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Seed coat lignification level is crucial in *Capsicum spp* seed longevity

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Abstract

Capsicum (pepper) is known for its poor seed germination, particularly in terms of seed longevity, which is usually much shorter than other Solanaceae. However, the molecular mechanisms involved are mostly unknown in these species. The present study examines the differences in seed longevity among *Capsicum* species and varietal types. Feral or less domesticated species, such as *C. chinense* and particularly *C. frutescens*, showed higher germination rates than the more domesticated *C. annuum* after accelerated seed aging treatments. In addition, variability was detected in the expression of genes involved the response to seed deterioration. The differences observed in *ASPG1* expression led us to study the seed protein profile in dry and germinating seeds. Seed storage protein mobilization during germination was faster in seed aging-resistant genotypes. Similarly, the transcriptional change observed for the orthologous gene of the trans-species regulator *AtHB25*, prompted us to study the structure and molecular components of the seed coat in peppers. All the *Capsicum* pepper accessions analyzed presented very lignified testa and we observed a positive correlation between the amount of this polyphenolic compound and seed viability. Our results provide essential information to explain the poor germination observed in pepper seeds and provides an experimental framework for future improvements in this important character.

Introduction

Seeds are necessary for survival of spermatophytes since they are part of the reproduction process of this clade. Germination will only occur when the environmental conditions are optimal and thus the seed must sometimes remain viable for long periods of time. However, this viability in not unlimited and the embryo may perish before optimum conditions occur. The maximum time that a seed can remain viable is variable among species and even between varieties. This parameter is also dependent on external biotic and abiotic factors and it is named seed longevity (Rajjou and Debeaujon 2008; Wang et al. 2018).

Seed longevity in the mostly widely employed and modern varieties of *Capsicum* peppers, i.e. blocky and fleshy types, is known for its short duration. In fact, seeds from this genus lose viability faster than other Solanaceae like tomatoes or eggplants, and seedbanks and breeders usually need to renew accessions every 4-5 years (Adebisi and Abdul-Rafiu, 2016; Priestly, 1986; Yildrim et al. 2020). Nevertheless, *Capsicum* peppers are very diverse genetically and differences in germination parameters can be found among varieties (Bosland and Votava, 2012; Yildirim et al. 2020).

In this regard, there are five cultivated species in the genus *Capsicum* and about 30 wild species originating from America (Pereira-Dias et al. 2019). Within the domesticated taxons, three species, namely *C. annuum* L. var. *annuum*, *C. chinense* Jacq. and *C. frutescens* L. are included in the *annuum* botanical complex and are the most important species deriving from America (DeWitt and Bosland, 2009, Pereira-Dias et al. 2019). Furthermore, they represent a range of domestication levels and seed germination capacity. Thus, *C. annuum* is the most economically important species and an intense domestication and breeding effort has been made to adapt this species to a plethora of agroclimatic conditions and culinary uses, due to its taste and flavour (Moreno-Peris et al. 2020 and Rodríguez-Burruezo et al. 2010). By contrast, several authors consider *C. frutescens* as a semi-domesticated taxon and, in fact, this genotype may grow in nature without assistance from growers (Bosland and Votava, 2012). Finally, *C. chinense* can be considered as an intermediate case, with many varietal types derived from domestication and breeding, but to a lesser extent than *C. annuum*. Moreover, this species displays reduced germination capacity (Bosland and Votava, 2012; Mavi, 2018; Pereira-Dias et al. 2019; Russo 2012). Thus, the knowledge about the seed viability of the different *Capsicum* species is limited and studies investigating the molecular mechanisms involved are scarce.

