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ORIGINAL ARTICLE

Characterization of the nucellus-specific dehydrin MdoDHN11 demonstrates its involvement in the tolerance to water deficit

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Abstract

Key message **MdoDHN11 acts in the nucellus layer to protect the embryo and the endosperm from limited water availability during apple seed development.**

Abstract Dehydrins (DHNs) are protective proteins related to several plant developmental responses that involve dehydration such as seed desiccation and abiotic stresses. In apple (*Malus* × *domestica* Borkh.), the seed-specific *MdoDHN11* was suggested to play important roles against dehydration during seed development. However, this hypothesis has not yet been evaluated. Within this context, several experiments were performed to functionally characterize *MdoDHN11*. In situ hybridization analysis during apple seed development showed that *MdoDHN11* expression is confined to a maternal tissue called nucellus, a central mass of parenchyma between the endosperm and the testa. The MdoDHN11 protein was localized in the cytosol and nucleus. Finally, transgenic *Arabidopsis* plants expressing *MdoDHN11* were generated and exposed to a severe water-deficit stress, aiming to mimic a situation that can occurs during seed development. All transgenic lines showed increased tolerance to water deficit in relation to wild-type plants. Taken together, our results provide evidences that MdoDHN11 plays important roles during apple seed development by protecting the embryo and the endosperm from limited water availability, and the mechanism of action probably involves the interaction of MdoDHN11 with proteins and other components in the cell.

Keywords Apple · Dehydrin · Seed development · Transgenic expression · Water-deficit stress

Introduction

Seeds are long-term storage structures commonly used by plants to propagate their descendants, given that these structures maintain their viability even under adverse environmental conditions. In flowering plants, the achievement of normal seed development is dependent on the coordination of growth and differentiation between three genetically different components: maternal tissues, embryo and endosperm (Hartmann et al. 2010). The last two are originated by the double fertilization of the egg and the central cell in the female gametophyte, and are responsible for the initiation of seed development (Sreenivasulu and Wobus 2013; Bleckmann et al. 2014). Although the maternal tissues of the ovule do not participate in the fertilization process, they undergo several reprogramming events in response to it (Sreenivasulu and Wobus 2013). One of these maternal tissues is a central mass of parenchyma cells called nucellus, which has nutritive and protective roles (Hartmann et al. 2010). During

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angiosperm seed evolution, plants have shifted between endosperm and nucellus as the main nutrient storage system (Xu et al. 2016). To reach physiological maturity, seeds need to acquire tolerance to desiccation, a process that involves critical water losses to prepare the seed for germination (Hartmann et al. 2010). For the proper seed development, several molecules are recruited to protect different seed components (González-Morales et al. 2016).

One of the protective proteins that stand out in the process of seed development includes a class of polypeptides belonging to the group II of late embryogenesis abundant (LEA) proteins called dehydrins (DHNs) (Banerjee and Roychoudhury 2016). These proteins are characterized by the presence of a highly conserved domain, the K-segment, followed or not by two other well-conserved domains, the Y- and S-segments. Under stress conditions, the K-segment is able to form an α-helical structure, which helps the stabilization of proteins and cellular membranes (Liu et al. 2017). Indeed, DHNs play important roles in plant developmental responses that involve dehydration tolerance such as seed desiccation and abiotic stresses (Hanin et al. 2011). Although many putative functions were reported for DHNs such as radical-scavenging activity and binding to membranes, metals, lipids and DNA, their precise mechanisms of action remain unknown (reviewed in Hanin et al. 2011; Graether and Boddington 2014; Liu et al. 2017). Given that DHNs are usually classified as intrinsically disordered proteins (IDPs), their function is commonly associated with this feature (Tompa et al. 2006). Their structure is prone to act as water replenishers and prevent an adverse increase in ionic strength, allowing DHNs to interact and protect membranes and other proteins during dehydration (Tompa et al. 2006; Banerjee and Roychoudhury 2016; Riley et al. 2019).

In apple (*Malus* × *domestica* Borkh.), one of the most economically important perennials worldwide, a high functional diversification was identified among its 11 *DHN* (*MdoDHN*) genes. Based on the presence of conserved domains (K, Y and S), MdoDHNs were assigned into four out of five DHN subclasses (Falavigna et al. 2015). *MdoDHN* genes were characterized according to their transcript expression in response to drought, cold, and abscisic acid treatment (Liang et al. 2012), as well as during bud dormancy progression, flowering and fruit ripening stages (Falavigna et al. 2015). However, no apple *DHN* was functionally characterized so far. *MdoDHN11*, a Y_3SK_2 dehydrin, is an interesting candidate to be further characterized due to its seed-specific transcript accumulation pattern and its probable protective role during apple seed tolerance to desiccation (Falavigna et al. 2015).

