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Patterns of protein carbonylation during *Medicago truncatula* seed maturation

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Abstract

Seeds mainly acquire their physiological quality during maturation, whereas oxidative conditions reign within cells triggering protein carbonylation. To better understand the role of this protein modification in legume seeds, we compared by proteomics patterns of carbonylated proteins in maturing seeds of *Medicago truncatula* naturally desiccated or prematurely dried, a treatment known to impair seed quality acquisition. In both cases, protein carbonylation increased in these seeds, accompanying water removal. We identified several proteins whose extent of carbonylation varied when comparing natural desiccation and premature drying and that could therefore be responsible for the impairment of seed quality acquisition or expression. In particular, we focused on PM34, a protein specific to seeds exhibiting a high sensitivity to carbonylation and of which function in dicotyledons was not known before. PM34 proved to have a cellulase activity presumably associated with cell elongation, a process required for germination and subsequent seedling growth. We discuss the possibility that PM34 (abundance or redox state) could be used to assess crop seed quality.

KEYWORDS

cellulase activity, *Medicago truncatula*, protein carbonylation, seed maturation and desiccation

1 | INTRODUCTION

Plant establishment depends on the success of seed germination, which mainly relies on physiological seed quality. Seed quality is an ability of the seed to germinate before (seed viability) and after storage (seed longevity), in a fast and homogeneous manner, providing proper and vigorous seedling establishment in a variety of environments (seed vigour). Seed quality also depends on environmental conditions prevailing during seed development on the mother plant and storage after seed harvest. When stored for a long time or under adverse conditions, seeds age. They lose vigour, become more

sensitive to the stresses occurring upon imbibition, generate abnormal seedlings, and may even die (Walters, 1998).

Seed quality is established in the latest phases of seed development on the mother plant. It relies on the synthesis of protective components such as late embryogenesis abundant (LEA) proteins and heat shock proteins. It also depends on the presence of antioxidant systems that allow the seeds to endure oxidative conditions that reign from maturation to germination, including after-ripening and storage, due to an accumulation of reactive oxygen species (ROS) and derived products (Bailly, 2004; Bailly, El-Maarouf-Bouteau, & Corbineau, 2008; Kranner, Minibayeva, Beckett, & Seal, 2010; Rajjou et al., 2012). Depending on their level of accumulation, ROS can either act as redox signals, involved in the regulation of growth and in adaptive responses to the environment, or as toxic compounds that cause extensive cellular damage leading to oxidative stress (Bailly, 2004; Das & Roychoudhury, 2014; Noctor, Reichheld, & Foyer, 2017; You & Chan, 2015). Among such cellular damage,

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; DAP, days after pollination; DNP, dinitrophenylhydrazine; KARI, ketol-acid reductoisomerase; LEA, late embryogenesis abundant; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SDR, small dehydrogenase reductase

oxidative conditions are known to lead to the carbonylation of biomolecules, notably proteins (Davies, 2005; Hansen, Roth, & Winther, 2009; Møller, Havelund, & Rogowska-Wrzesinska, 2017), which can affect their structure and activity.

Protein carbonylation is thought to be an irreversible phenomenon targeting Arg, Lys, Thr, and Pro amino-acid residues that are highly sensitive to a direct carbonylation by ROS. Carbonylation can also result from reaction of proteins with reactive carbonyls such as sugar or lipid oxidation end products as methylglyoxal, hydroxynonenal, or malonaldehyde (Kranner et al., 2010; Møller et al., 2017; Møller, Rogowska-Wrzesinska, & Rao, 2011). Protein carbonylation was observed in mature seeds, in after-ripened seeds, in germinating seeds, and in aged seeds of different species (Bailly et al., 2008; Barba-Espín et al., 2011; Job, Rajjou, Lovigny, Belghazi, & Job, 2005; Kranner et al., 2010; Muller, Carstens, Linkies, Torres, & Leubner-Metzger, 2009; Oracz et al., 2007; Rajjou et al., 2008; 2012). Importantly, protein carbonylation in seeds produced in optimal environmental conditions does not preclude but on the contrary may accompany seed quality establishment and expression. In these conditions, carbonylation is nonetheless selective and targets storage proteins and some metabolic proteins. However, in aged seeds, stored for a long time or in adverse conditions, such protein carbonylation events can occur massively and are surmised to be associated with decreased seed vigour (Bailly et al., 2008; Rajjou et al., 2008).

Legumes are the second most important source of human food and animal forage, notably because their seeds and young plants are exceptionally rich in proteins. They are also essential nowadays in cropping systems for the ecosystemic service they provide, contributing to nitrogen enrichment of soils and thereby to sustainable agriculture (Lithourgidis, Dordas, Damalas, & Vlachostergios, 2011). Key breeding objectives include improving biotic and abiotic stress resistance and enhancing yield components and seed quality, which are currently being realized through the development of genomic tools (Pandey et al., 2016; Tayeh et al., 2015; Verdier et al., 2013). In many plant species, proteomics approaches have widely been deployed to investigate the role of seed proteins in establishment of seed quality, notably concerning tolerance to desiccation and longevity (Boudet et al., 2006; Châtelain et al., 2012; Huang, Møller, & Song, 2012; Rajjou et al., 2012; Villegente et al., 2017; Wang, Liu, Song, & Møller, 2015; Wang, Møller, & Song, 2012; Yacoubi, Job, Belghazi, Chaibi, & Job, 2013); for a recent review in legumes, see Rathi, Gayen, Gayali, Chakraborty, and Chakraborty (2016). In particular, several studies highlighted the impact of protein carbonylation on seed physiological quality of a number of plant species, for example, in *Arabidopsis thaliana* (Job et al., 2005; Rajjou et al., 2008; Thu-Phuong, Cueff, Hegedus, Rajjou, & Bentsink, 2015), rice (Yin et al., 2017; Zhang et al., 2016; Zhang, He, Li, & Yang, 2017), wheat (Gietler, Nykiel, Orzechowski, Fettke, & Zagdańska, 2017), elm (Li, Wang, Xue, Pritchard, & Wang, 2017), sunflower (El-Maarouf-Bouteau, Meimoun, Job, Job, & Bailly, 2013; Morscher, Kranner, Arc, Bailly, & Roach, 2015; Oracz et al., 2007), apple (Debska, Krasuska, Budnicka, Bogatek, & Gniazdowska, 2013; Krasuska, Ciacka, Debska, Bogatek, & Gniazdowska, 2014), beech (Kalemba & Pukacka, 2014), upas (Bai et al., 2011), or westerner redstar (Terskikh et al., 2008). However,

there are presently only very few reports for legume seeds, for example, in mung bean (Verma, Mishra, Sangwan, & Sharma, 2015) or in pea (Barba-Espín et al., 2011).