Several mechanisms involved in seed longevity have been well-studied in model plants, such as *Arabidopsis thaliana*. Two main strategies have been identified: protection and repair. It has been demonstrated that DNA, lipid and protein repair systems are crucial. For instance, it is known that the prevention of lipid oxidation increases seed viability, as observed for the germination of seeds produced by mutant plants lacking the homogentisate phytyl transferase (*VTE2*) gene, involved in tocopherol biosynthesis. These plants display a reduction in the level of vitamin E and produce seeds with reduced viability (Sattler et al. 2004). Another example is the L-isoaspartyl methyltransferase (PIMT) protein, a repair enzyme that catalyses the conversion of L-isoaspartyl residues, detrimental for protein folding and activity, to L-aspartyl forms. PIMT1 over-accumulation increases both seed longevity and germination capacity (Ogé et al. 2008).

Regarding protection, the seed coat is the first barrier against the adversities of the environment (Mohamed-Yasseen et al. 1994). The testa isolates the embryo from many types of stress, such as UV radiation, temperature, moisture, oxygen, attack by pathogens and predators, among others. Therefore, its structure and composition are key factors in determining the longevity of the seed (Rajjou and Debeaujon, 2008). The seed coat begins to form by the differentiation of ovule integuments in the early stages of embryo development. At the end of seed maturation, the testa acquires the proper consistency by accumulating biopolymers. The most important polymers found in the testa are lignin, polysaccharides, suberin and cutin (Sano et al. 2016). Suberin is a lipid polyester located on the external part of the seed coat in some species. It confers impermeability and it has been demonstrated to play an important role in protecting the embryo in Arabidopsis (Renard et al. 2020, 2021). The mucilage, missing in pepper, is a viscous polysaccharide whose hygroscopic properties allow it to absorb and maintain water around the seed, providing favorable conditions for germination. The cuticle is associated with the endosperm in numerous species, where it regulates permeability to external compounds and therefore affects seed viability (De Giorgi et al. 2015). Finally, lignin is formed in the spaces between the cellulose microfibrils by the oxidative coupling of free lignin monomers secreted directly into the plant cell wall. The canonical monolignols are the non-methoxylated p-coumaryl alcohol, the monomethoxylated coniferyl alcohol and the dimethoxylated sinapyl alcohol that form H- (hydroxyphenyl), G- (guaicyl) and S- (syringyl) units in the lignin polymer, respectively. However, seed coat lignins also include the non-canonical C- (caffeyl) units which derive from an unusual monomer, caffeyl alcohol. Seed coat cells develop heavily lignified secondary cell walls to reinforce the outer surface of the seed mechanically and to make it impermeable to liquids and gasses (Barros et al. 2015). Thus, a loss-of-function mutant in the Arabidopsis *AtLAC15* gene, encoding a laccase involved in seed lignin synthesis, presents higher seed coat permeability (Liang et al. 2006).

On the other hand, many transcription factors have been described to participate in the regulation of the different developmental stages and layers of the testa (Haughn and Chaudhury, 2005), but not many of them have been found to be involved in seed longevity. AtHB25 is a member of the homeobox family and it is considered as a trans-species regulator of seed viability through the regulation of the expression of both gibberellin and lipid polyester biosynthesis enzymes (Bueso et al. 2014 and Renard et al. 2021).

Finally, in addition to the protection and repair mechanisms, it has been reported that the efficiency in seed protein storage mobilization is crucial for seed germination over time. For instance, a positive correlation between the expression of the aspartic protease *ASPG1* gene and seed viability has been demonstrated (Shen et al. 2018).

This work explores the variability of the seed longevity among *Capsicum* peppers and, to our knowledge, this is the first study aimed at analysing the genes and mechanisms involved in this trait in *Capsicum* peppers, which is of paramount interest for plant physiologists, geneticists, breeders and seedbank managers.