In the present work, the functional characterization of the *MdoDHN11* gene was carried out. To accomplish this goal, the spatio-temporal *MdoDHN11* gene expression was analyzed in apple seeds, the subcellular localization of the encoded protein was demonstrated, as well as the performance of transgenic *Arabidopsis* plants expressing *MdoDHN11* under a severe water-deficit stress. These assays were performed aiming to better understand the functional role of *MdoDHN11*, and the data gathered in this work confirmed its protective relevance during long-term water deficit, in a similar manner to a situation that can occurs during seed development.

Materials and methods

Plant material

Apple fruits were harvested in an experimental orchard located at Embrapa Uva e Vinho (− 29.165762, − 51.535147 and 614 m above sea level) in Southern Brazil. Apple trees were maintained using standard orchard management practices. Plant material consisted of 2-year-old 'Imperial Gala' trees grafted on M.7 rootstocks. Flowers at the anthesis stage were tagged in the field. Apple fruits were harvested at 22, 51, and 83 days after anthesis (DAA), which corresponded to fruits with 20, 40, and 60 mm diameter, respectively (Fig. 1a, d, g). At each sampling point, at least six individual whole fruits were analyzed. Seeds were immediately removed and fixed in 4% formaldehyde under vacuum (600 mmHg) for 30 min followed by overnight incubation at 4 °C (McDowell and Trump 1976). Samples were dehydrated through a graded series of ethyl alcohol from 30 to 100% and stored at − 20 °C until use.

Seed morphological assay

The fixed and dehydrated seed samples were embedded in 2-hydroxyethyl methacrylate resin as described in Gerrits and Smid (1983). The 5 µm sections were obtained using a Leica RM 2255 microtome. The metachromatic reagent Toluidine Blue O was used to stain seed structures by submerging the slides with sections in the reagent for 1 min (Feder and O'brien 1968). The stained tissues were photomicrographed in bright field using a Leica DM1000 LED microscope coupled with a Leica MC170 HD camera system. Photomicrographs were merged and the background color was adjusted to white.

Nucleic acid extraction and cDNA synthesis

DNA was purified from young leaves (100 mg) using modified protocols scaled down to 2 mL centrifuge tubes (Lodhi et al. 1994; Lefort and Douglas 1999). Total RNA was isolated (Zeng and Yang 2002; Falavigna et al. 2014) and DNase-treated using the TURBO DNA-free Kit (Ambion). The SuperScript™ III Reverse Transcriptase (Thermo Fisher