To gain an insight into the role of protein carbonylation on the physiological quality of seed legumes, we used the legume model species *Medicago truncatula*. To this end, we first analysed whether proteins are carbonylated during seed maturation, a phase during which legume seed quality is greatly improved as early shown by Ellis, Hong, and Roberts (1987). We found that protein carbonylation accompanies water removal by targeting a small set of proteins. Carbonylated proteins were separated by two-dimensional (2D) polyacrylamide gel electrophoresis, and some were identified by peptide mass fingerprinting. Then, to help identifying proteins for which carbonylation might be detrimental for seed quality, we assessed the impact of an artificial and premature drying of the seeds, a treatment known to impair the acquisition of seed quality (Bailly et al., 2001; Châtelain et al., 2012; Ellis et al., 1987; Huang et al., 2012; Sanhew & Ellis, 1996; Wang et al., 2014; 2015). We observed that whereas the identity of most of the carbonylated proteins remained the same, the extent of their carbonylation varied substantially. We discuss the possible role of the specific carbonylation of these proteins in the acquisition of seed quality in legume seeds, focusing our attention on a protein known as PM34, specifically accumulated in seeds. We identified that this protein bears a cellulase activity that may be required for the cell elongation processes occurring during seed germination and seedling growth.

2 | MATERIALS AND METHODS

2.1 | Plant material

Three independent sets of maturing seeds of *M. truncatula* A17 produced in controlled conditions (Châtelain et al., 2012) were used for this study. Briefly, plants were grown in a growth chamber at 24/21 °C and 16 hr photoperiod at 200 $\mu\text{mol m}^{-2} \text{s}^{-2}$ photosynthetically active radiation. Maturing seeds were removed from the pods at different times of development (28, 32, 40, and 48 days after pollination [DAP]). During maturation, the seed water content decreased from about 1.29 g/g dry weight at 28 DAP to 0.11 g/g dry weight at maturity (Châtelain et al., 2012). Immature seeds (28 to 40 DAP) were used either fresh (referred to as fresh seeds) or after an artificial drying (referred to as dried seeds) to reduce their water content to a value similar to that measured in mature seeds. The drying treatment was applied for 3 days at 20 °C and 44% relative humidity, generated by using a saturated salt solution of K_2CO_3 . After this treatment, the water content in dried seeds was similar to that measured in mature and naturally desiccated seeds. It is noted that such a treatment applied to mature seeds does not change their water content (Châtelain et al., 2012).

2.2 | Protein carbonylation state

For the determination of the protein carbonylation state, the seed powder obtained by grinding the seeds (60 to 200 seeds, for resolution in one-dimensional [1D] or 2D gel electrophoresis, respectively)

in liquid N₂ was first delipidated to avoid unspecific protein carbonylation through reaction with reactive carbonyls during sample preparation. Delipidation was achieved by suspension of the seed powder in 10 ml of dichloromethane. Following incubation for 2 hr at room temperature (20 to 22 °C) under constant agitation, the dichloromethane was removed. This step was repeated three times, the last incubation step being performed overnight. The pellet was dried and weighted. Then the proteins were solubilized in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM nordihydroguaiaretic acid (2 ml per 50 mg of pellet). After the determination of protein concentration according to Bradford (1976), proteins were precipitated with cold acetone (1.5 ml of protein extract/7 ml of acetone). Finally, they were suspended, treated with dinitrophenylhydrazine to label carbonyls with dinitrophenylhydrazone (DNP), and separated on 1D or 2D gels as described (Job et al., 2005). After electrophoresis, proteins were either stained in the gels with Coomassie Brilliant Blue or transferred to polyvinylidene fluoride membranes and further probed with the anti-DNP antibody coupled to enhanced chemiluminescence detection of carbonylated proteins (Job et al., 2005; Korolainen, Goldsteins, Alafuzoff, Koistinaho, & Pirttila, 2002).

For the determination of global abundance and extent of protein carbonylation in seed extracts, the ratio of the intensities of protein luminescence (carbonylated soluble proteins) to Coomassie Blue staining (total soluble proteins) detected in the lanes of digitalized images of 1D gels and blots was calculated (Quantity One; Bio-Rad). For the determination of abundance and extent of carbonylation of individual spots, digitalized images of 2D gels and blots were analysed using the PD-Quest 7.1 software (Bio-Rad). After optimization of the parameters for background subtraction and spot detection (Boudet et al., 2006), volumes of the spots were normalized using total spot intensity. Then, the fold change was calculated between mature seeds and 32D seeds (seeds harvested at 32 DAP then dried). Student's *t* test was applied to determine significant differences in the fold change between mature and 32D seeds (Factor 2, *p* < 0.05), using gels and blots from three independent experiments.

2.3 | Identification of carbonylated proteins

Proteins were identified by peptide mass fingerprinting. For that purpose, spots of interest were excised from the 2D gels and washed with 100 µl of 25 mM NH₄HCO₃, followed by dehydration with 100 µl of 50% acetonitrile in 25 mM NH₄HCO₃. Proteins were reduced by incubation with 10 mM dithiothreitol (1 hr, 57 °C) and alkylated with 100 µl of 55 mM iodoacetamide (45 min at room temperature). Gel spots were further washed with NH₄HCO₃ and dehydrated as described above. The proteins were digested overnight at 37 °C by addition of 10 µl of trypsin (3 ng/µl; Promega, Madison, WI, USA). One microliter of the resulting peptide mixture was spotted onto a microtiter plate AnchorChip matrix-assisted laser desorption/ionization (MALDI) target plate (Bruker, Bremen, Germany). Deposits were covered with 1 µl of 1 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) as a MALDI matrix, diluted in a 4:1 (vol/vol) mixture of ethanol and acetone.