Materials and methods

Plant material and growth conditions

Nine accessions of different species from *Capsicum* genus were obtained from the *Capsicum* breeding team of the Instituto Universitario de Conservación y Mejora de la Agrodiversidad Valenciana (Valencia, Spain). The *C. annuum* accessions were Bola, Pasilla, Piquillo, Chile Serrano and California Wonder breeding line 286.12.1. The *C. chinense* accessions were Habanero and Ecu-994. The *C. baccatum* accession was Bol-58 and the *C. frutescens* accession was Bol-144. This collection encompassed a range of different geographical origins and morphological traits. In order to work with new and standardized lots of seeds, plants from all the accessions were grown in greenhouses throughout the experiment. To achieve this, seedlings were transplanted in the COMAV greenhouses, located at the Campus de Vera of the Universitat Politècnica de València, 50-60 days after sowing. The cultivation was carried out in 10 L pots with coconut fibre in the spring-summer season, since this period is the best weather for pepper growth and reproduction for the Mediterranean climate (Nuez et al. 2003). In the greenhouse, the temperature was about 18-30˚C and plants grew under natural photoperiod. Plants were pruned in four stems and supported with strings to facilitate the vertical growth. Drip irrigation was applied every 8 hours for 3 min (4L/h) with 1 g/L of a commercial fertiliser 15N-2,2P-24,9K (BASF, Barcelona, Spain) diluted in the water.

Accelerated aging treatment and germination studies

Seeds were harvested when the fruit was at the fully ripe stage, so the seeds of all accessions were harvested at the same physiological maturity. The accelerated aging treatment was performed with hydrated seeds and 100% relative humidity (RH) atmosphere at 41ºC during 48h. Seeds contained 0.05–0.8 g H_2O/g dry weight before treatment and 0.13-0.16 g H_2O/g dry weight after treatment. After the treatment, seeds were grown and germinated on Murashige and Skoog (MS) plates with 1% sucrose (w/v), 10 mM MES and 1% agar. The pH was adjusted to 5.7 with Tris buffer (Renard et al. 2020). All germination analyses were performed after 7 days of sowing with using around 100 seeds per biological replicate. The experiments were repeated four times. The results are the average of these four experiments with 100 seeds per line. A Student's t test was used to analyse the significant differences ($P < 0.05$).

Orthologous gene identification and primer design

To identify orthologous pepper genes corresponding to Arabidopsis genes implicated in seed longevity, we utilized Plaza by Ghent University. More specifically, we used the Integrative Orthology Viewer (https://bioinformatics.psb.ugent.be/plaza). This software selects the orthologous gene of a species after performing four tests, "Tree-based ortholog", "Orthologous gene family", "Anchor point" and "Best-Hits-and-Inparalogs" (Van Bel et al. 2018). As shown in Table 1, we identified 13 unique candidate orthologs in *Capsicum*. However, *ABI3*, a gene essential for seed maturation, showed two potential candidates in *Capsicum*. The genes analysed were: *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), *ABA INSENSITIVE 5* (*ABI5*), *HOMEOBOX PROTEIN 25* (*HB25*), *PROTEIN-L-ISOASPARTATE METHYLTRANSFERASE* (*PIMT1*), *SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 2-6* (*SnRK2.6*), *8-OXOGUANINE-DNA GLYCOSYLASE 1* (*OGG1*), *VITAMIN E DEFICIENT* 1 (*VTE1*), *VITAMIN E 2* (*VTE2*), *LEAFY COTYLEDON 1* (*LEC1*), *LATE EMBRYOGENESIS ABUNDANT 14* (*LEA14*), *ASPARTIC PROTEASE IN GUARD CELL 1* (*ASPG1)*, *GALACTINOL SYNTHASE 2* (*GOLS2)* and *FUSCA3* (*FUS3)*. To design primers to amplify the cDNA, we identified the sequences from orthologous genes in *Capsicum annuum, Solanum tuberosum and Solanum lycopersicum* in Plaza as well. The genomic information of *Capsicum chinense* was found in Sol Genomics (https://solgenomics.net/) doing a BLAST (Basic Local Alignment Tool). A BLAST search was done for each exon using the exons of the orthologous *C. annuum* genes previously found in Plaza. We performed the multiple alignment of the coding sequences (CDS) using ClustalW (gap penalty=8) (https://www.genome.jp/tools-bin/clustalw) to find the most conserved regions where primers were designed. As shown in Figure S1, primers were designed in the most conserved sequence of the gene with no nucleotide changes, and when this was not possible, degenerate primers were designed. Table S1 shows the direct and reverse primers designed for each gene. Before carrying out a differential gene expression analysis, we checked the primer efficiency in the three accessions and all were comprised between 96% and 120%. The R^2 value ranged from 0.99 to 1.00.