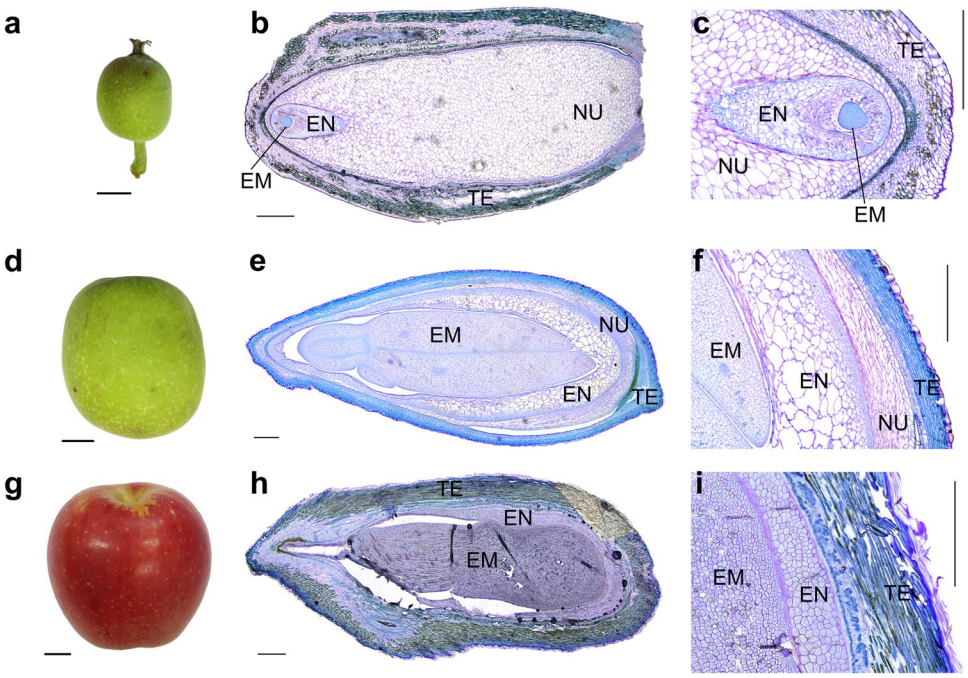

Fig. 1 Morphoanatomical analysis of seeds during apple development. **a**–**c** 20 mm fruits (22 DAA). **d**–**f** 40 mm fruits (51 DAA). **g**–**i** 60 mm fruits (83 DAA). Representative images of the used apples are present in **a**, **d** and **g**, with a scale bar equal to 1 cm. Light micrographs of apple seed cross sections stained with Toluidine blue O are shown in **b**, **c**, **e**, **f**, **h** and **i**, with a scale bar of 500 µm. Enlarged images of the peripheral seed structure are presented in **c**, **f** and **i**. *EN* endosperm, *EM* embryo, *NU* nucellus, *TE* testa (color figure online)

Scientific) was used for cDNA synthesis, according to manufacturer's instructions.

In situ hybridization

Seed samples were embedded in paraffin and longitudinal and transversal sections (8–10 µm) were prepared and mounted on silanized microscope slides as described (Malabarba et al. 2017). A gene-specific fragment of 225 bp (*MdoDHN11*_ISH_probe in Supplementary Table 1) from *MdoDHN11* was amplified from cDNA obtained from seeds of 'Imperial Gala' fruits and cloned into the pGEM®-T Easy vector (Promega), according to manufacturer's instructions. The resulting recombinant vector was confirmed by sequencing. Sense and antisense labeled probes were generated by digoxigenin-labeling using the DIG RNA Labeling Kit (SP6/T7) (Roche). After detection of the hybridization signals by immunostaining, slides were washed, dehydrated, and mounted using Entellan® (Merck). Photomicrographs were obtained as described above.

Subcellular localization of MdoDHN11

To identify the subcellular localization of MdoDHN11, a transient expression assay in *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) leaf protoplasts was performed. The complete coding sequence of *MdoDHN11* was amplified using Platinum® *Pfx* DNA polymerase (Thermo Fisher Scientific) from cDNA obtained from 'Imperial Gala' seeds using gene-specific primers (Supplementary Table 1). The amplicon was cloned into pENTR™ Directional TOPO® (Thermo Fisher Scientific), generating the TOPO-*MdoDHN11* construct. This construct was used to fuse the coding sequence of *EYFP* (enhanced yellow fluorescent protein) to the 5′ portion of *MdoDHN11* using the p2YGW7 vector (Karimi et al. 2007) and Gateway® LR Clonase™ II Enzyme Mix (Thermo Fisher Scientific). The resulting vector with the 35S::*EYFP-MdoDHN11* insert was used in protoplast transformation. Protoplast isolation and transformation were performed essentially as described in Wu et al. (2009). Negative controls were composed of untransformed cells, whereas positive controls were cells transformed with 35S::*EGFP* (enhanced green fluorescent protein). Transformed protoplasts and negative controls were incubated under light for 20 h at 24 °C prior to imaging. Cell nuclei were stained using 1 µg/mL DAPI (4′,6-diamidino-2-phenylindole). Fluorescence microscopy was performed using an Olympus FluoView 1000 confocal laser-scanning microscope (UFRGS Electron Microscopy Center, Porto Alegre, Brazil) equipped with a set of filters capable of distinguishing chlorophyll autofluorescence, DAPI, GFP and YFP fluorescence. The assay was repeated twice to confirm the obtained results.

