RESEARCH ARTICLE

Retarded germination of *Nicotiana tabacum* seeds following insertion of exogenous DNA mimics the seed persistent behavior
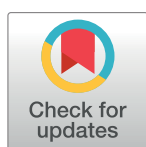
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Abstract

Tobacco seeds show a coat-imposed dormancy in which the seed envelope tissues (testa and endosperm) impose a physical constraint on the radicle protrusion. The germination-limiting process is represented by the endosperm rupture which is induced by cell-wall weakening. Transgenic tobacco seeds, obtained by insertion of exogenous genes codifying for seed-based oral vaccines (F18 and VT2eB), showed retarded germination with respect to the wild type and modified the expression of endogenous proteins. Morphological and proteomic analyses of wild type and transgenic seeds revealed new insights into factors influencing seed germination. Our data showed that the interference of exogenous DNA influences the germination rather than the dormancy release, by modifying the maturation process. Dry seeds of F18 and VT2eB transgenic lines accumulated a higher amount of reserve and stress–related proteins with respect to the wild type. Moreover, the storage proteins accumulated in tobacco F18 and VT2eB dry seeds have structural properties that do not enable the early limited proteolysis observed in the wild type. Morphological observations by electron and light microscopy revealed a retarded mobilization of the storage material from protein and lipid bodies in transgenic seeds, thus impairing water imbibition and embryo elongation. In addition, both F18 and VT2eB dry seeds are more rounded than the wild type. Both the morphological and biochemical characteristics of transgenic seeds mimic the seed persistent profile, in which their roundness enables them to be buried in the soil, while the higher content of storage material enables the hypocotyl to elongate more and the cotyledons to emerge.

Introduction

In angiosperms, double fertilization enables the triploid endosperm to develop as reserve tissue, to supply nutrients for the embryo during germination and seedling [1]. The mechanisms involved in protein folding and mobilization upon seed imbibition regulate seed dormancy and the crucial steps of seedling emergence. Storage proteins are synthesized during seed maturation and are conserved in specialized tissues, such as in the endosperm and/or in the parenchyma of cotyledons [2]. The synthesis/storage and degradation of reserve proteins are tightly regulated. The way storage proteins are protected during seed maturation from uncontrolled proteolysis involves the deposit of reserve proteins into membrane-bounded organelles as vacuoles or protein bodies (PB) [3]. However, although the structural features of reserve proteins protect them from proteinases deposited in the same compartments, storage proteins such as legumins, albumins, some lectins and vicilins undergo limited proteolysis within the storage vacuoles [4, 5].

In addition to proteins, the endosperm accumulates lipids such as triacylglycerol, which are transformed into sucrose at the onset of seed germination [6]. On the other hand, proteomic characterization of the cress micropylar endosperm revealed the presence of proteins involved in protein folding, protein defense and stability [7]. This study also suggested that cress micropylar endosperm proteins may have a regulatory function as well as being the source of nutrition for the embryo [7].

Seed germination is defined by the emergence of the radicle through surrounding structures which in *Tobacco* correspond to the seed coat (testa) and micropylar endosperm [8, 9]. The dormancy break (which allows seeds to survive unfavorable conditions) occurs in dry tobacco seeds during after-ripening, a status characterized by physiological changes making the seeds ready for germination. After-ripening triggers active transcription and biochemical reactions which could lead to dormancy release [10–12]. It has also been shown that dormancy alleviation depends on non-enzymatic reactions associated with ROS (reactive oxygen species) which cause the formation of peroxy-lipids, carbonylated proteins, and oxidized mRNA. This selective oxidation of mRNA and proteins gradually occurs during storage, and influences the first few hours of imbibition leading to the maintenance or release of germination inhibition [13–17].

The uptake during imbibition leads to embryo cell elongation and radicle protrusion [18, 19]. When a radicle emerges from the micropylar endosperm, cells undergo cell cycle in order to form seedlings [20]. In *Arabidopsis*, germination and seedling growth have been shown to be accompanied by a significant reduction in stored metabolites, in parallel with the reactivation of metabolic pathways [21].

Edible vaccines (EVs) are represented by transgenic or xenogenic plants containing selected genes responsible for the expression of immunogenic proteins in their genome [22]. The fact that transgenic tobacco seeds obtained by insertion of genes codifying for the FedA (the main protein of the F18 adhesive fimbriae) and the B subunit of verocytotoxin from O138 verocytotoxic *E.coli* serotypes (VTEC) [23–25] displayed retarded germination with respect to the WT, prompted us to investigate the possible mechanisms regulating seed maturation and seedling.

EV has been known for years in plants, but how it may influence seed development and seed germination is hardly known since few careful investigations focused on this issue. The aim of this study was to evaluate the changes in morphological and proteomic traits induced by unintended effects of EV transgene integration into the plant genome in *N. tabacum* seeds, following a comparative approach with their near isogenic counterpart, and to correlate these changes with germination and seedling modifications.

We found that early germination stages of F18 and VT2eB transgenic seeds were delayed compared to the wild-type (WT). In addition, changes were also observed both in the shape of seeds and in the behavior of the reserve tissues. Light and transmission electron microscopy investigations revealed alterations in the embryo size and development. Modifications were also observed in the timing of reserve mobilization, which could also be related to the delay in seed germination. In addition, one dimensional (1D) gel electrophoresis showed that WT also differs from F18 and VT2eB seeds in terms of protein expression and that this difference is more acute in dry seeds compared with imbibed seeds. Data from 1D, two dimensional (2D) gel electrophoresis and mass spectrometry revealed that transgenic seeds accumulated a higher amount of reserve and chaperone proteins, together with proteins involved in seed dehydration and proteolytic enzymes.

The modulation of seed germination, related to changes in the expression of reserve and chaperone proteins and the ecological implications are discussed.

Materials and methods

Tobacco seeds

Transgenic *N. tabacum* lines *(cv. Xanthi)* transformed for the B subunit of VT2eB toxin (VT2eB) and for the major subunit FedA of the F18 adhesive fimbriae (F18) were considered (GenBank Accession number: VT2eB-X81417; F18-M61713). Briefly, the transgenic *N. tabacum* lines were obtained by agroinfection using pBIpGLOB binary vectors (DDBJ accession no. AX006477; S1 Fig) as described by Reggi et al.[26]. The encoding sequences, for VT2eB and for the major subunit FedA of the F18 adhesive fimbriae genes, were placed under the control of the soybean basic 7S globulin promoter for seed-specific expression [24]. Six homozygous transgenic lines of tobacco, three harboring respectively the Vt2e-B gene and three the FedA subunit of F18 fimbriae gene, were compared with the wild-type *N. tabacum (cv. Xanthi)* which was considered as the negative control (WT). All plants were grown in parallel and WT and transgenic seeds were collected in the same years from mother plants. The third generations (R3) of the experimental transgenic lines were randomly selected from a cultivation of a total of eighty F18 positive plants and one hundred and forty VT2eB positive plants, derived from the selected homozygous transgenic plants cultivated in a greenhouse in a period of 14 months.

Evaluation of exogenous genes

Six independent lines (three transformed for VT2e-B and three transformed for F18 expression) of the third generations (R3) were evaluated for the presence of VT2e-B and F18 genes, respectively, by polymerase chain reaction (PCR) in two different experiments using each time about one thousand seeds. The reaction conditions were developed for a final volume of 50 μl, using 50 ng of template represented by genomic DNA purified from VT2eB and F18, respectively. For the VT2eB gene detection specific oligonucleotides were used (F:5' atgaagaagatgttta tagcgg; R:3' aacgggtccacttcaaatgatt). Thermal cycling was carried out using an initial denaturation step of 95˚C for 5 min, followed by 30 cycles of denaturation at 95˚C for 1 minute, annealing at 50˚C for 1 minute and 20 seconds, and elongation at 72˚C for 1 min 30 s. Cycling was completed by a final elongation step of 72˚C for 5 minutes. For the F18 gene detection, specific oligonucleotides were used (F: 5'atgaaaagactagtgtttatttctttttg; R: 3'cgaatgcgccaatgaatgtt- catt). Thermal cycling was carried out using an initial denaturation step of 95˚C for 5 min, followed by 25 denaturation cycles at 95˚C for 1 minute, annealing at 56˚C for 1 minute and 20 sec, and elongation at 72˚C for 1 min 30 s. Cycling was completed by a final elongation step of 72˚C for 5 minutes.