Seed RNA extraction and RT-qPCR

The extraction of seed RNA was performed at a specific seed development stage: advanced cotyledonary (Manzur et al. 2015). This stage was at 30 DAF (days after fertilization) in the Piquillo accession and 25 DAF in Bol-144 and Ecu-994 accessions. The method utilized is described in Oñate-Sánchez and Vicente-Carbajosa (2008). Subsequently, RNA was purified with the Nucleo spin kit (Macherey-Nagel). About 1000 ng RNA were reverse transcribed using the Maxima first-strand cDNA synthesis kit for qRT-PCR (Thermo Fisher Scientific) according to the manufacturer's instructions. Each reaction was performed in triplicate in a total volume of 20 µl. qRT-PCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific) with the 5 PyroTaq EvaGreen qPCR Mix Plus (ROX; Cultek S.L.U., Madrid, Spain) according to the manufacturer's protocol. Data are the mean of three biological samples and relative mRNA abundance was calculated using the comparative ΔCt method described in Pfaffl (2001). The housekeeping gene utilized was the pepper orthologue of the tomato Ef1-alpha (López-Gresa et al. 2017). Standard curves were performed to calculate the efficiency of the reaction from a series of primer dilutions and a mixture of cDNA from each accession. Four-point standard curves of a dilution series (1:1, 1:5, 1:25 and 1:125) were utilized to calculate the R^2 value and primer efficiency.

Protein extraction and SDS-PAGE

Proteins were extracted from dry seeds and seeds 6 days after imbibition (50 mg). Seeds were frozen in liquid nitrogen in a mortar and were ground into powder with a pestle. The powder was quickly and thoroughly suspended in 400 µL of PBS with the protease inhibitor cocktail (cOmplete, Roche Ref. 04693159001). Following centrifugation for 15 min, the supernatant was transferred to a new tube and the protein content was quantified using the Bradford method. Extracted proteins (20 µg) were resolved on a 12% SDS-PAGE gel. The gel was stained using Coomassie Blue R-250.

Histological staining

Seeds were hydrated and fixed in a solution of 2.5% paraformaldehyde and 0.5% glutaraldehyde, as described by Karnovsky (1965). Before the infiltration in LR White, the seeds were washed in phosphate buffer, post-fixed with 2% osmium, washed with water and dehydrated using an ethanol series (30%, 50%, 70%, 90%). The infiltration was carried out with mixes of 100% resin and ethanol (2 parts EtOH 90% + 1 part resin, 1 part EtOH 90% + 2 parts resin, 1 part EtOH + 2 parts resin). After complete polymerization in capsules in the absence of oxygen at 55/60°C, LR White embedded materials were sectioned with a Leica RM2125RTS microtome to obtain 4 µm sections.

Sections were stained with 0.1% toluidine blue O (w/v) in distilled water. Sections (16 µm of thickness) were generated with Microm HM 520 Cryostat and stained with phloroglucinol (3% in ethanol) and mixed with one volume of HCl (37N).

Lignin quantification

Polyphenolic compounds were extracted from seeds of each accession (8 replicates) with acetyl bromide, as described in Moreira-Vilar et al. (2014). Briefly, a protein-free cell wall sample (20 mg) was digested with 25% acetyl bromide (v/v in glacial acetic acid) and incubated at 70ºC for 30 min. Then, the sample was quickly cooled in an ice bath and mixed with 0.9 ml of 2 M NaOH, 0.1 ml of 5 M hydroxylamine-HCl, and 1 volume of glacial acetic acid. After centrifugation, the absorbance of the supernatant was measured at 280 nm.