Arabidopsis transformation

To generate transgenic *Arabidopsis* plants expressing *MdoDHN11*, the TOPO-*MdoDHN11* construct previously obtained was used to clone *MdoDHN11*'s CDS into the pH7WG2D.1 vector under CaMV 35S promoter (Karimi et al. 2007) using Gateway® technology (Thermo Fisher Scientific). The resulting vector harboring the 35S::*MdoDHN11*

cassette was confirmed by sequencing and used in the transformation of *Agrobacterium tumefaciens* (strain EHA105). Wild-type *Arabidopsis* Col-0 plants were transformed using the floral dip method (Clough and Bent 1998). Transformed seeds were sown in individual plastic pots. Given that the vector encodes *EGFP* under the control of the *rolD* promoter (Karimi et al. 2007), a GFP fluorescence screening was performed in 3-week-old plants using a Leica M165FC stereomicroscope. Plants lacking GFP fluorescence were discarded. Total RNA was isolated from leaves and cDNA was synthesized as previously described. To assess transgene expression, real-time PCR was performed using *MdoDHN11* gene-specific primers (Supplementary Table 1) as described Falavigna et al. (2014, 2015). Actin 2 (*AtAct2*) and constitutive photomorphogenic 1 (*AtCOP1*) were used as reference genes (Supplementary Table 1). Amplicons from each line were sequenced to confirm their transgenic nature. Plants without transgene expression were also discarded. Nine independent lines were obtained and further analyzed.

Water-deficit stress

A preliminary water withhold assay was performed to determine how long wild-type *Arabidopsis* plants could survive without water in our growth conditions (Falavigna et al. 2018). Wild-type plants were unable to recover after 25 days without water (Falavigna et al. 2018). In the present work, pot capacity of wild-type and transgenic T1 line plants was achieved and maintained prior to the water deficit treatment. All pots have had their weight adjusted to approximately 160 g (soil, plant, pot and water) before starting the stress assay. The water deficit experiment was performed by transferring unstressed 2-month-old transgenic and wild-type plants to trays without water for 42 days. During the treatment, all pots were independently weighed once a week to measure water loss. After 6 weeks of treatment, water was added to the trays, allowing water influx to the pots through diffusion. Plant recovery was evaluated after 7 days of rehydration. Pictures were taken for visual analysis.

Results

Morphoanatomical analysis of apple seeds

Morphoanatomical analyses of 'Imperial Gala' seeds were performed to better characterize the apple seed structure during its development. During the early development of the seed (seeds from 20 mm fruits), the nucellar tissue increased and the seed coat (testa) differentiated, while the young embryo (globular embryo) remained immersed in the developing endosperm (Fig. 1b, c). In seeds from 40 mm fruits, the further development of the endosperm and the embryo (torpedo embryo) was observed, limiting the nucellus to a small number of cell layers in contact with the integument already differentiated in the seed coat, with mesotestal characteristics (Fig. 1e, f). In seeds from 60 mm fruits, a well-developed embryo was observed and the endosperm was reduced to a few cell layers in contact with the testa (Fig. 1h, i).

Spatial and temporal accumulation of *MdoDHN11* transcripts and subcellular localization of the encoded protein

Transcript accumulation of *MdoDHN11* was previously analyzed in apple during bud dormancy, flowering and fruit development, and its main expression was identified in seeds (Falavigna et al. 2015). Gene expression data from this previous work was reanalyzed aiming to identify which *MdoDHN* gene presented the highest steady-state mRNA levels during apple seed development (Supplementary Fig. 1). When considering seeds from 40 mm fruits, *MdoDHN11* presented the highest transcript levels among all *MdDHN* genes. This result prompted us to further characterize the *MdoDHN11* spatial and temporal expression patterns using in situ hybridization (ISH). The antisense *MdoDHN11* probe signal was clearly visible in the nucellus layer in seeds from 40 mm fruits (Fig. 2a). ISH slides hybridized with the sense *MdoDHN11* probe were used as control and showed no hybridization signals (Fig. 2b).