Seed germination

Wild type, F18 and VT2eB seeds of *N. tabacum* (L.) were sown in batches in soil and allowed to germinate under long-day conditions at 24°C ± 1 (14 h day/10 h night). The seeds were grown for three weeks and monitored every day.

Wild type, F18 and VT2eB seeds of *N. tabacum* (L.) were also sown on sterile wet paper and allowed to germinate in a culture room at 24°C under continuous light. Seeds were cultured for five days and the early stages of the germination process (3–5 days) were monitored in three different experiments (about 160 seeds were considered for each experiment), by a Leica light microscope DM RB, using a Leica N PLAN 2.5X objective. Images were collected using a Leica video camera MC 170 HD.

Light and transmission electron microscopy

Seeds of WT, F18 and VT2eB were imbibed for 24 hours using distilled water. For fixation and embedding, both whole seeds and the separated embryo and seed coats were considered. Samples were fixed in 50mM Hepes, pH 7.4, 2% formaldehyde and 0.2% glutaraldehyde, overnight at 4°C and then repeatedly rinsed in 50mM Hepes, pH 7.4, dehydrated with increasing concentrations of methanol and embedded in LR white resin (Sigma). Semi-fine sections (2μm) and ultra-thin sections (80 nm), were obtained using a Reichert Jung Ultracut E microtome. Semi-fine sections were stained by 1% toluidine blue and observed with a Leica DMRB light microscope. Ultra-thin sections were stained with 3% uranyl-acetate and observed with an EFTEM LEO 912AB transmission electron microscope (Zeiss) working at 80 kV. Five imbibed seeds for 3 different embedding experiments were analyzed for EM and light microscopy for each sample. The observations were performed on longest embryos for WT and transgenic lines.

The size of the seeds and embryos were measured using ImageJ. The values were processed for statistical analysis (t-test) by Microsoft Excel.

Seed protein extraction

Wild type, F18 and VT2eB seeds of *N. tabacum* were imbibed in distilled water for 24 hours at 4°C. Dry and hydrated wild type, F18 and Vte2B tobacco seeds were frozen in liquid nitrogen and ground to powder. The homogenate was resuspended using the extraction buffer (EB): 8 M urea, 40 mM Tris-HCl, 20 mM DTT, 2% Tween-20, 5 mM PMSF and incubated for 1 hour at 4°C, vortexing every 10 minutes to facilitate protein extraction. The homogenates were subsequently centrifuged at 15°C for 30 minutes at 18.000 *g* (13.000 rpm) in an ALC A21-C rotor. The resulting supernatants were collected and stored at -20°C as crude extracts. Aliquots of crude extracts were protein assayed (Bradford), using BSA as a standard protein.

One- and two-dimensional electrophoresis

Proteins (20 μg/lane) were resolved in denaturing 15% polyacrylamide one-dimensional (1D) gels in a discontinuous buffer system following Laemmli [27]. MiniVe Vertical Electrophoresis System (GE Healthcare, USA) was used for 1D electrophoresis. Proteins were stained with Coomassie brilliant blue R250. 1D-electrophoresis were replicated five times.

2D electrophoresis was performed using the Immobiline-polyacrylamide system, as previously described [28, 29]. IEF was carried out on non-linear wide-range immobilized pH gradients (IPG) (pH 4–7; 18 cm long IPG strips; GE Healthcare, Uppsala, Sweden) and performed using the Ettan™ IPGphor system (GE Healthcare). Strips for analytical runs were rehydrated with 60 μg of protein in 350 μl of EB and 0.2% (v/v) carrier ampholyte for 1 h at 0 V and for 8

h at 30 V, at 16°C. The strips were then focused according to the following electrical conditions at 16°C: 200 V for 1 h, from 300 V to 3500 V in 30 min, 3500 V for 3 h, from 3500 V to 8000 V in 30 min, 8000 V for 3 h, 10.000 V until a total of 80.000 Vh was reached.

MS-preparative strips were rehydrated with 500 μg of protein in 350 μl of EB and 2% (v/v) carrier ampholyte, for 1 h at 0 V and overnight at 30 V, at 16°C. Then IEF was achieved setting the following voltage steps at 16°C: 200 V for 8 h, from 200 V to 3500 V in 2 h, 3500 V for 2 h, from 3500 V to 5000 V in 2 h, 5000 V for 3 h, from 5000 V to 8000 V in 1 h, 8000 V for 1 h, from 8000 V to 10.000 V in 1 h, 10.000 V until a total of 100.000 Vh was reached.

After IEF, strips were subjected to two equilibration steps: the first was for 12 min in 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.05 M Tris–HCl pH 6.8, 2% (w/v) DTE; and the second one was for 5 min in 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.05 M Tris–HCl pH 6.8, 2.5% (w/v) iodoacetamide, and bromophenol blue in trace.

The second dimension was carried out, at 10°C, on house-made 9–16% polyacrylamide linear gradient gels at 40 mA/gel constant current, until the dye front reached the bottom of the gel. Analytical and MS-preparative gels were stained with ammoniacal silver nitrate [30, 31] and with MS-compatible silver staining [32], respectively. They were then scanned using an ImageScanner III (GE Healthcare).

Image analysis and statistics

Image analysis was performed on analytical 2D gels using ImageMaster 2-D Platinum v6.0 (GE Healthcare). For each condition tested, image analysis was performed on three different spot maps from three biological replicates for a total of nine analyzed gels. An intra-class quality and experimental control was performed by comparing the gels and then a differential inter-class analysis was performed to detect any statistically significant quantitative and qualitative differences. Based on a fold change of at least ±2 in relative volume (%V) ratio, and on statistically analysis as reported in the tables, differentially expressed proteins were detected among the three conditions examined.

Protein identification by mass spectrometry

Protein identification was performed by peptide mass fingerprinting [33, 34] using an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA). Bands and spots of interest were manually excised, destained, as previously described [34, 35], and acetonitrile dehydrated. Before protein digestion, 1D gel-resolved proteins were reduced and alkylated as previously reported [35]. 1D and 2D-gel resolved proteins were rehydrated in trypsin solution (Sigma Aldrich, Italy), and in-gel protein digestion was performed by an overnight incubation at 37°C. A total of 1.25 μl of each protein digest was directly spotted onto the MALDI target and air-dried. Then 0.75 μl of the matrix solution (a saturated solution of alpha-cyano-4-hydroxycynnamic acid in 50% v/v acetonitrile and 0.5% v/v trifluoroacetic acid) was added to the dried samples and allowed to dry again.

Mass spectra were acquired using the above-mentioned mass spectrometer in reflector positive mode with a laser frequency set to 100 Hz. Spectra were analyzed by Flex Analysis v.3.0. Peptide mass fingerprinting (PMF) database searching was carried out in NCBIprot database set for *Viridiplantae* (Green Plants) using Mascot (Matrix Science Ltd., London, UK, http://www.matrixscience.com) with the following settings: experimental and theoretical peptide fingerprinting patterns with a Δmass less than 100 ppm, trypsin as the digestion enzyme with one missed cleavage allowed, carbamidomethylation of cysteine and oxidation of methionine as fixed and variable modifications, respectively. For protein identifications, the number of

matched peptides, the extent of sequence coverage, and the probabilistic score were considered [36].