Tocopherol analysis

The quantification of endogenous levels of tocopherols in pepper seeds was performed at the metabolomics facility of the Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC, Valencia, Spain) as previously described by Stahl et al. (2019) with slight modifications. Briefly, 50 mg of seeds per sample were homogenized with liquid nitrogen and extracted in 1.4 mL 100% methanol supplemented with 1.4 µL of internal standard (1 mg/ml nonadecanoic acid methylester in $CHCl_3$) for 15 min at 70 °C. The extract was centrifuged for 10 minutes at 14000 rpm. The supernatant was transferred to a glass vial and 1 ml of $CHCl_3$ and 1ml of water were added. The mixture was vortexed for 15 seconds and centrifuged for 15 minutes at 14000 rpm. 600 µl of the lower organic phase were dried in vacuum for 6–16 h. For derivatisation, dry residues were redissolved in 70 µL MSTFA (N-methyl-N-[trimethylsilyl]trifluoroacetamide) with 6 µL of a retention time standard mixture (3.7% [w/v] mix of fatty acid methyl esters ranging from 8 to 24C) and incubated for 30 minutes at 37 ºC. Sample volumes of 2 µL were injected in splitless mode in a 6890 N gas chromatograph (Agilent Technologies Inc. Santa Clara, CA) coupled to a Pegasus 4D TOF mass spectrometer (LECO, St. Joseph, MI). Gas chromatography was performed on a BPX35 (30 m × 0.32 mm × 0.25 µm) column (SGE Analytical Science Pty Ltd., Australia) with helium as the carrier gas at a constant flow of 2 ml/min. The liner was set at 230 °C. The oven program was 85 °C for 2 min, 8 °C/min ramp until 360 °C. Mass spectra were collected at 6.25 spectra s^{-1} in the *m/z* range 35–900 and ionization energy of 70 eV. Chromatograms and mass spectra were evaluated using the CHROMATOF program (LECO, St. Joseph, MI). The absolute contents of the three tocopherols were calculated using a commercially available tocopherol mix (Sigma ref. W530066).

Statistical analysis of the results

The mean and the standard error (SE) were calculated and differences of the means were considered to be statistically significant when $P<0.05$ using a Student's t test. All the experiments were repeated at least three times.

Results

The analysis of germination after accelerated aging treatments reveals variability in seed longevity between *Capsicum spp*

Natural seed aging is a complex trait that requires years to evaluate. To facilitate the study of this important trait, the scientific community has developed several artificial and accelerated, but validated methods to mimic seed aging (Zinsmeister et al. 2020). In the present study, we grew the *Capsicum annuum* accessions, Bola, Pasilla, Piquillo, Chile Serrano and 286.12.1, the *Capsicum chinense* accessions, Habanero and Ecu-994, the *Capsicum frutescens* Bol-144 and the *Capsicum baccatum* Bol-58 at the same time and under the same conditions. Then, we collected the ripe fruits and the seeds were dried following the standard procedures used at the COMAV-UPV seedbank for this crop: 1 week at room temperature, followed by 1 week within a sealed glass can together silica gel (to ensure the removal of any excess of moisture). These seeds were subjected to an accelerated aging treatment (Renard et al. 2021) during 48 h and sown. As shown in figure 1, the percentage of germination without treatment was higher than 80% in all the accessions, which confirms that all the accessions were grown under optimal conditions and their seeds were well-dried and prepared, ensuring a reliable and reproducible experiment. By contrast, the rate of germination after the treatment revealed different responses to seed aging among accessions, from very sensitive to very tolerant. Thus, the germination rate of most accessions after treatment was reduced to 50%. However, Piquillo, the most sensitive accession, showed seed germination rates of 15%, while the most tolerant were Ecu-994 (77% germination) and Bol-144 (72% germination), whose seed germination was not affected by the accelerated aging treatment.