For the identification of proteins by peptide mass fingerprinting, measurements were performed on a MALDI time-of-flight/time-of-flight mass spectrometer (Autoflex III; Bruker, Bremen, Germany) equipped with a smartbeam laser (355 nm, 200 Hz). Positive ion spectra were recorded in reflector mode, on the *m/z* range 800–3,000. Fragmentation (mass spectrometry [MS]/MS) spectra were acquired on the most intense ions using the LIFT™ technology (Bruker, Bremen, Germany). MS profiles—completed by the MS/MS spectra acquired on selected *m/z* ions—were confronted to the *M. truncatula* contigs available at the J. Craig Venter Institute (release from March 25, 2011; 774,768 sequences; <http://www.jcvi.org/cms/home/>) using the Mascot server 2.2 program (Matrix Science). The mass tolerance was set at 50 ppm for parent ions (MS mode) and 0.8 Da for fragment ions (MS/MS mode). One missed cut per peptide was allowed. The carbamidomethylation of cysteine was set as a fixed modification, and the oxidation of methionine was set as a variable modification. Proteins were considered as valid on the basis of the significance threshold score provided by Mascot (*p* < 0.05). Protein identification by MS was performed within the Biopolymers, Structural Biology platform (BIBS, www.bibs.inra.fr) at INRA, Nantes (INRA UR1268 BIA, Nantes, F-44316 Nantes, France) by Hélène Rogniaux and Marion Hamon.

2.4 | Recombinant PM34 production

PM34, a protein carbonylated in *M. truncatula* seeds, was produced as a recombinant protein following the procedure previously described by Alkhalfioui, Renard, and Montrichard (2007). Total RNA from *M. truncatula* seeds, imbibed for 14 hr, was extracted with the RNeasy plant kit (Qiagen) and reverse transcribed using the Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer's instructions. Complete PM34 coding region was amplified by PCR using KOD HiFi DNA polymerase (Novagen) with a sense primer: gac gac gac aag ATG ACT ACT GGT GGA CAA and an antisense primer: ga gga gaa gcc cgg CTA CGC ATT CAC AAC AGT, harboring a 5' extension (in lower case) for an insertion in the pRSF2 vector. This vector was engineered to express target proteins fused to an N-terminal His-tag. After a preliminary denaturation step of 5 min at 94 °C, DNA amplification was performed during five cycles (94 °C × 1 min, 52 °C × 1 min, and 72 °C × 1 min) and 30 cycles (94 °C × 1 min; 72 °C × 1 min). A final elongation step was run for 10 min at 72 °C. The amplicon was purified with the Qiaquick gel extraction kit (Qiagen) and inserted into the plasmid pRSF2 of the ligation-independent Ek/LIC cloning kit (Novagen). The recombinant plasmid was introduced in Nova Blue *Escherichia coli* strain for multiplication and then subsequently transferred into BL21 (pLysS) cells for the production of the recombinant protein. Protein purification on Q-Sepharose and on Ni₂⁺-chelating Sepharose (Amersham) was carried out according to the manufacturer's instructions. Salts were finally eliminated through filtration on Centricons (exclusion 10 kDa) and buffer exchange.

2.5 | Cellulase activity

Cellulase activity of PM34 was tested according to Lin et al. (2009) using 1% agarose plates containing 0.2% carboxymethyl cellulose.

Two types of tests were carried out. In the first test, total proteins extracted from nontransformed bacteria (control) or the bacteria expressing PM34 were loaded on the gel. In the second test, purified bovine serum albumin or PM34 were used. After an incubation of the plate at 37 °C for 72 hr, the remaining carboxymethyl cellulose was stained with 0.2% (wt/vol) Congo red for 30 min. The plate was observed after several washes with 500 mM NaCl.

3 | RESULTS AND DISCUSSION

3.1 | Soluble protein patterns in maturing seeds

In the presently used growth conditions, maturation of *M. truncatula* seeds lasted from 28 to 48 DAP, 48 DAP corresponding to the fully mature seeds (Châtelain et al., 2012). We first examined the pattern of soluble proteins in maturing seeds (referred to as fresh seeds), resolved in 1D gels (Figure 1) or 2D gels (Figure 2) and stained with Coomassie Blue.

The left panel of Figure 1a shows that the protein pattern in the 1D gels remained almost the same from early maturation (28 DAP) to the end of maturation (48 DAP). This is also visible, when the protein patterns from 32 and 48 DAP seeds are compared in 2D gels (Figure 2). This result was expected as it was previously shown that during *M. truncatula* seed development protein synthesis mainly occurs before maturation (Châtelain et al., 2012). Among the few proteins that are still accumulated during maturation, some LEA proteins likely playing a role in seed longevity establishment have been found (Châtelain et al., 2012).

It is noteworthy that immature and mature seeds do not exhibit the same degree of physiological quality. Indeed, seed longevity is much shorter for immature seeds artificially dried than for fully mature seeds naturally desiccated (Châtelain et al., 2013). As an example, after controlled deterioration at 35 °C and 75% relative humidity for 9 days, only 22% of immature seeds harvested at 32 DAP survived, whereas 100% of the mature seeds survived under these conditions (Châtelain et al., 2013). Therefore, although *M. truncatula* seeds harvested at 32 and 48 DAP did not substantially differ by their protein composition, they might differ by the quality of their proteins and possibly by their level or mode of carbonylation, which could be responsible for the observed difference in seed quality.

3.2 | Global protein carbonylation state

To assess this above hypothesis, we characterized the extent of protein carbonylation in *M. truncatula* seeds during maturation. For immature seeds, the analysis was performed either before (fresh seeds) or after an artificial drying (dried seeds), to reach a water content similar to that of mature seeds (Châtelain et al., 2012). The protein carbonylation was monitored as previously described (Job et al., 2005). Carbonylated proteins were labelled with DNP, and the level of global protein labelling was assessed in mature seeds at 370 nm to determine whether it was in the range of values generally reported. Values of 3.7 and 6.2 nmol of carbonyls per mg of protein were obtained in two independent experiments. The mean value is in the range of values reported for mild oxidative conditions (Møller et al., 2011).