Results and discussion

Transgenic seeds showed changes in the timing of germination and in the behavior of reserve tissues

The presence of VT2eB and F18 genes in the two lines of transgenic seeds was assessed by PCR. Our results confirmed the stable integration of the two exogenous genes in R3 generation of both lines (S1 Fig).

Preliminary experiments of seed germination on soil revealed a significant delay of F18 and VT2eB seedlings compared to WT, in four independent experiments. Whereas the WT seeds exposed the photosynthetic cotyledons in 4 days, transgenic lines F18 and VT2eB only reached the same developmental stage after 7 and 8 days, respectively (S2 Fig).

To analyze the dynamics of seedlings in more detail, seeds of WT and transgenic lines germinated on wet filter paper, under controlled light and temperature conditions. Seeds were observed every 24 hours for five days. *Nicotiana* seed germination involved two steps: testa (seed coat) rupture followed by endosperm rupture [9]. Since the mycropilar endosperm was considered a germination constraint of *Solanaceae* seeds, the rupture of the mycropilar endosperm and the consequent emission of the radicle primordium was considered as the germination point in *N. tabacum* (visible germination, see Fig 1A, stage 3). Seven additional developmental stages were considered for the analysis (Fig 1A).

Germinating seeds of wild type and transgenic lines, observed at different time points after sowing (3 to 5 days), confirmed that the germination time of F18 and VT2eB strains was delayed with respect to WT, in two repeated experiments (Fig 1B and 1C). In the WT a considerable number of seeds had already developed roots three days after sowing (Fig 1A and 1B; about 50% considering stages 4, 5, 6). In contrast most of the F18 seeds underwent testa rupture but not micropylar endosperm rupture, while VT2eB seeds were mostly between stages 2 and 3, suggesting that these seeds are able to initiate the micropylar endosperm rupture (Fig 1A and 1B). The germination process, analyzed up to five days after seed sowing, showed that whereas most of the F18 germinating seeds were still in stage 7, in WT seeds, root and shoot meristems actively participated in seedling growth (Fig 1C). VT2eB seeds distributed in steps 7–10, thus supporting the idea that the VT2eB seedling was delayed in the early stages of the process but to a lesser extent than the F18 mutant.

To explain these differences, morphological analyses of WT, F18 and VT2eB seeds were carried out (Fig 2).

The sizes of dry and imbibed seeds (24 h in water) were calculated by measuring both their major (length) and minor axis (width) using ImageJ. Usually, dry seeds are egg shaped, with a prominent hilum at the germinating end (Fig 2A–2C, arrow). The insertion of exogenous DNA induced changes in both the seed size and shape. In dry WT, the seed was significantly longer than the transgenic seeds (Fig 2D: $p < 0.01$), while the width was less influenced by the exogenous DNA insertion, and significant differences were observed only between the two transgenic seed lines (Fig 2D; $p < 0.01$). Considering the length/width ratio as the value describing the shape, dehydrated seeds of F18 and VT2eB were significantly rounder than the WT (Fig 2B and 2F; $p < 0.01$) and VTe2B seeds were significantly rounder, with respect to F18 (Fig 2C and 2F; $p < 0.01$). The differences in seed size and shape were attenuated following the imbibition process (Fig 2E).

The water input accounts for a general increase in seed size; in particular the imbibed WT became rounder while the VTe2B seeds lost their round form compared to the dry seeds
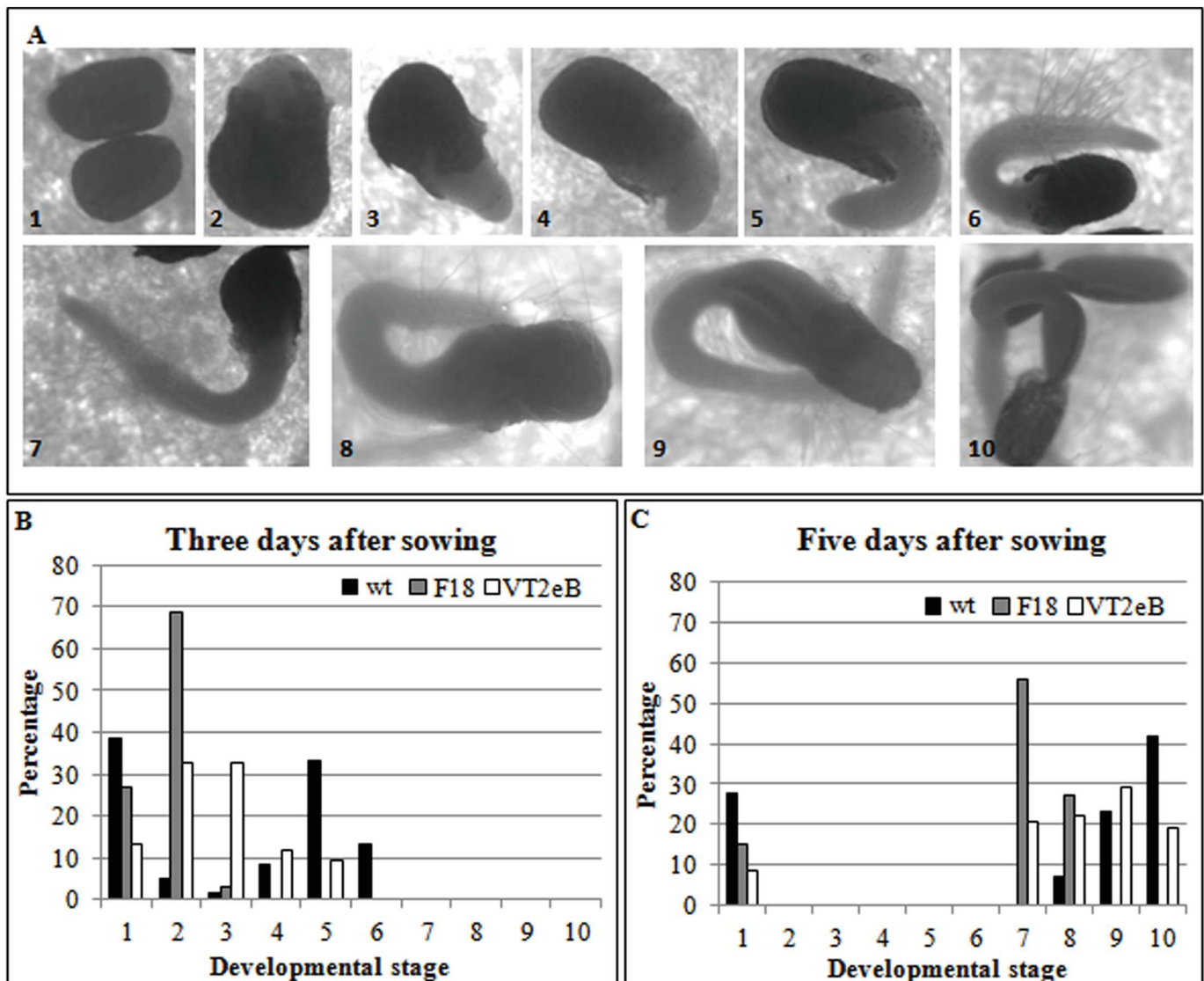
Fig 1. Time course experiments on tobacco seeds germination. A The observation of seeds germinated on wet filter paper for several days revealed ten different seed developmental stages. **B** The graphs described developmental behavior of WT and transgenic seeds three and five days after sowing. The analysis of data showed that the germination process was delayed in transgenic seeds and that VT2eB seedlings were less affected than F18 line.