RT-qPCR analysis shows differential expression in seed longevity genes among *Capsicum* spp

We carried out a transcriptional analysis of genes putatively involved in seed longevity in the pepper accessions Ecu-994 and Bol-144, since they were the most tolerant accessions and in Piquillo, which is the most sensitive accession. RNA was extracted from seeds in the advanced cotyledonary stage, when seeds begin maturation and longevity is acquired (Verdier et al. 2013). In order to study the differences between the three accessions regarding seed viability, we focused on those genes that are more expressed in both tolerant accessions (Bol-144 and Ecu-994), as compared to the more sensitive accession Piquillo (Figure 2). This differential pattern was observed for *VTE2*, *HB25*, *ASPG1, PIMT* and *LEC1*. Other genes that could contribute the resistance phenotype of one of the accessions are *GOLS2*, a galactinol synthase (2-fold increase in Ecu-994) and *ABI3* (*CAN.G771.56*), more highly expressed in Bol-144. However, for the other *ABI3* ortholog (*CAN.G771.55*), we could not detect the expression in any of the accessions.

Seed storage protein mobilization is less efficient in the Piquillo accession.

The *ASPG1* gene encodes an aspartic protease that plays an important role in seed dormancy, viability and germination. These proteases are responsible for the mobilization of SSPs (seed storage proteins) to provide energy during the germination process (Shen et al. 2018). According to the expression analysis, the *ASPG1* gene was 7.9 and 3.4 times more expressed in Bol-144 and Ecu-994, respectively, as compared to Piquillo (Figure 2). These results prompted us to investigate the protein profiles of the selected accessions before and during germination. We extracted the proteins from dry seeds and from seeds 6 days after imbibition (during the germination process). The dry seed protein profile did not differ substantially between the accessions. However, after 6 days of imbibition, the Piquillo seeds, despite having started the process of germination, did not efficiently degrade putative globulins (≈35 kDa) or albumins (≈20 kDa) (Tan-Wilson and Wilson, 2012), in contrast to Bol-144 and Ecu-994, which degraded both kinds of proteins (Figure 3).

Tocopherol accumulates in Bol-144

Tocopherols (vitamin E) are part of the battery of antioxidant molecules that plants have available. Specifically, tocopherols are responsible for preventing oxidative damage to lipids and are crucial for seed longevity in several species (Sattler et al. 2004, Hwang et al. 2014 and Chen et al. 2016).

The amount of α, γ and δ tocopherols was analyzed in dry seeds in order to determine whether the levels of this molecule are correlated with seed longevity among the accessions. The results indicate that, despite the transcriptomic results, where we observed that *VTE2* is more highly expressed in varieties with longer seed longevity (Figure 2), Ecu-994 does not contain more tocopherols than Piquillo (Figure 4). However, Bol-144 seeds accumulated more total tocopherols, accruing almost 700 µg/g dw seed (Figure 4). Gamma tocopherol was the more abundant form in seeds in the three accessions and specifically Bol-144 accumulated higher levels than Piquillo and Ecu-994 (+200 µg/g dw and +300 µg/g dw, respectively) (Figure 4). These differences could explain the longer viability of these seeds.

Histological analysis of seed coats shows differences in the levels and structure of lignification among the accessions

The seed coat is a structure specialized in embryo protection and seed dispersal. The accumulation of different biopolymers and their locations are specific for each species and occasionally differences can be found even between cultivars, ecotypes or accessions within the same species.

There are no reports in the literature characterizing the composition of the pepper seed coat. Therefore, we performed thin histological sections that we stained with toluidine blue to better visualize seed coat structures (Figure 5A). We could recognize a cuticle surrounding a thin endosperm. This cuticle was quite similar in the three accessions. The outer integument includes an inner palisade layer. In the Piquillo accession, this integument was much less lignified than in Bol-144 and Ecu-994. The Piquillo accession also presented a subepidermal layer very characteristic of a well-organized palisade. By contrast, in the less domesticated *C. frutescens*, the palisade layer was thinner and was formed by only one row of cells. In addition, another lignified layer was found between the palisade layer and the endosperm-surrounded cuticle in this species (Figure 5A). Compared to *C. annuum* and *C. frutescens*, *C. chinense* Ecu-994 showed an intermediate structure and distribution of layers within its coat. The presence of lignin was detected by staining with phloroglucinol (Figure 5B). In addition, the determination of polyphenolics extracted from dry seeds using the acetyl bromide method suggested that the amount of lignin in the domesticated species *C.annuum* is lower than in *C. frutescens* and *C. chinense* (Figure 5C).