Carbonylated proteins were detected by Western blotting using the anti-DNP antibody and enhanced chemiluminescence. Figure 1b shows the results obtained with the 1D blots. It appears that a few proteins are carbonylated during early maturation (fresh seeds at 28 DAP), the number and the intensity of the bands corresponding to carbonylated proteins increasing progressively from early to late maturation. The global extent of protein carbonylation was determined for each seed extract by the ratio of the intensities of protein luminescence (carbonylated soluble proteins; Figure 1b) to Coomassie Blue staining (total soluble proteins; Figure 1a). This ratio shows that the level of protein carbonylation increased progressively during maturation with a marked difference of about twofold between 28 and 48 DAP (Figure 1c, fresh seeds in green). To see whether an anticipated drying had an impact on the extent of protein carbonylation, immature seeds artificially dried were analysed similarly. Here, the pattern of carbonylated proteins in seeds prematurely dried at 28 DAP and onwards was similar to that of mature seeds naturally desiccated, albeit some variations in band intensity could be observed (Figure 1b). Moreover, the global level of protein carbonylation, as expressed in percent of total proteins, was also found to be similar to that observed in mature seeds (compare dried seeds in grey in Figure 1c). These data also document that after a premature drying, the level of protein carbonylation increased twofold in seeds harvested at 28 DAP (Figure 1c). Collectively, the current data suggest that the observed protein carbonylation increase during natural desiccation or premature drying is presumably due to a flaw in the detoxification of ROS and/or in the repair of oxidative damage in cells of low water potential. Nonetheless, carbonylation appeared to target a common set of proteins whatever the mode of drying, with the exception of one protein band of about 17 kDa. This protein was found to be carbonylated only in dried seeds (Figure 1b). Note, however, that the protein bands detected in 1D gels or corresponding Western blots correspond to abundant proteins. Thus, a difference at the level of minor proteins cannot be excluded.

To further address this question, we compared the total soluble and carbonylated soluble protein patterns in 2D gels and corresponding Western blots (Figure 2). These patterns were very similar in the two seed extracts. Indeed, 49 and 52 major carbonylated protein spots were detected on the blots of 32D seeds and mature seeds, respectively. Thus, as in the 1D blots, the pattern of carbonylated proteins in the 2D blots is very similar and seems to differ only by the extent of carbonylation of some individual spots and the presence of few carbonylated spots in the acidic zone of the blots of 32D seeds, which are not visible on the blots of the mature seeds. However, the proteins responsible for these later signals could not be identified with certainty presumably because the blue spots in this zone were either too faint or too diffuse. We further verified whether the luminescent signals could be assigned with certainty to protein spots in 32D and mature seed protein patterns. This led us to select the 38 fluorescent spots depicted in Figure 2 for further analyses. Also, some spots appear as much highly carbonylated in 32D seeds (Nos. 8, 9, 10, 12, 13, 22, 23, 28, and 30) or in mature seeds (Nos. 34 and 35). We also note that contrary to the 1D analysis (Figure 1), no carbonylated spot around 17 kDa is observed for the 32D seeds (Figure 2). A possible explanation is that the 17 kDa protein has an isoelectric point below