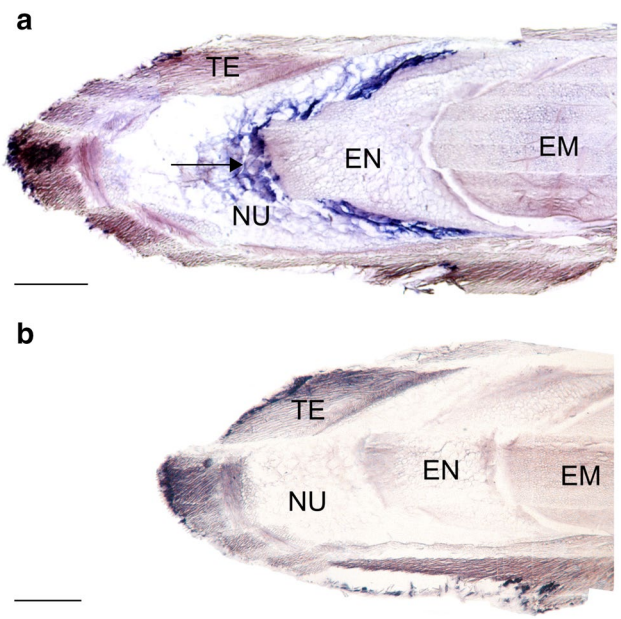

Fig. 2 Spatial and temporal accumulation of *MdoDHN11* transcripts in apple seeds. Hybridization signal (black arrow) is visible in the nucellus layer (**a**) compared to the control slide (**b**). *EN* endosperm, *EM* embryo, *NU* nucellus, *TE* testa. Scale bar 500 µm (color figure online)

To gain insights about MdoDHN11 function, EYFP was fused to MdoDHN11 to identify its subcellular localization. Transient expression analyses in *Arabidopsis* leaf protoplasts revealed that MdoDHN11 was present in the nucleus and cytosol (Fig. 3). Positive controls showed similar subcellular localization, whereas negative controls showed only DAPI and chlorophyll fluorescence.

MdoDHN11-expressing *Arabidopsis* plants and evaluation of water deficit tolerance

MdoDHN11 transcript accumulation during apple seed development suggested an adaptive role against dehydration (Falavigna et al. 2015), which is characteristic during the acquisition of seed desiccation tolerance (González-Morales et al. 2016). Aiming to test if the function of MdoDHN11 may be related to protection against dehydration, transgenic *Arabidopsis* plants expressing *MdoDHN11* were generated and submitted to a severe water-deficit stress. Nine independent transgenic lines expressing *MdoDHN11* were obtained and analyzed. All transgenic plants expressed the *EGFP* reporter gene as well as the *MdoDHN11* transgene (Fig. 4a). Transformed and wild-type plants were grown under normal conditions for 2 months and then their water supply was removed. Wild-type plants displayed a higher water loss rate during water-deficit stress than transgenic lines, reaching almost half of the initial water soil content in the end of the treatment (Fig. 5). Similar water losses were identified among transgenic plants, achieving around 60% of water soil content after 42 days of treatment. The exception was DHN11 plant #9, which showed an intermediate rate between wild-type and transgenic plants, especially after 21 days of treatment. Despite these differences, similar water soil contents in all plant pots were obtained after 7 days of rehydration (inset, Fig. 5). All plants flowered during the experiment. None of the wild-type plants survived the stress assay, while all transgenic lines were able to cope with the imposed water deficit (Fig. 4b). After the stress treatment, all transgenic lines followed their normal growth cycle, producing fertile flowers, siliques, and seeds.

Discussion

Several studies in many plant species, including apple (Liang et al. 2012; Falavigna et al. 2015), have reported the relationship between dehydrins (DHNs) and several plant adaptive responses involving dehydration, especially seed development and abiotic stresses (reviewed in Hanin et al. 2011; Graether and Boddington 2014; Banerjee and Roychoudhury 2016). Specifically in apple, previous studies from our group suggested that MdoDHN11 is one of the proteins recruited to protect seeds during development (Falavigna et al. 2015). However, this hypothesis has not yet been evaluated. Within this context, we performed the functional characterization of *MdoDHN11*.

All main seed components, i.e., embryo, endosperm, nucellus and testa, were identified in apple seeds (Fig. 1). In apple, about 4–6 weeks after fertilization, the endosperm becomes cellular and soon fills much of the developing ovule