Discussion

Both interspecific and intraspecific variability of seed longevity in crops is widespread, but poorly studied. Species with seeds lacking mechanisms to tolerate desiccation and storage at low temperatures are named recalcitrant and their seed longevity is very short. In comparison, the longevity in orthodox seeds is longer in general. However, there are important differences between some species, such as sunflower, chives and lettuce with a P50 (half-viability period) lower than five years, whereas species like maize germination is still over 80% after 12 years of storage (Nagel and Börner, 2009). *Capsicum* peppers, especially *C. annuum*, is an economically important crop, although its poor seed longevity is one of the drawbacks, in comparison to tomato plants. The reason for this short viability is unknown, but it is likely a consequence of the domestication syndrome (Ensslin et al. 2017) that may have fostered this negative trait, since seed longevity was not the criteria for selection. Thus, in this particular case, although domestication has improved traits in cultivated species, such as seed germination and uniformity rates and the decrease of seed dormancy, it could be detrimental for seed longevity. The results of our study support this hypothesis, since accessions from species with historically less intensive breeding efforts, such as *C. frutescens* and *C. chinense*, generate seeds with longer viability.

In order to generate a list of candidates for further functional analysis of the molecular mechanism underlying these differences in seed longevity, we carried out gene expression analyses in seeds. Very few molecular mechanisms controlling seed longevity have been established in crops, thus we focused on those described in Arabidopsis. We began with studies of the expression of homologs of Arabidopsis genes known to play a role in seed longevity. Gene sequences are widely conserved among the species in the *Capsicum* genus and, therefore, primers to amplify genes from different species can be designed, as reported by Pereira-Dias et al. (2019). In this regard, the final stage of the maturation process is crucial for seeds to acquire longevity and, thus this stage was selected for the transcriptomic study. This stage is marked by the accumulation of protective molecules, such as sugars and LEA proteins. Sugars maintain the integrity of membranes and proteins during the formation of the glassy state (Sano et al. 2016). For instance, galactinol is one of the few molecules whose role in seed longevity has been demonstrated in multiple species like tomato, cabbage and Arabidopsis (De Souza Vidigal et al. 2016). We observed a two-fold increase in the expression of *GOLS2* during seed maturation in Ecu-994, suggesting that this accession could accumulate more galactinol, thus providing one of the possible mechanisms to preserve seed longevity in *C. chinense*. However, this may not be a general mechanism in pepper species since, in *C. frutescens* Bol-144, the expression of this galactinol synthase was similar to *C. annuum* Piquillo.

Vitamin E is another of the few molecules with a described function in seed longevity in different species. One of the pioneering studies using molecular biology technics to investigate seed longevity demonstrated that vitamin E is essential for this trait due to its ability to reduce the oxidation of reserve lipids in Arabidopsis (Sattler et al. 2004). In addition, three different studies concluded that tocopherols play an important role in extending seed viability in rice (Chen et al. 2015, Hwang et al. 2014, Lee et al. 2020). In our transcriptomic study, the expression of *VTE1* expression was similar in the three accessions analysed. Although the *VTE2* expression was two-fold higher in feral species, compared to Piquillo (Figure 2), only Bol-144 accumulated more γ-tocopherol during seed maturation (Figure 4), suggesting that this antioxidant could be a specific mechanism of *C. frutescens* to avoid lipid oxidation over time and such differences could explain the longer viability of this genotype.