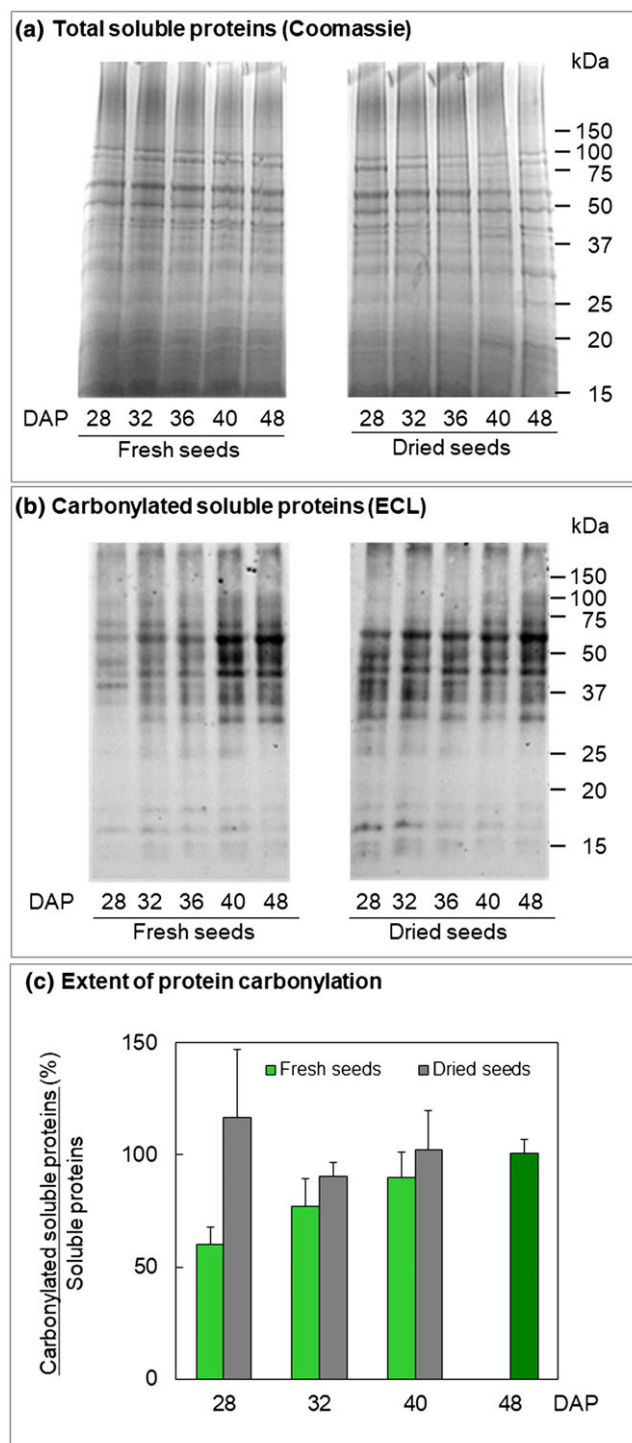


FIGURE 1 Proteins are carbonylated in *Medicago truncatula* seeds during natural desiccation or in case of an anticipated drying. Seeds were harvested during maturation from 28 to 48 days after pollination (DAP; referred to as fresh seeds), 48 DAP corresponding to fully mature seeds, and a part of immature seeds was artificially dried (referred as dried seeds) at 20 °C and 44% relative humidity for 3 days. Then, soluble proteins were extracted from fresh or dried seeds. (a) Pattern of total soluble proteins separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and stained with Coomassie Blue. (b) Pattern of carbonylated soluble proteins labelled with dinitrophenylhydrazine, after transfer onto polyvinylidene fluoride membrane and detection with an anti-dinitrophenylhydrazine antibody. (c) Extent of protein carbonylation in the soluble protein extracts determined by the ratio of the signal detected on Western blot normalized to the soluble proteins stained in blue. After drying, the water content in dried seeds was similar to that measured in mature and naturally desiccated seeds. There is a unique dark green bar in panel c corresponding to mature seeds harvested at 48 DAP, because they are both freshly harvested (fresh seeds) and dried but by natural desiccation (dried seeds). Three independent experiments were performed, using two different sets of maturing seeds. Analyses of variance were carried out using the GLM procedure of SAS software (V9.4). Significant effects between stages and/or between fresh and dried seeds were searched for the level of protein carbonylation. Mean comparisons were also performed using the Student–Newman–Keuls test. A significant twofold increase in the carbonylation level was found between (i) 28- and 48-DAP-old seeds and (ii) 28F and 28D for fresh and dried 28-DAP-old seeds ($p < 0.05$). ECL = enhanced chemiluminescence

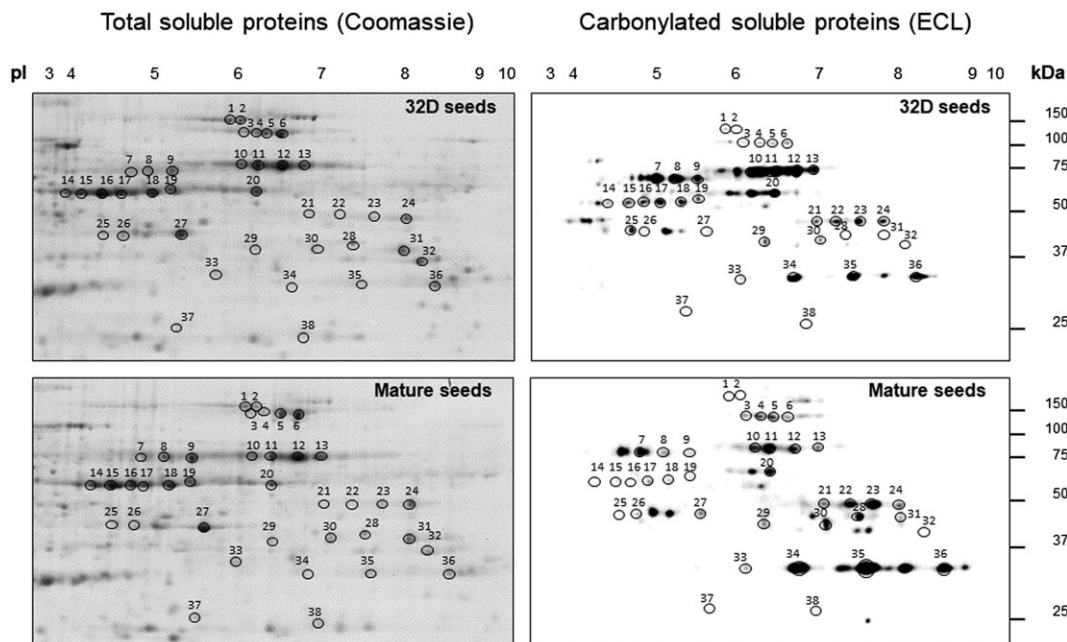


FIGURE 2 Proteins carbonylated in *Medicago truncatula* seeds prematurely dried or naturally desiccated are similar but differ by their extent of carbonylation. Soluble proteins were extracted from seeds harvested either at a mature stage (mature seeds) or at an immature stage (32 days after pollination) and artificially dried (32D seeds), labelled with dinitrophenylhydrazine and separated in two-dimensional gels. Left panels: pattern of the soluble proteins stained with Coomassie Blue. Right panels: pattern of carbonylated soluble proteins after transfer onto polyvinylidene fluoride membrane and detection with the anti-dinitrophenylhydrazine antibody. ECL = enhanced chemiluminescence

pH 3 or above pH 10 (i.e., outside of the pH zone analysed in 2D). Another possibility is that the signal is due to an artefact. Some artefacts in carbonylated protein detection were indeed reported to occur on Western blots (Luo & Wehr, 2009; Meyer, Baum, Vollmer, & Pischetsrieder, 2012).

3.3 | Identity of carbonylated proteins

Carbonylated proteins were functionally identified by peptide mass fingerprinting to gain clues about the physiological mechanisms underlying seed quality. All the numbered spots (Figure 2), with the exception of those already known to correspond to 1-Cys peroxiredoxin (1-Cys Prx; Spot Nos. 37 and 38; Alkhalifioui et al., 2007; Châtelain et al., 2012), were punched out from the gels for identification (Table S1). Although most of the spots could be matched by their electrophoretic mobility in different 2D gels, they were analysed in both seed extracts (32D and mature seeds) to better ascertain their identities. Among the 36 spots analysed, 12 spots gave no results (Table 1; not identified) very likely because the oxidative modification in these proteins was too massive for the generated tryptic peptides to be recognizable. In the remaining 24 spots, which gave only one significant protein hit, proteins were successfully identified either in one or in both gels corresponding to 32D and mature seeds (as indicated with a cross in Table 1). The matching spots gave almost the same results for 32D and fully mature seeds with the exception of Spot No. 7 that gave different results (Table 1). Collectively and taking into account that some spots contained the same protein, 13 different proteins—that is, having different names—were identified, some of them represented by different isoforms (Tables 1 and S1).

Among the proteins identified in this study, several corresponded to enzymes belonging to glycolysis: aldolase, glyceraldehyde phosphate dehydrogenase, and phosphoglycerate kinase; tricarboxylic acid cycle and associated: malate dehydrogenase and malic enzyme; carbon metabolism: ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco); amino-acid synthesis: ketol-acid reductoisomerase (KARI); antioxidant systems: 1-Cys Prx; and the small dehydrogenase reductase family (SDRs): PM34, also named glucose and ribitol dehydrogenase (Alexander, Alamillo, Salamini, & Bartels, 1994). We also identified different isoforms of vicilin (P54) and three LEA proteins, namely, PM10, previously named PM51 (Alkhalifioui, Renard, Vensel, et al., 2007), BP8 protein also named PM18 (Châtelain et al., 2012), and a maturation polypeptide. PM10, BP8, and the maturation polypeptide belong to the Group 4 (Cuevas-Velazquez, Reyes, & Covarrubias, 2017) of LEA proteins (Châtelain et al., 2012).