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(Fig 2F; $p<0.05$). Conversely, the shape of the F18 seeds did not change during the imbibition (Fig 2F).

The seed size analyses also highlighted that a number of WT seeds (about 8%) showed the testa breaking and the growth of the radicle primordium as soon as 24 hours after imbibition (type 2, Fig 1). In contrast, this stage was not observed in the transgenic lines. In addition, once isolated from the seed coat, WT embryos appeared significantly longer than the transgenic embryos (Fig 2G; $p<0.01$ WT versus F18; $p<0.05$ WT versus VT2eB). Radicle protrusion depends on embryo expansion which is driven by water uptake. In this early phase of germination, cell proliferation does not occur [18, 37].
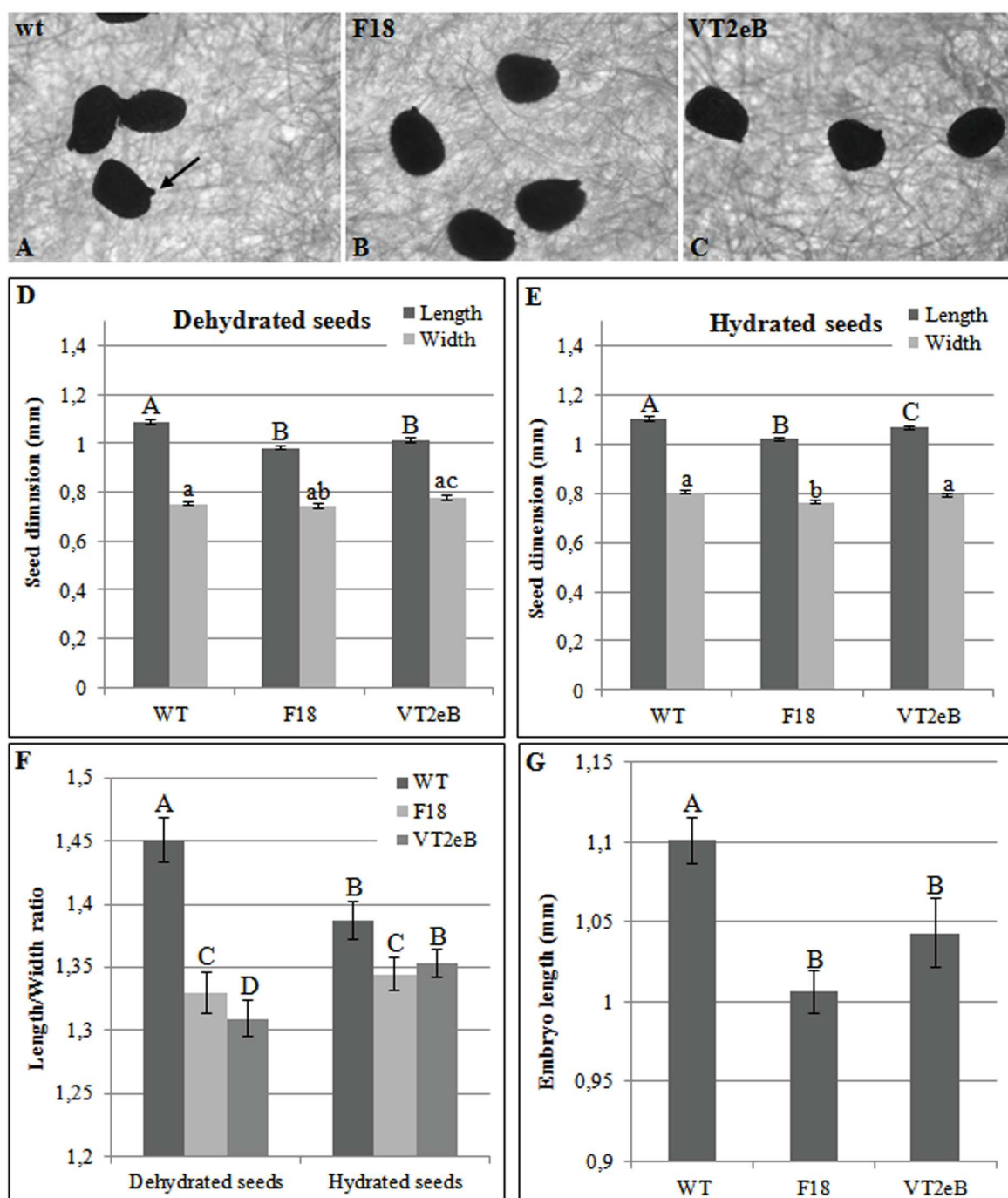
Fig 2. Morphological analysis of dry and hydrated seeds. A, B, C WT, F18 and VT2eB dry seeds (respectively) are egg shaped, with a prominent hilum at the germinating end (arrow). However, in transgenic lines, seeds were more rounded than WT. D, E The size of dry and imbibed seeds, calculated by measuring their major (length) and minor axis (width). The length of WT seeds was significantly higher than the transgenic seeds, while the width was less influenced by the exogenous DNA insertion, and significant differences were observed only between the two transgenic seed lines. F The seed shape was described considering the length/width ratio. Dehydrated seeds of F18 and VT2eB were significantly more rounded -than the WT, and VTe2B seeds were significantly more rounded than F18. During hydration, VT2eB seeds took on a similar shape to WT, while F18 seeds remained more rounded than the WT. G Measurement of the embryo length following the hydration process. WT embryos were significantly longer than the transgenic embryos.

The differences observed in embryo elongation among WT and F18 /VT2eB seeds could be related to the modification of water uptake. Water uptake occurs in three phases involving a rapid initial imbibition (phase I), a subsequent slowdown (phase II) and a further uptake, starting late in phase II and continuing in phase III [18]. Phases I and II support the initial embryo elongation and testa rupture. The endosperm rupture occurs in phase III and is ABA and β-1,3 glucanase dependent [18]. After 24 h of imbibition, tobacco seeds were at the onset of phase II [18] and thus the embryo elongation observed in WT tobacco seeds was only due to hydration. In mutant seeds, this phase of water uptake appeared to be impaired. Since in oilseeds water is absorbed almost exclusively by carbohydrates and proteins [18], carbohydrates and/or storage proteins may have been altered in F18 and VT2eB mutants, thus triggering the reduced embryo elongation.

To investigate the correlations between differences in the size and shape of the seeds and their anatomy, the endosperm and the isolated embryos were studied by light microscopy after 24h of water imbibition (Fig 3, embryo, and Fig 4, endosperm).

In order to facilitate the embedding, the seed coat was removed from both the WT and the transgenic lines. Interestingly, in transgenic seeds, both the embryo and the endosperm cells appeared isodiametric, with evident nuclei (Figs 3E, 3H and 4B; arrows), apparently without large vacuoles and with dark bodies which are present in higher numbers in VT2eB seeds (Figs 3D, 3E, 3G, 3H, 4B and 4C; arrowheads). TEM analyses of mutant embryos showed that dark bodies were indeed protein bodies (PBs), which appeared to be filled with electron-dense material (Fig 4F and 4I). These PBs are surrounded by numerous vacuoles ascribable to lipid bodies (LBs; Fig 4F and 4I). In the WT embryos, isodiametric cells were confined in the apex of the root (meristematic cells) and in the cotyledons (Fig 3A; square brackets), while the hypocotyl cells appeared elongated along their major axis (Fig 3A, arrows), suggesting that both the distension (in accordance with water uptake dependent elongation) and the differentiation arose in WT embryos, whereas the same processes were retarded in transgenic seeds. In addition, in the WT, most of the cells revealed a lack of storage vacuoles (both PBs and LBs; Fig 3B and 3C) and showed a dense cytoplasm with prominent nuclei (Fig 3B and 3C, white arrows) and a large central vacuole (Fig 3A–3C). This thus suggested that the storage material supporting germination and early seedling growth had already been mobilized and that embryo cells had undergone root growth.

