipid and sucrose metabolisms (Table 2; Figs 7 and 8, blue bold spots). Enolase appeared significantly increased in both seed mutants with respect to WT although to a greater extent in VT2eB transgenic line (Table 2; Figs 7 and 8). This enzyme is involved in glycolysis. It catalyzes the reversible dehydration of 2-phosphoglycerate (2PGA) to phosphoenolpyruvate (PEP) and plays an important role during adaptation to anaerobiosis [94]. PEP generated through the enolase reaction in the cytosol is also a central metabolite in plant primary and secondary metabolism. It is involved in the tricarboxylic acid (TCA) cycle occurring into the plastid stroma, and acts as a precursor for the biosynthesis of aromatic amino acids in the shikimate pathway and for the biosynthesis of fatty acids [95–97], branched chain amino acids [98] and isoprenoids [99]. The alteration in the carbohydrate and lipid metabolism could affect seed germination in F18 and VT2eB. It is known the carbohydrate content controls the entry of water into the seed during imbibition [47]; therefore the modification of sugar metabolism could affect imbibition thus contributing to the delay in the storage material mobilization observed in transgenic comparing to WT seeds in tobacco.

In addition, glucose and ribitol dehydrogenase homologs 1 (GRDs) are involved in the carbohydrate metabolism and increased in transgenic with respect to WT seeds (Table 2; Figs 7 and 8). An increase in the expression of GRD was observed in seeds and tissues after heat, salinity and anoxic stresses, suggesting a role in the accumulation of sugars with an osmo-protective function [100–103]. The increase of these proteins in transgenic lines of tobacco (F18, VT2eB) could interfere with the carbohydrate metabolism and thus with the water uptake during imbibition, thereby inducing a delay in the reserve mobilization observed by morphological analyses. In addition, proteomic analyses of dry and after-ripening wheat seeds showed that imbibition of after-ripening seeds led to a substantial repression of glucose/ribitol dehydrogenase compared to dry seeds, thus suggesting that suppression of GRDs could be related to germination [71]. The presence of high GRD content in F18 and VT2eB with respect to WT tobacco seeds could contribute to the delay in transgenic seed germination.

Aspartic proteinase was only detected in transgenic seeds (Table 2; Figs 7 and 8). Aspartic proteinase was involved in the proteolytic processes of storage proteins during seed maturation and participates in the mobilization of storage proteins during seed germination [104–107]. In *Arabidopsis* seeds, these enzymes colocalize in the PBs with the seed storage protein 2S albumin and the vacuolar marker α-mannosidase [108]. In addition, in *Arabidopsis* seeds, proteolytic processing of 2S albumins occurs inside multivesicular bodies (MVBs) before the storage proteins reach the PBs. Golgi-derived vesicles carrying aspartic protease are different from vesicles carrying storage proteins. These vesicles fuse with the same MVBs where proteolysis of 2S albumins occurs [109]. The presence of aspartic proteinase only in tobacco F18 and VT2eB seeds suggests that the maturation process leading to the proteolysis of storage proteins had not been completed in the transgenic seeds and that this enzyme was still present in dry seeds. This is in line with the absence of limited proteolysis observed in 1D and 2D gel analyses (Table 2; Fig 6).

Other proteins significantly enhanced in transgenic tobacco seeds, such as the MPL-like protein, Methyonil tRNA formyltransferase and an uncharacterized protein LOC104224147, were less characterized (Table 2; Figs 7 and 8, red bold spots). The MPL-like protein is a low-molecular-weight polypeptide called a major latex protein (MLP) which is abundant in the latex from the opium poppy (*Papaver somniferum*) [110, 111]. This protein was later found in other plants, such as tobacco [112, 113]. The function of MLPs is unknown and they have been associated with fruit and flower development and in pathogen defense responses [114]. The MLPs expression pattern is similar to some of the intracellular pathogenesis-related (IPR) proteins [115]. No relation between the expression of all these proteins and seed germination has been reported, and it is possible that their increase could be related to the response induced by exogenous DNA insertion and exogenous EV protein expression.

## Conclusions

Tobacco transgenic seeds, created by the insertion of DNA codifying EV, showed a different germination and seedling ability compared to the WT, suggesting that exogenous DNA insertion interfered with endogenous protein expression and with germination. Morphological and proteomic analysis revealed new insights into the traits that influence germination. The findings highlight that the assumptions of germination are determined during seed maturation, in terms of storage protein accumulation and processing and of carbohydrate metabolism, which regulates water uptake during the early phases of germination. In addition, morphological and proteomic seed modifications support the theory that seed shape and storage protein content are related to seed dormancy and persistence in soil, which in turn are important in terms of the role of biodiversity and conservation played by seeds.

## Supporting information

**S1 Fig. Detection of VT2e-B and F18 genes in the transgenic tobacco plants. A, B** pBIpGLOB binary vectors maps for F18 and VT2eB. **C** DNA samples from WT and transgenic lines were analyzed by PCR using specific primers for the detection of VT2e-B and F18 genes in R3 generation. The analyses confirmed the stable integration of the exogenous genes in both lines of tobacco plants.
(TIF)

**S2 Fig. Soil seed germination.** The graph shows the mean time of seedling of WT and transgenic lines seeds grown in soil. Seedling time was significantly delayed in transgenic seeds

compared to WT.
(TIF)

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