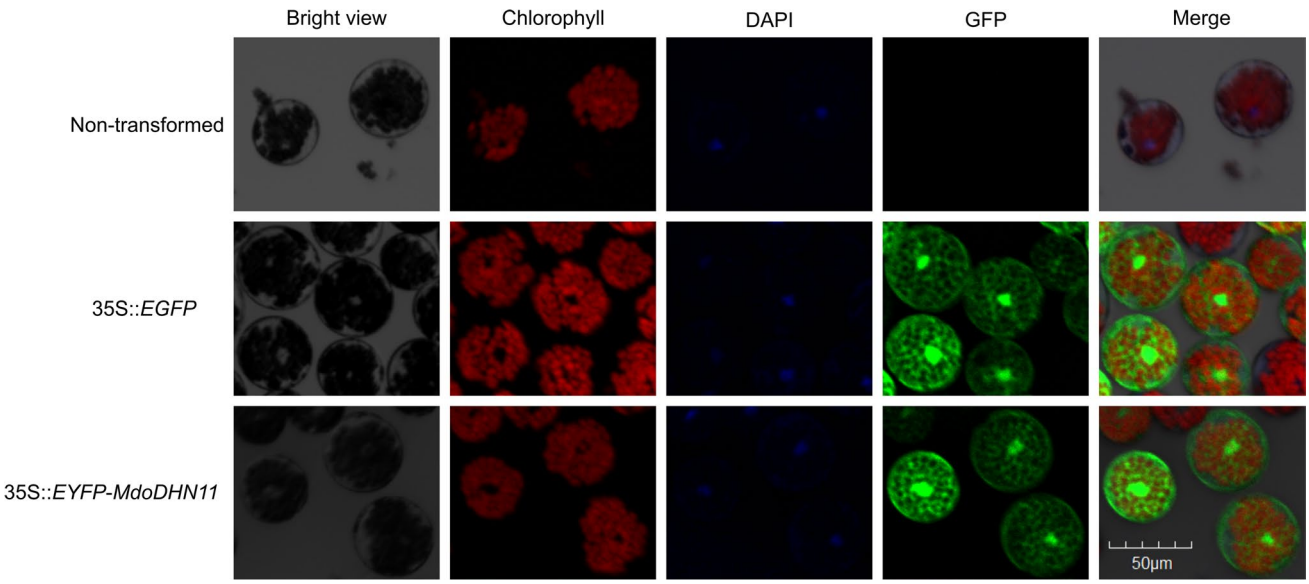

Fig. 3 Subcellular localization of MdoDHN11 in *Arabidopsis* leaf protoplasts. Negative and positive controls were composed by untransformed cells and cells transformed with 35S::*EGFP*, respectively. All images were captured with a confocal laser scanning system. Green EYFP or EGFP, red chlorophyll, blue DAPI (color figure online)