The analysis of the endosperm cells by TEM confirmed that the storage material comprised both PBs (Fig 4, PBs) and LBs (Fig 4, LBs), and that in the WT seeds, the storage material had partially mobilized already after 24 hours of water imbibition, as LBs were no longer present. It is possible that lipids were the first to be mobilized, as an energetic source to support embryo growth, as some PBs could still be observed in endosperm cells (Fig 4A and 4D; arrow). Ultrastructural observations also revealed changes in the behavior of PBs. Whereas the PBs of WT seeds appeared as uniform, amorphous electron-dense material, the PBs of transgenic endosperm cells showed a core electron-dense structure surrounded by a lower dense matrix (Fig 4; white arrowheads and black arrows, respectively). The differences in PB ultrastructure in WT, compared with F18 and VT2eB suggested that different processes of protein folding occurred in order to package the storage proteins in the PBs [38]. The accumulation of misfolded proteins might affect the packaging and the accessibility of proteins to the processing enzymes, leading to changes in resource availability (see below in 2D-elecrophoretic analyses).

The disappearance of LBs in the WT endosperm cells, following water imbibition, suggested that these materials were mobilized early during seed germination compared to F18 and VT2eB. It has been shown that significant amounts of triacylglycerol accumulated in the endosperm cells of *Arabidopsis* seeds and that the carbohydrates derived from this lipid are required for embryo development [6]. In addition, in tobacco seeds, it has been shown that oil
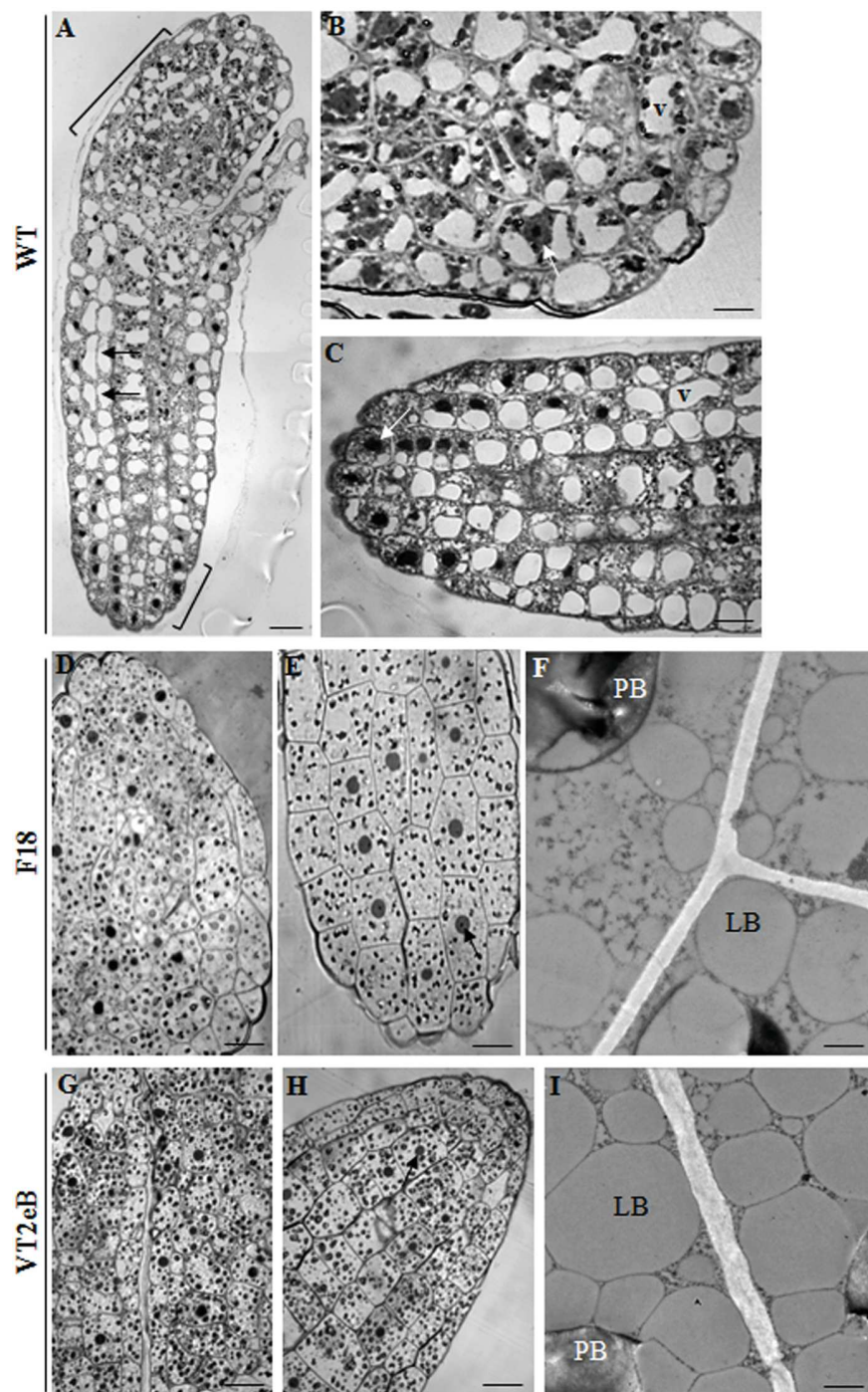
Fig 3. Anatomy of embryos by light and TEM microscopy. A-C WT embryo. Isodiametric cells were observed in the root meristems and in the cotyledons (square brackets). In the hypocotyl, cells appeared elongated along the major axis (black arrows), suggesting that the differentiation processes had already occurred. Most of the embryo cells showed a dense cytoplasm with prominent nuclei (white arrows), a large central vacuole (v), and the lack of storage vacuoles. This suggests that the reserve material was already mobilized, and embryo cells undergo root growth. Scale bar: 10 μm. **D, E** In F18 seeds, all the embryo cells appeared isodiametric, with evident nuclei (arrows), apparently without large vacuoles and with dark bodies ascribable to protein bodies (PBs). Scale bar: 10 μm. **F** TEM analyses showed the presence of both PBs and lipid bodies (LPs) in embryo cells. Scale bar: 300 nm. **G, H** In VT2eB seeds, all the embryo cells appeared isodiametric, with evident nuclei (arrows) and appeared not to have large vacuoles. There were more dark