Interestingly, some of these proteins were also identified as carbonylated proteins in pea when seeds were treated with H₂O₂ to accelerate their germination (Barba-Espín et al., 2011). This suggests that their carbonylation is required for the success of germination. Carbonylation being thought to be a denaturing modification, it may trigger loss of function. Inhibition of the glycolytic pathway is thought to favour the pentose phosphate pathway that is involved in the breaking of seed dormancy (Bewley & Black, 1994) and sustains early germination (Job et al., 2005). This pathway probably provides NADPH necessary to awake the metabolism (Buchanan & Balmer, 2005). A potential alteration of the glycolytic pathway in both 32D and mature *M. truncatula* seeds may also contribute to decrease the concentration of triose phosphates, which could otherwise be transformed in oxidative conditions in the highly reactive methylglyoxal carbonyl. Rubisco activity can lead to an accumulation of 3-

TABLE 1 Carbonylated proteins identified in maturing seeds

Spot no.	Name	32D seeds	Mature seeds	Gene number	Protein MW (Da)	Fold	p value
1	Not identified					0.9	0.306
2	P54-1	x	x	1g071710	97,082	1.3	0.342
3	Not identified					0.9	0.380
4	Not identified					0.8	0.331
5	Not identified					3.1	0.023
6	Malic enzyme	x	x	8g102620	68,722	1.3	0.194
7	P54-1	x		4g079780	64,820	0.6	0.071
7	Ketol-acid reductoisomerase		x	1g071710	56,443		
8	PM10 (LEA)	x	x	4g123950	58,282	1.1	0.413
9	PM10 (LEA)	x	x	4g123950	58,282	2.6	0.046
10	P54-1		x	1g071710	56,443	2.3	0.112
11	P54-1	x	x	1g071710	56,443	1.5	0.041
12	Not identified					1.4	0.175
13	P54-1	x		1g071710	56,443	1.8	0.080
14	P54-2	x	x	7g079730	53,461	0.2	0.000
15	P54-2		x	7g079730	53,461	0.2	0.001
16	P54-2	x	x	7g079730	53,461	0.2	0.000
17	P54-3	x	x	7g079820	55,121	0.9	0.180
18	P54-3	x	x	7g079820	55,121	0.9	0.283
19	P54-4	x	x	7g079740	54,585	1.1	0.329
20	Rubisco	x	x	7g021770	53,239	1.0	0.470
21	Not identified					0.6	0.079
22	Not identified					2.1	0.023
23	Not identified					1.7	0.050
24	Not identified					1.5	0.168
25	Not identified					0.6	0.028
26	Phosphoglycerate kinase	x	x	2g066120	43,104	0.4	0.093
27	BP8 (LEA)	x	x	Contig_83897	38,579	2.3	0.035
28	Fructose-bisphosphate aldolase	x	x	5g069050	40,125	2.1	0.073
29	Malate dehydrogenase	x		1g043040	39,438	1.2	0.367
30	Not identified					1.5	0.062
31	Maturation polypeptide (LEA)	x		1g061730	43,905	1.4	0.161
32	Glyceraldehyde phosphate dehydrogenase	x		4g103920	40,715	1.9	0.088
33	PM34-1	x	x	4g131740	32,249	1.7	0.090
34	Not identified					2.4	0.009
35	PM34-2	x	x	7g113650	32,995	4.2	0.035
36	PM34-2	x		7g113650	32,995	1.0	0.474
37	1-Cys peroxiredoxin	x	x			3.7	0.197
38	1-Cys peroxiredoxin	x	x			3.0	0.050

Note. Spots of carbonylated proteins from 32D and mature seeds of *Medicago truncatula*, separated in 2D gels as depicted in Figure 2, were punched out for identification by peptide mass fingerprinting (successful identification in each seed extract is indicated by a cross). Significant fold change in carbonylation is indicated in bold. Calculations were effected from 2D blots of carbonylated proteins from three independent experiments, using two different sets of maturing seeds (Table S2). 32D seeds = seeds collected at 32 days after pollination then dried; Fold = ratio of the normalized volume of carbonylated spots (normalized to the total volume in the 2D blots) for mature seeds/32D seeds; Gene number = identification in Medicago database (<http://www.jcvi.org/cms/home/>); LEA = late embryogenesis abundant; Mature seeds = mature seeds harvested at 48 days after pollination; Name = name of the protein; p value = p values of the fold change values (Student's t test, $p < 0.05$); Protein MW = protein molecular weight; Rubisco = ribulose-1,5-bisphosphate carboxylase/oxygenase; Spot no. = spot number on the gels and blots of Figure 2.

phosphoglycerate, which could be converted in triose phosphates. Rubisco carbonylation might thus represent another way to limit the accumulation of triose phosphates and thereby methylglyoxal in both 32D and mature *M. truncatula* seeds. The carbonylation of KARI identified in Spot No. 7 as a carbonylated protein only in 32D seeds in this study may alter the accumulation of branched chain amino acids.

Interestingly, disruption of the Val degradation pathway was reported to affect physiological seed quality in *A. thaliana* (Gipson et al., 2017). However, there were two different proteins identified in this Spot No. 7 (Table 1). Therefore, it is difficult to ascertain that detection of this carbonylated Spot No. 7 was due to the carbonylation of KARI or of the other protein identified within this spot (P54-1; Table 1).

Regarding the LEA proteins, they may be carbonylated while forming shields around the protein targets they protect, as do the heat shock proteins (Cabisco, Piulats, Echave, Herrero, & Ros, 2000). LEA proteins are thought to play an important role in the protection of soluble proteins against various abiotic stresses (Tunnacliffe & Wise, 2007). Thus, one can imagine that a premature drying, which would impair the complete accumulation of these protective proteins, would have as a consequence the carbonylation of some soluble proteins due to a lack of protection. Such an event would otherwise occur to a lower extent in mature seeds, once proteins are protected by LEA proteins. Regarding 1-Cys Prx, its abundance has been shown to alter seed quality, either negatively as in *A. thaliana* (Haslekås et al., 2003) or positively as in *Nelumbo nucifera* seeds (Chen et al., 2016). Carbonylation of this protein may therefore contribute to the expression of seed quality in *M. truncatula*.