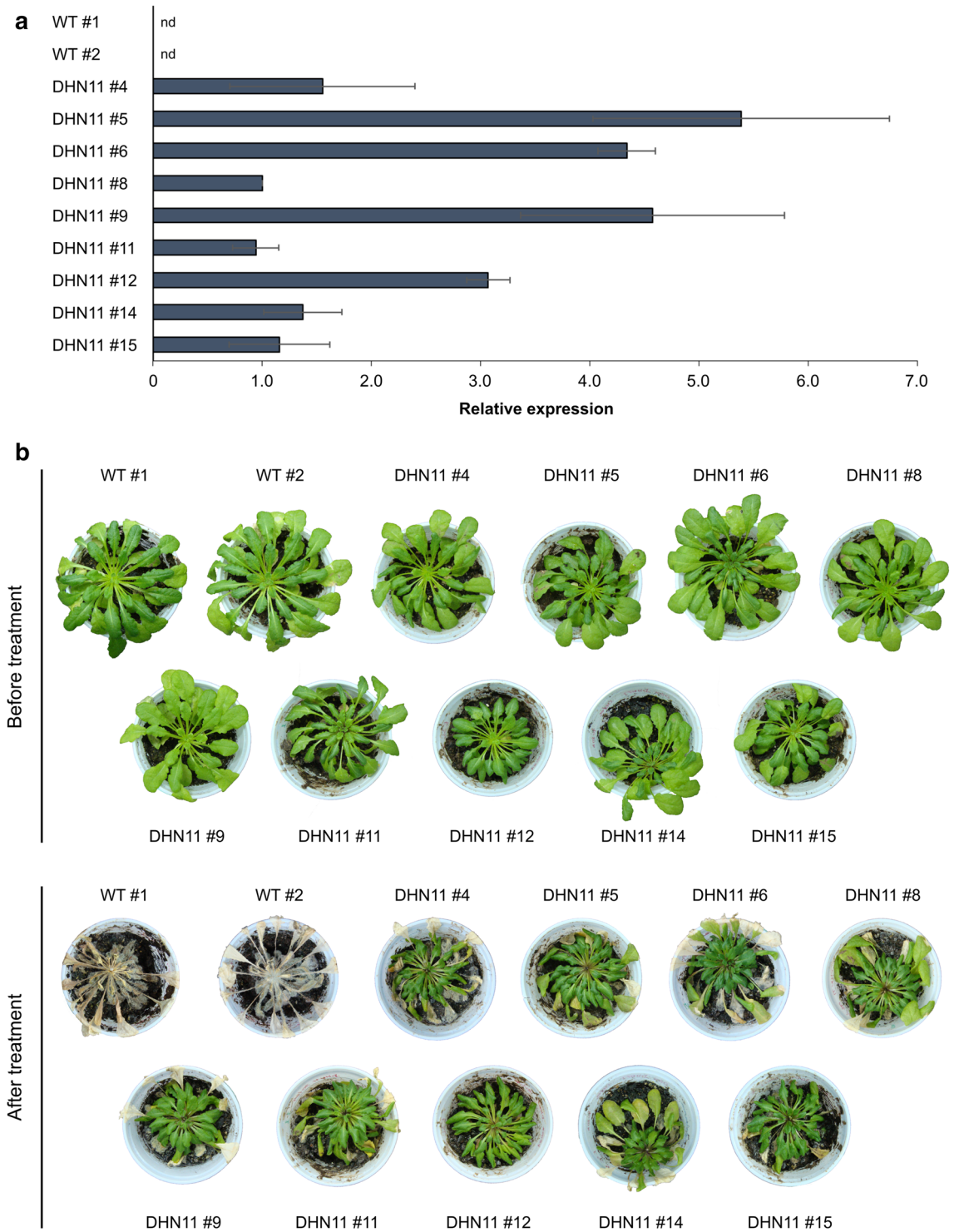

Fig. 4 Evaluation of transgenic *Arabidopsis* plants expressing *MdoDHN11*. **a** Real-time PCR of *MdoDHN11* expression in *Arabidopsis* leaves. Gene expression was plotted relatively to DHN11 #8. Standard error bars are shown. *nd* not-detected. **b** Phenotype of transgenic and wild-type plants exposed to water-deficit stress. The stress treatment was composed of removing the water supply for 6 straight weeks, followed by rewatering for 1 additional week (color figure online)

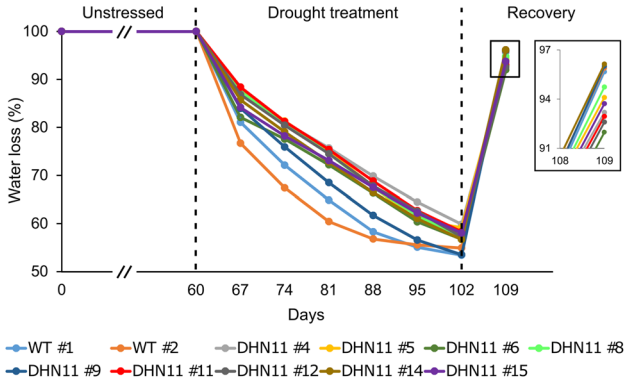

Fig. 5 Quantification of water loss rates of each pot. Pot capacity was maintained during the unstressed growing condition. The water deficit treatment consisted in removing the water supply of 2-month-old wild-type and transgenic plants for 42 days. For rehydration, water influx was allowed through diffusion. Inset shows the recovery phase (color figure online)

as it grows at the expense of the nucellus. Afterwards, the embryo gradually consumes the endosperm and occupies most of the seed (Dennis 2003). Embryo development from globular to torpedo, at the expense of the endosperm, was clearly visible through the stages analyzed (Fig. 1). To the best of our knowledge, the photomicrographs of the morpho-anatomical analyses are, up to now, the best images reporting apple seed development in the literature, especially considering seeds from 40 mm fruits. These images may help the better understanding of analyses such as the ISH assays presented here.

MdoDHN11 transcripts accumulated in the nucellus layer, in the border with the endosperm of fruits with 40 mm diameter (Fig. 2). Due to the maternal origin of this layer, the accumulation of *MdoDHN11* may represent a protective source for the embryo and the endosperm during seed development. Within this context, DHNs, as IDPs, are able to shift their conformational status under limited water availability and bind partly dehydrated surfaces of other proteins, protecting stored proteins and nutrients to support seed survival during maturation and desiccation (Tompa et al. 2006; Graether and Boddington 2014). Such activity would need the location of MdoDHN11 to be in the cytosol, which was demonstrated in our subcellular localization assays (Fig. 3). Recently, a subcellular localization study of all *Arabidopsis* DHN proteins showed that these proteins are localized in the cytosol or in the cytosol and nucleus (Candat et al. 2014). Phylogenetic analyses of DHNs from seven species, including apple and *Arabidopsis*, demonstrated that MdoDHN11 is present in a seed-specific clade together with *Arabidopsis* AtDHN14 and AtDHN45 (Falavigna et al. 2015). Interestingly, these two AtDHNs have the same subcellular localization of MdoDHN11, nucleus and cytosol (Candat et al. 2014). However, the nuclear localization is likely to be due to diffusion rather than targeting to nucleus (Candat et al. 2014). Taken together, these results fit well with our subcellular localization data and add additional evidence of the protective role of MdoDHN11 during apple seed development.