bodies ascribable to PB than in the F18 embryo. Scale bar: 10 μm. **i** TEM analyses showed the presence of BPs and LPs in embryo cells. Scale bar: 300 nm.
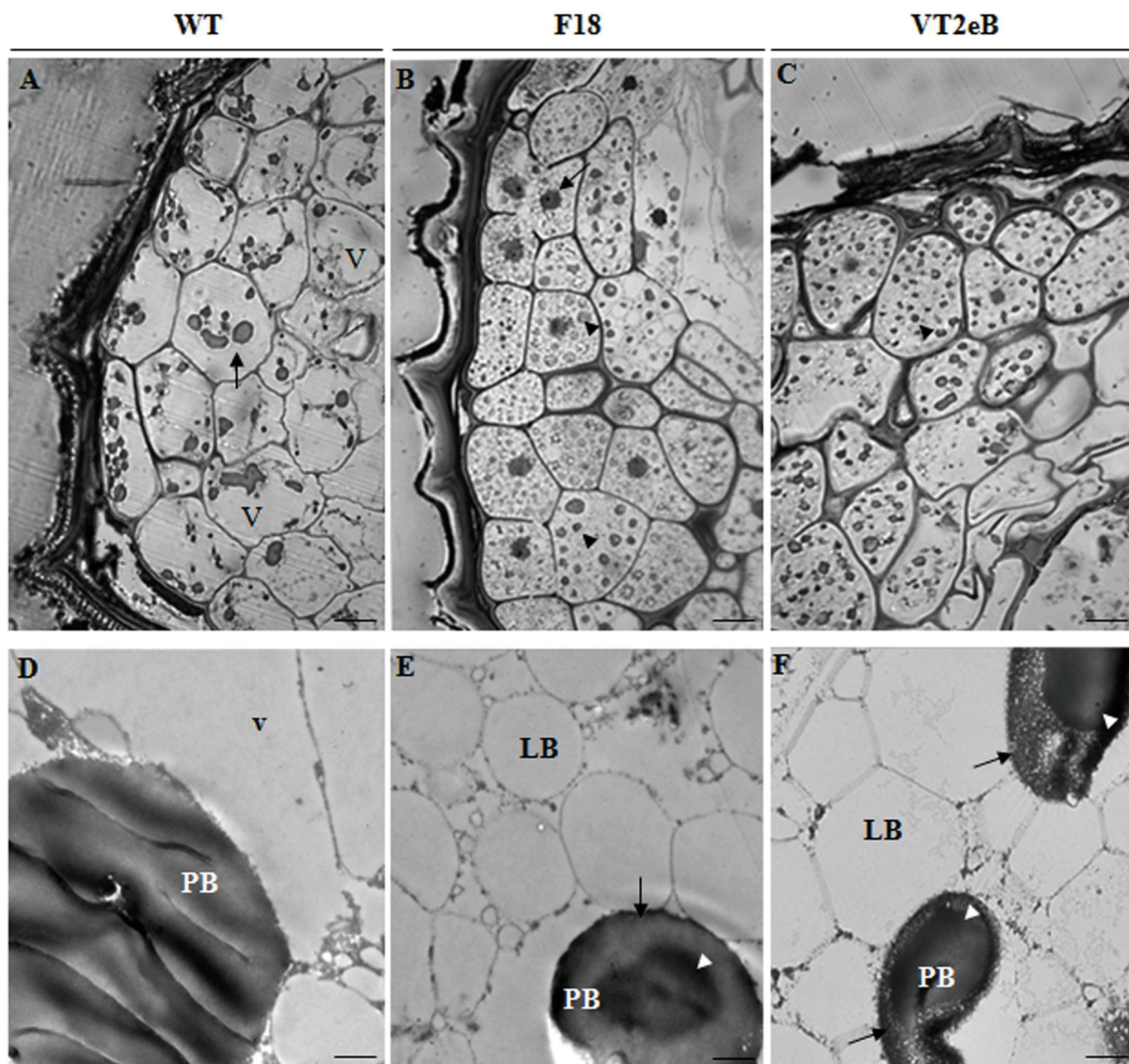
Fig 4. Anatomy of the endosperm by light and TEM microscopy. A In the WT endosperm cells, under an light microscope cells appeared isodiametric, with large vacuoles (v) and with dark bodies ascribable to PBs (arrow). Scale bar: 10 μm. **B, C** In transgenic seeds endosperm cells showed prominent nuclei (arrow) and storage material (arrowheads). Large vacuole were not observed. Scale bar: 10 μm. **D-F** TEM analyses showed that in WT seeds the storage material was partially mobilized: PBs were still observed in endosperm cells, while LBs were no longer present. Large vacuoles were observed within the cells (v). In transgenic seeds both PBs and PLs were present. In WT seeds, PBs appeared to contain uniform, amorphous electrondense material, the PBs of transgenic endosperm cells showed electron-dense structures surrounded by a lower dense matrix (white arrowheads and black arrows, respectively). Scale bar: 300 nm.

mobilization occurs during phase II of water uptake [18] which we also observed in WT seeds, since most LBs disappeared from the endosperm and embryo cells (Figs 3A–3C, 4A and 4D).

Therefore, in transgenic seeds the delay in the mobilization of reserve materials could reflect the hydration modification on which the use of the reserve material depends or it could be related to the accumulation of misfolded proteins during seed maturation. The observation of WT and transgenic dry seed morphology suggested that the delay in the early stages of germination in transgenic lines could be related to changes in seed maturation and after-ripening processes, which in turn enable the appropriate seed imbibition and germination.

The relationships between seed shape and delayed germination have been observed in many ecological studies dealing with seed persistence and dormancy [reviewed in 39]. Thompson et al. [40] found in 97 species of British flora that small and rounded seeds tend to persist in soil longer than elongated and/or flattened seeds. This pattern was confirmed for floras from different continents and ecological contexts [41–43] although with some exceptions for Australian and New Zealand flora [44, 45]. A highly significant relationship between seed morphology and the level of dormancy was observed for weed species by Gardarin and Colbach [46]. Large and elongated seeds may experience selection for a faster germination in order to avoid predation, while small and rounded seeds can be more easily buried in soil and delay germination. A negative correlation has also been reported between spherical seed and germination rate [47]. Seed shape has been found to be related to specific gene controlling hormone synthesis, metabolism or signaling pathways [48] and a relationship has been observed between the earliness of germination and the seed lipid content [46].

In addition, proteomic analyses reported in this study (see below) showed for the first time a relationship between seed persistent syndrome and storage protein content and processing. Our results suggest common mechanisms underpinning seed morphology and germination mechanisms.

Protein analysis by 1D- and 2D-gel electrophoresis

To investigate the molecular basis of the differences observed by light and electron microscopy and to reveal whether modifications in transgenic lines were ascribable to alterations in seed maturation or germination, the protein profile of WT dry seeds was compared with that of F18 and VTe2B transgenic lines. One dimensional gel electrophoresis revealed qualitative differences in the protein profile. While the intensity of some polypeptides seemed to be the same in the two samples, others increased in transgenic seeds (Fig 5).

Polypeptides with a molecular mass of 55 kDa and about 20 kDa were enhanced in F18 and VTe2B with respect to the WT (Fig 5, compare bands 11–13 and 19–21 with bands 1–2; compare bands 16 and 24 with the band 6; compare bands 18 and 26 with the band 7). In addition, no polypeptide with a molecular weight ranging around 14 kDa in the F18 and VT2eB, was observed in the WT seeds (Fig 5, bands 17 and 25). On the other hand, one polypeptide with a molecular mass of 43 kDa (Fig 5, band 3) and three polypeptides with molecular mass of between 6.5 and 14 kDa (Fig 5, bands 8–10) were observed in WT and were not present in F18 and VT2eB dry seeds.