3.4 | Extent of carbonylation of individual proteins

To help discriminating among the carbonylated proteins those for which carbonylation is required for seed quality establishment from those for which this modification is detrimental, we determined the level of protein carbonylation in 32D and mature seeds. We first compared the abundance of the 38 selected proteins in 32D and mature seeds using the volume of the spots in blue pixels in the corresponding gels and Student's *t* test (see the fold change column in Table S2). The abundance of six spots was found to change significantly (Factor 2, $p < 0.05$), being more abundant in mature seeds than in 32D seeds, the three spots of LEA proteins (Spot Nos. 8 and 9 [PM10] and Spot No. 27 [BP8]), the Spot Nos. 18 and 19 (P54), and the Spot No. 23 (not identified). The results regarding PM10 and BP8 are in accordance with those reported by Châtelain et al. (2012) showing an accumulation of these two proteins during late maturation.

Then, the extent of carbonylation of the 38 selected spots in 32D and mature seeds was compared by the volume of luminescent spots in the corresponding blots (Table S2). This allowed distinguishing two categories of spots: spots whose carbonylation level did not change significantly between 32D and mature seeds and spots whose carbonylation level was significantly different (Factor 2, $p < 0.05$), such as Spot Nos. 5, 9, and 14–16 (P54), Spot Nos. 22, 23, and 27 (BP8), and Spot Nos. 34 and 35 (PM34; see the fold change column in

Tables 1 and S2). The level of carbonylation was higher in 32D seeds than in mature seeds for the Spot Nos. 14–16 (P54) but lower for all the other spots, notably Spot No. 35 (PM34).

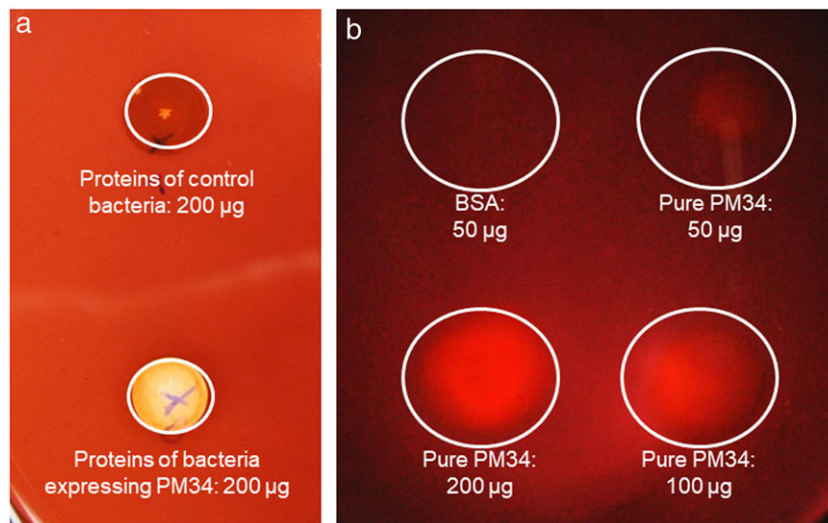
3.5 | Glucose/ribitol dehydrogenase and cellulase activities of PM34

PM34 caught our attention because although present as two isoforms in three spots (Nos. 33, 35, and 36), it was not an abundant protein as judged by Coomassie Blue staining, and it proved to be highly sensitive to carbonylation, the Spot No. 35 exhibiting notably a marked carbonylation increase (4.2-fold) during maturation (Tables 1 and S2). In plants, this protein is encoded by a small number of genes whose coding sequence is highly conserved. It specifically accumulates in seeds of several species. Furthermore, in rice, its mRNA, stored in the dry seed, is translated during germination (Sano et al., 2012), suggesting that this protein has a function in rice seed quality. In *M. truncatula* genome, three genes of PM34 were found, which are specifically expressed during maturation with a high pic of messenger accumulation at 24 and 36 DAP (<https://mtgea.noble.org>). The protein was also found to be present in maturing seeds as two isoforms (PM34-1 and PM34-2; Table 1 and Figure 2) that were also found to be present in germinating seeds, but under a reduced form (Alkhalifiou, Renard, Vensel, et al., 2007). The three isoforms of the protein share 70% to 86% of identity (Figure 3) and harbour two conserved residues (Cys59 and Cys113). The exact function of PM34 in dicotyledonous seeds has not been demonstrated yet. Because PM34 presents some homology with glucose and ribitol dehydrogenase found in bacteria, a glucose dehydrogenase activity was proposed for this protein in wheat embryos (Alexander et al., 1994). However, this enzyme activity could not be demonstrated in sugar beet seeds (Catusse et al., 2011). Using PM34 as a recombinant protein (see Section 2), we found that, as in sugar beet, *M. truncatula* PM34 was not active on monosaccharides such as glucose, galactose, and mannose and alcohols such as ribitol (results not shown). It is interesting however that high concentrations of ribitol characterize a strategy of desiccation tolerance in lichens (Sadowsky, Mettler-Altmann, & Ott, 2016) and green algae (Holzinger & Karsten, 2013). Furthermore, it is interesting to observe that barley lines exhibiting salinity tolerance during seed germination expressed a high level of glucose/ribitol dehydrogenase (Witzel



FIGURE 3 Alignment of *Medicago truncatula* PM34 protein sequences. The protein sequences deduced from the coding regions of PM34-1 (Medtr4g131740), PM34-2 (Medtr 7g113650), and PM34-3 (Medtr 1g082760) were aligned. Identical and conserved amino-acid residues appear in red and blue, respectively. Residues that are identical in two similar sequences appear in green

FIGURE 4 *Medicago truncatula* PM34 has a cellulase activity. (a) Soluble proteins (200 μ g) extracted from control bacteria or bacteria overexpressing PM34 were loaded on an agarose gel containing carboxymethyl cellulose. (b) Bovine serum albumin (BSA; 200 μ g), used as a control, or different quantities of purified PM34 were loaded on the gel. After an incubation for 72 hr at 37 °C in the dark, the remaining carboxymethyl cellulose was stained with Congo red. The white areas correspond to the zones where carboxymethyl cellulose has been digested by PM34



et al., 2010). In agreement, this protein was detected in imbibed salt-stressed alfalfa seeds (Yacoubi et al., 2013). Also, Nosarzewski, Downie, Wu, Douglas, and Archbold (2012) pointed out a role of the ribitol conversion to ribulose or sorbitol in *A. thaliana* during recovery from drought stress, whereas Aharon and O'Connell (2002) reported that glucose and ribitol dehydrogenase is involved in desiccation tolerance of strawberry achenes. It is therefore tempting to suggest that the expression of a PM34 active towards alcohols as ribitol could afford an osmoprotective function important for maintenance/expression of desiccation tolerance in *M. truncatula* seeds, although we were not able to measure this activity with the recombinant protein.