To gain insights about the *MdoDHN11* capacity to confer protection during restricted water availability, the *MdoDHN11* gene was constitutively expressed in *Arabidopsis* and these plants were submitted to a severe water deficit assay. During the treatment, wild-type plants showed higher water loss rates than transgenic lines (Fig. 5). Similar findings were observed in water-deficit stress studies using transgenic plants ectopically expressing *DHN*s from *Medicago truncatula*, *Musa acuminata* and *Prunus mume* (Shekhawat et al. 2011; Xie et al. 2012; Bao et al. 2017). The slow water losses showed by transgenic lines may be partly explained by their lower leaf area (Fig. 4b), which can consequently lead to a lower transpiration rate. However, no clear relationship could be drawn between water loss rate and total plant leaf area (Figs. 4, 5). As an example, DHN11 plant #4 had the slowest water loss but was not the smallest plant. Another explanation may be directly linked to the DHN ability to bind water, which would help the maintenance of the original cell volume, preventing massive water losses and thus cellular collapse (Hanin et al. 2011).

The sole plants able to survive the water deficit treatment were the ones expressing the *MdoDHN11* transgene (Fig. 4). Interestingly, all transgenic plants fully recovered less than 2 h after rehydration, suggesting that MdoDHN11 was able to maintain root integrity and functionality. Other reports have shown better root growth and development of plants constitutively expressing *DHN*s in comparison to wild-type plants under water-deficit stress-inducing agents such as PEG or mannitol (Shekhawat et al. 2011; Yang et al. 2014). Studies with *DHN* genes from several other plant species also pointed out the DHN ability to enhance tolerance to water deficit and other osmotic stresses (Cheng et al. 2002; Xing et al. 2011; Shekhawat et al. 2011; Xie et al. 2012; Ruibal et al. 2012; Yang et al. 2014; Bao et al. 2017). However, it is worth to mention that constitutive expression of *DHN*s in plants is not a guarantee of increased tolerance to abiotic stresses (Hanin et al. 2011). As an example, transgenic tobacco plants ectopically expressing a *Craterostigma plantagineum DHN* did not show increased drought tolerance (Iturriaga et al. 1992). One possible explanation is that the introduced gene was not sufficient to increase osmoprotection and may only work in cooperation with other molecules (Iturriaga et al. 1992). Indeed, overexpression of dehydrin *RAB18* in *Arabidopsis* did not improve stress tolerance, while its co-expression with dehydrin *Cor47* increased freeze tolerance (Puhakainen et al. 2004).

The improved water deficit tolerance showed by transgenic *Arabidopsis* plants expressing *MdoDHN11* is evidence

that MdoDHN11 is an important player in the plant response to limited water availability (Fig. 4). However, we cannot rule out that other MdoDHNs, especially MdoDHN1 (Supplementary Fig. 1), may also contribute for this trend during seed development. The protective role of DHNs may occur due to their disordered protein structure allied with their inability to denature (Graether and Boddington 2014). During dehydration, these features allow them to prevent massive water losses as well as to bind to other proteins and to interact with membranes, protecting tissues and cells under stress (Tompa et al. 2006; Kovacs et al. 2008; Banerjee and Roychoudhury 2016). If we consider the seed-specific expression of *MdoDHN11* confined to the nucellus layer, its probable function during apple seed development may be related to protect embryo and endosperm from limited water availability. Most likely, MdoDHN11 mechanism of action involves its interaction with proteins and other components present in the cell.

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Author contribution statement VSF, LFR, MMP and GP conceived the work and the experimental design; VSF, JM, VB and JEAM performed the ISH and the seed morphological assays; VSF and CPS performed the subcellular localization assay; VSF performed field samplings, nucleic acid extractions, real-time PCR assays, gene amplifications and cloning, *Arabidopsis* transformation, water deficit assay, and drafted the manuscript; LFR, MMP and GP revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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