In hydrated seeds, the difference in the protein profile was less pronounced (Fig 5), since only the polypeptide with a molecular mass of 43 kDa, and three comprised between 6.5 and 14 kDa were present in the WT and were not observed in the F18 and in the VT2eB transgenic seeds (Fig 5, asterisks). The presence of low molecular weight polypeptides only in WT seeds suggested that after 24h of imbibition, the protein degradation in mutant seeds had not started, thus confirming the delay in storage mobilization. In *Arabidopsis*, the embryo elongation and subsequent seedling growth were associated with the increase in proteins involved with RNA
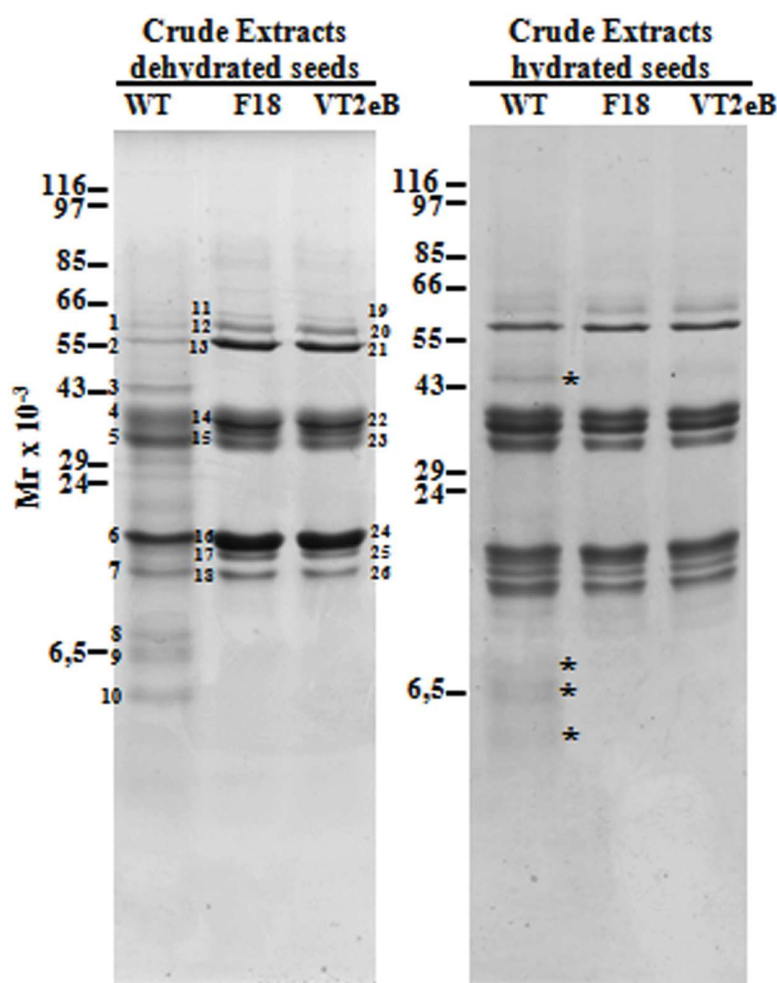
Fig 5. 1-D gel electrophoresis of dry and hydrated seeds crude extracts. Qualitative differences in the polypeptidic profile was observed: while the intensity of some polypeptides seemed to be equivalent in the WT and in transgenic seeds, others increased in transgenic lines (polypeptides 11–13, 14–15 and 16–18 in F18 dry seed and 19–21, 22–23, 24–26 in VT2eB dry seed). On the other hand, low molecular weight polypeptides were present only in WT seeds (bands 8–10 in dry seed). In hydrated seeds the difference in the polypeptidic profile was less pronounced, and only polypeptides with a molecular mass of 43 kDa, and comprised between 6.5 and 14 kDa were observed in the WT (asterisks). In moist WT seeds, the polypeptides corresponding to band 2 and those corresponding to bands 6–7 had a higher intensity than the dry WT seeds. The band numbers correspond to polypeptides identified by MALDI TOF/TOF MS analysis showed in Table 1. The band numbers of the enhanced polypeptides are in bold.

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translation, cell wall modification and protein degradation, which occur in imbibed seeds [18, 49]. The lack of protein degradation in mutant seeds suggested that some processes supporting metabolic events may be altered. In addition, biochemical analyses showed a lower number of reserve proteins in WT compared to the F18 and VT2eB tobacco dry seeds (Fig 5), thus the process allowing storage protein accumulation also appeared to be altered in mutant seeds.

In moist WT seeds, the polypeptides corresponding to band 2 and between bands 6–7 (Fig 5) showed a higher intensity compared to dry seeds, suggesting *de novo* synthesis of these proteins during seed hydration. Proteomic analyses of *Arabidopsis* seeds during germination revealed that the accumulation of cruciferin (the main seed storage protein in *Arabidopsis*)

occurred by *de novo* synthesis during after-ripening in order to provide an additional source of amino acids and nitrogen to seedlings [50]. These data suggest a modification in the processes controlling the accumulation of storage proteins and the storage mobilization, thus contributing to the delay in F18 and VT2eB seed germination.

During seed germination, *de novo* transcription is not required and early germination events depend on the mRNA and protein stored during seed maturation, highlighting the hypothesis that germination has already been prepared during maturation [19, 49, 51]. To investigate whether the delay in early seed germination observed following exogenous DNA insertion could be due to changes in maturation rather than germination, the differences in the protein expression of WT, F18 and VT2eB dry seed crude extracts were analyzed in greater detail by 2D gel electrophoresis. The 2D gel analysis showed that a number of spots significantly varied in the transgenic seeds with respect to the WT (Fig 6 for WT and Figs 7 and 8 for F18 and VT2eB, respectively; varied spots were colored).

This thus suggests that the insertion of exogenous genes induced changes in the protein expression before the seed activation, and that these variations could lead to changes in seed imbibition and in the timing of reserve mobilization.

Protein identification

To unravel the possible role of differentially expressed proteins in regulating the germination time of transgenic seeds with respect to the WT, polypeptides from dry seeds were excised from 1D and 2D gels and subjected to MALDI TOF/TOF MS analysis.

Mass spectrometry identified most of the polypeptides separated by 1D gel (Table 1), showing that enhanced polypeptides in the transgenic seeds are in fact storage proteins, belonging to the vicilin, legumin and globulin protein families (band numbers of the enhanced polypeptides are in bold), suggesting that a higher amount of reserve proteins accumulated in F18 and VT2eB dry seeds with respect to the WT. However, the polypeptides corresponding to bands 4 and 5 of the WT, comigrating with bands 14–15 and 22–23 of the F18 and VT2eB lines (Fig 5), respectively, were identified both as storage proteins and as centromere-associated protein E-like isoform X2, a mitotic kinesin that is required for the stable microtubule capture at kinetochores [52, 53]. However, in the F18 mutant, 14–15 bands showed only storage proteins and there was no centromere-associated protein E-like isoform X2. On the other hand, in VT2eB seeds this kinesin was only present in the 23 band (Table 1).