Interestingly, PM34 seems highly related to an enzyme of the SDR superfamily of enzymes that has been described as being able to eliminate reactive carbonyls in *A. thaliana* (Yamauchi, Hasegawa, Taninaka, Mizutani, & Sugimoto, 2011). We tested whether PM34 could have a similar function in *M. truncatula* seeds. However, the recombinant PM34 was not active on carbonyls that could accumulate in seeds (Kranner et al., 2010; Møller et al., 2011) such as acrolein, glyoxal, methylglyoxal, butyraldehyde, or nonenal (data not shown).

Because PM34 also shares some similarities with a SDR possessing a cellulase activity in fungi (Schuster, Kubicek, & Schmoll, 2011), we tested the hypothesis that this protein could have such an activity in *M. truncatula* seeds. For this purpose, we used carboxymethyl cellulose immobilized in agarose plates (see Section 2). After the action of PM34 or control proteins, the remaining carboxymethyl cellulose was stained with Congo red (Figure 4). The results allowed establishing that recombinant PM34 possesses a cellulase activity, responsible for the disappearance of carboxymethyl cellulose in the agarose plate (Figure 4). What might be the role of the PM34 cellulase in seed quality? One possibility would be that full cell wall disassembly and tissue weakening in the endosperm cap that is necessary for radicle protrusion may require the action of enzymes such as the PM34 cellulase. Alternatively, cellulase activity could be required for cell elongation that triggers radicle elongation at the time of germination then organ growth, through cell wall loosening. Furthermore, as this protein is found in the current study to be highly carbonylated in desiccated seeds, it could be that the maintenance of the PM34 cellulase in a carbonylated and inactive state during seed

maturation is a mean to prevent the seed to commit to premature germination until the most favourable conditions are met.

However, further experiments, notably by gene inactivation or overexpression, are needed to establish the role of this enigmatic protein in seed quality.

4 | CONCLUSIONS AND PERSPECTIVES

Thanks to pioneering work in *A. thaliana* and sunflower, we already knew that proteins are carbonylated in mature seeds and that the level of protein carbonylation increases with seed storage and ageing (Job et al., 2005; Oracz et al., 2007; Rajjou et al., 2008). Here, we document that protein carbonylation mainly occurs at the end of development in seeds of *M. truncatula* (from 28 to 48 DAP), accompanies the water removal during natural desiccation and concerns only a set of specific proteins. Thus, it does not impair the acquisition of desiccation tolerance and longevity, two components of seed quality, which are acquired at the same time (Châtelain et al., 2012). Although protein carbonylation may not be a detrimental process when it occurs during natural desiccation, it could be harmful for seeds prematurely dried. An explanation could rely on the extent or the mode of carbonylation in specific proteins. Proteins that play an important role in seed physiology may be highly carbonylated in seed prematurely dried but not in seeds naturally dried at maturity. For example, they would not be protected in immature seeds because protection and antioxidant systems are not fully established at this stage, whereas they would be protected in mature seeds when these systems are fully in place. Because enzymes eliminating reactive carbonyls during maturation contribute to seed viability and quality (Nisarga et al., 2017; Shin, Kim, & Gynheung, 2009), reaction of proteins with reactive carbonyls should be reduced in mature seeds as compared with immature seeds. In contrast to protein carbonylated by ROS, proteins carbonylated by reactive carbonyls are resistant to proteolysis (Berlett & Stadtman, 1997). Aggregates of these proteins may impair proper cell metabolism functioning in seeds prematurely dried. Most importantly, although carbonylation is commonly thought to be an irreversible modification

of the proteins that lead to denaturation and loss of function, the impact of carbonylation on protein stability or function has not been assessed in most of the cases (Møller et al., 2011). In addition, a process of protein decarbonylation has been reported (Wong, Marcocci, Liu, & Suzuki, 2010) rendering direct carbonylation reversible (Wong, Bansal, Marcocci, & Suzuki, 2012).

Thus, one cannot exclude that protein carbonylation has different roles in seeds depending on the mode of carbonylation and the targets. During seed maturation, protein carbonylation probably contributes to slow down metabolic activity by modifying or denaturing enzymes such as those involved in glycolysis as already suggested (Job et al., 2005). PM34 cellulase may belong to this category of enzymes. In addition, carbonylation may also participate to the regulation of protein turnover, contributing to elimination of proteins at the end of maturation that are no longer necessary for the next programme of seed biology, that is, germination, allowing by this way a switch in metabolism.

In the model legume *M. truncatula*, the duration of the maturation phase is the most influenced by environmental conditions, although the duration of this phase is crucial for subsequent seed and seedling performance (Righetti et al., 2015). This is also true and long known for seeds of legumes crops (Ellis et al., 1987; Sanhewe & Ellis, 1996). Thus, a possibility to maintain crop legume seed quality in Europe is to sow earlier in the season or northwards and thereby limit the drought episodes at the end of their biological cycle that will become more frequent as a consequence of climate change (Alqudah, Samarah, & Mullen, 2011; Gray & Brady, 2016). Another possibility is to select varieties having versions of crucial proteins less sensitive to carbonylation due to their structure or their abundance. PM34 being highly sensitive to carbonylation could be a marker of the events encountered by the seeds during their development and storage. PM34 abundance could also be used as a marker to assess or to improve crop seed quality.

In future work, it would be of interest to test whether PM34 has a glucose/ribitol dehydrogenase activity depending on its redox state. It would also be interesting to analyse the impact of an extinction or an overexpression of PM34 genes on seed physiology and quality. In *M. truncatula*, as mentioned above, there are only three genes that are highly identical. A RNAi vector targeting the three genes could be used to generate a deficient mutant. It would finally be interesting to compare the mode of carbonylation of proteins, including PM34, in seeds either prematurely dried or naturally desiccated. This could be done by immunoblotting using antibodies raised against several reactive carbonyls such as malonaldehyde and hydroxynonenal (Mano, Nagata, Okamura, Shiraya, & Mitsui, 2014).

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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