These data highlight that, unlike mutant seeds, the proteins needed for the control of mitosis checkpoints were already expressed in the WT dry seeds. Although previously proteomic and genetic analyses have not reported the specific accumulation of proteins involved in the cell cycle in seeds, the presence of the centromere-associated protein E-like isoform X2 (Table 1) in tobacco dry WT seeds suggests that some cell cycle control proteins may be present to enable the rapid elongation of the roots after their protrusion from the micropylar endosperm. This hypothesis is supported by data on barley seeds: cell cycle effectors are present in dry seeds before the cell cycle begins and some embryo cells are stopped in the G2 phase of the cell cycle [54]. In addition, proteins involved in the cell cycle, including also cytoskeleton components, were conserved in *Arabidopsis* dry seeds [50], thus highlighting the important role of these proteins for germination. The lack of the centromere-associated protein E-like isoform X2 in the F18 suggests that some pathways allowing the synthesis of cell cycle proteins may be modified in transgenic tobacco seeds before dehydration. This implies that exogenous DNA insertion may also affect some maturation pathways, such as the synthesis of non-storage proteins (i.e. centromere-associated protein E-like isoform X2 and actin; Tables 1 and 2). The presence of the centromere-associated protein E-like isoform X2 in bands 4 and 5 in WT seeds
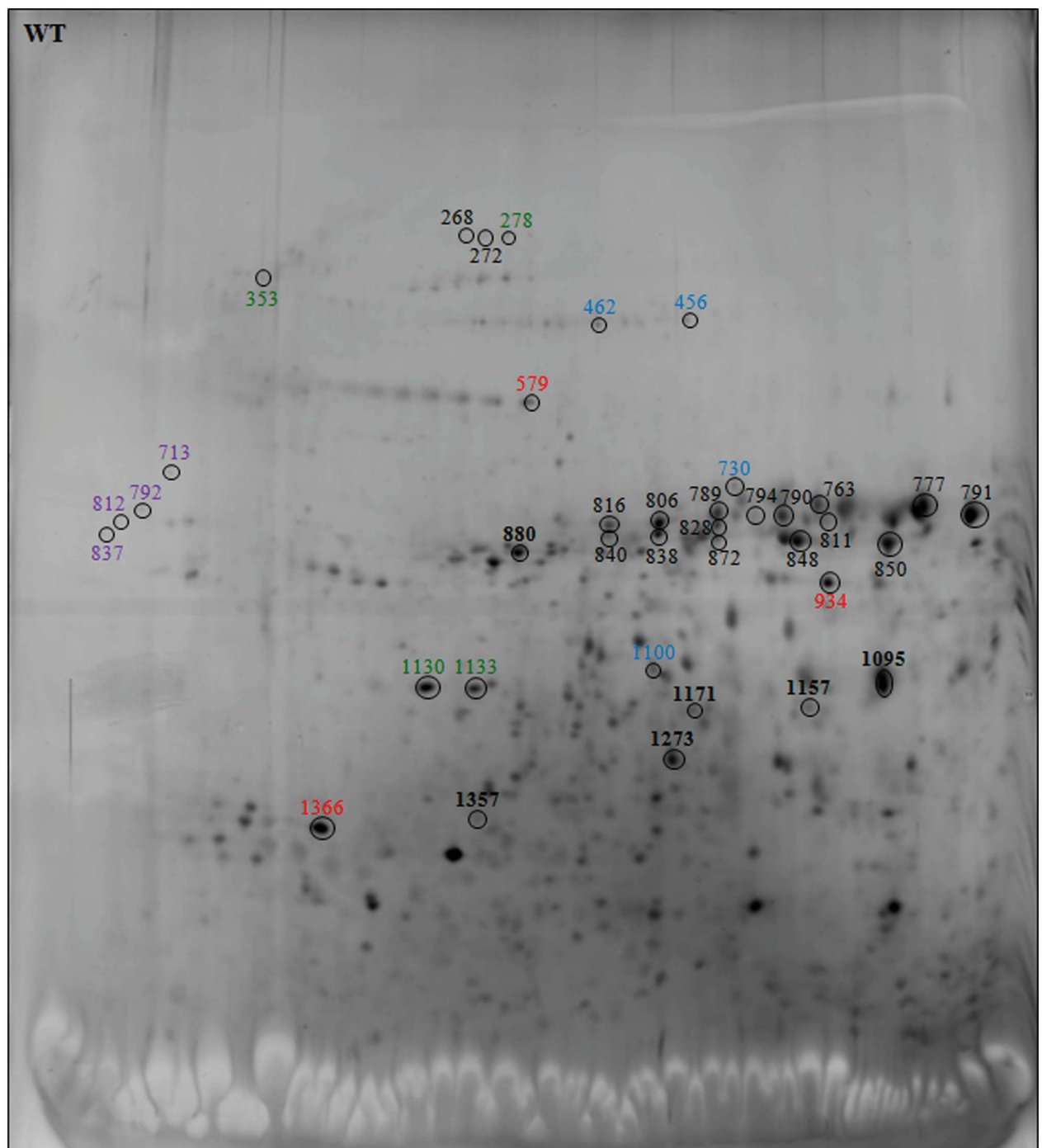
Fig 6. 2D gel electrophoresis of WT seeds. Polypeptides that changed in transgenic lines with respect to the WT were highlighted with a circle. The number of spots corresponds to polypeptides identified by MALDI TOF/TOF MS analysis. Storage proteins are highlighted in black, Chaperone proteins in green, LEA proteins in violet, enzymes in blue, and other proteins in red.
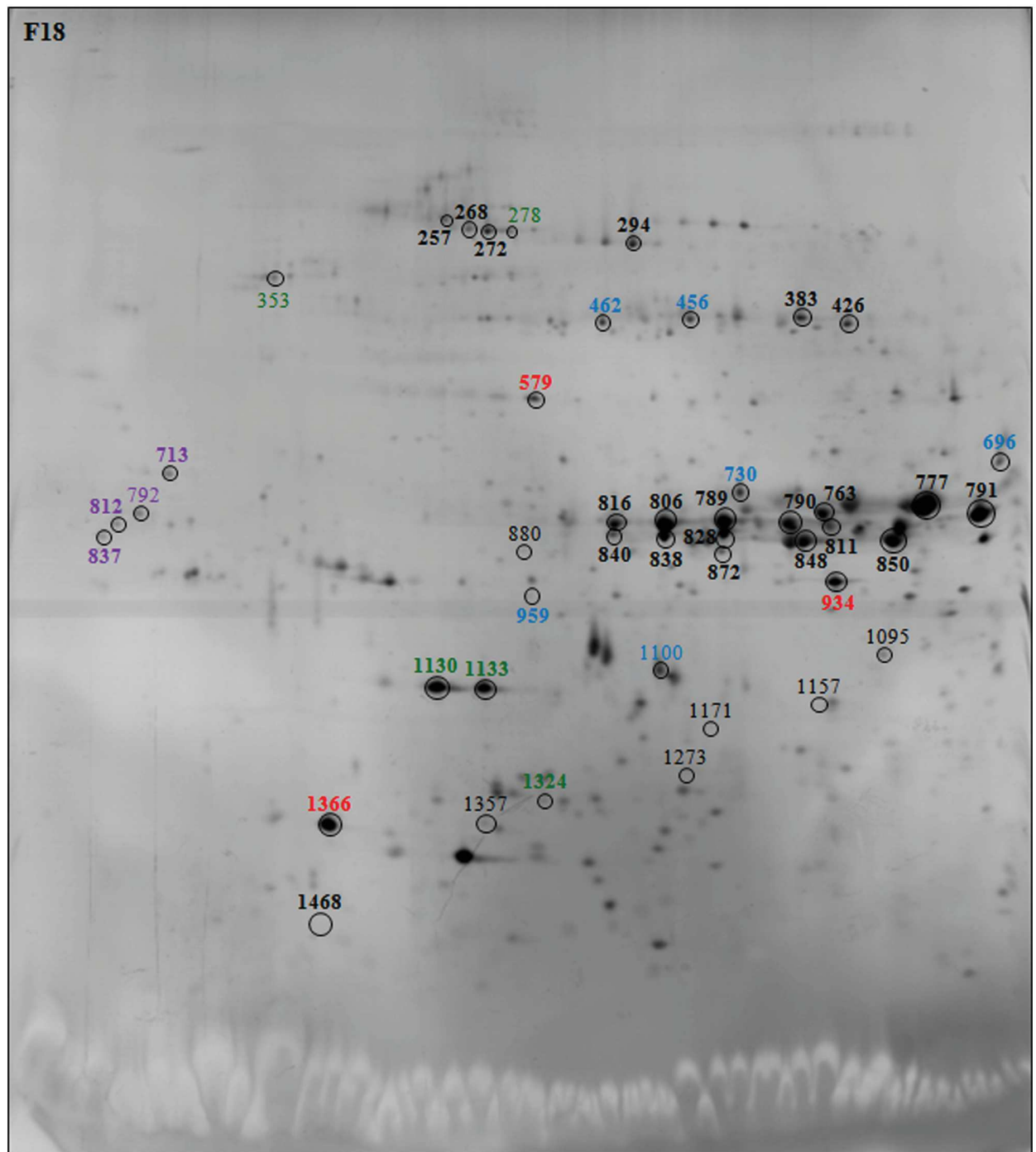
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Fig 7. 2D gel electrophoresis of F18 seeds. Polypeptides changed in transgenic line with respect to the WT were evidenced by circle. The number of spots correspond to polypeptides identified by MALDI TOF/TOF MS analysis. Storage proteins are highlighted 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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