As mentioned, a new mechanism to conserve seed viability has been recently described. The mobilization of seed storage proteins is a crucial process for germination over time and plants ensure their prompt availability by accumulating active proteases or their mRNAs to ensure the availability of amino acids for fast protein synthesis (Bai et al. 2020 and Sano et al. 2015). These kinds of proteases that function by degrading seed storage proteins are commonly cysteine proteases, but serine or aspartic metalloproteases are also involved (Tan-Wilson and Wilson, 2012). ASPG1 was first described to function in guard cells, but recently it has been demonstrated that this aspartic protease is important for seed longevity (Shen et al. 2018). Thus, we checked its expression in the selected accessions and we found that the expression was lower during seed maturation in the sensitive accession in response to the accelerated aging treatment when compared with the resistant accessions (Figure 2). In addition, we observed a positive correlation between the expression of this protease and the degradation of putative globulins and albumins during the germination of the selected accessions (Figure 3). These results suggest that the *C. frutescens* Bol-144 and *C. chinense* Ecu-994 accessions mobilize seed storage proteins more efficiently at the end of dormancy and germination, which would provide them with an advantage during germination, as they would be better able to cope with molecular deterioration that they could undergo during storage. An open question to address is if this is a specific event and whether it could be used as a marker to screen the viability of the seeds of other species and genotypes of the *Capsicum* genus.

The role of the seed coat in preserving seed viability as a primary defense against adverse environmental conditions has been described in multiple studies over the years. These studies, however, addressed mainly basic aspects, such as color, thickness or embryo position with respect to the seed coat. Currently, there is a lack of knowledge regarding the relationship between the longevity of crop seeds, the compounds that form the seed coat and the molecular mechanisms that are involved in their biosynthesis. In one of the few studies reported to date, it was demonstrated that the transcription factor AtHB25, a trans-species regulator of seed longevity, stimulates the accumulation of different lipid polyester monomers in the seed cuticles of tomato and wheat, providing seed coat impermeability (Renard et al. 2021).

Histological analysis of the seed coats in the pepper accessions did not display differences in the endosperm-associated cuticle. However, *C. annum* presented lower amount of lignin in the outer integument of the seed coat, as compared to feral species (Figure 5). The role of lignin in seed longevity has been demonstrated in Arabidopsis (Renard et al. 2020) and our study suggests that it could be an important factor in many species, since this biopolymer is predominant in seed coats. This lignified layer could hinder the diffusion between the environment and the embryo. These results could be decisive, since they would establish that lignin accumulation in the pepper seed coat as an important parameter to extend seed vigor and longevity in commercialized pepper seeds.

Author contributions

EB and ARB conceived the project, supervised the experiments and wrote the article.

RS, AF, MAN and LY advised on the experiments.

GB, MB, JR and IMA performed most of the experiments.

EMP grew the plants and checked the maturation stage of the seeds and embryos.

AE performed tocopherol determinations.

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Figure legends

Figure 1. Pepper accessions present variability in germination after accelerated aging treatment. Habanero, Pasilla, Bola, Ecu-994, 286.12.1, Piquillo, Chile Serrano Bol-58 and Bol-144 seeds were subjected to an accelerated aging treatment (100% relative humidity (RH) atmosphere at 41ºC during 48h) and sown on MS plates. The percentage of germination was recorded after 7 days in both control (dark grey) and aged seeds (light grey). The results are the average of four experiments with 100 seeds per line. Error bars indicate the standard error and the asterisks indicate significant differences ($P < 0.05$) compared to control germination from fresh seeds (Student's t test).

Figure 2. Gene expression analysis during seed maturation stages of *C. Annuum* orthologs involved in seed longevity . Gene expression analysis of *GOLS2, ABI3* (*CAN.G771.56*), *VTE1, VTE2, PIMT1, OGG1, LEC1, LEA14, ASPG1, SnRK2.6* and *FUS3* in maturing seeds. Expression values are relative to the housekeeping gene EF1α and normalized to Piquillo. Results are the average of the analysis of three biological samples. Error bars indicate the standard error and the asterisks indicate significant differences ($P < 0.05$) compared to control germination from fresh seeds (Student's t test).

Figure 3. Seed storage protein degradation during germination. SDS-PAGE gel electrophoresis of proteins extracted from seeds of the different accessions and stained with Coomassie Blue. In the left panel, the protein profile from dry seeds is shown. In the right panel, the protein profile from seeds after 6 days of imbibition is shown. Results are representative of 4 experiments. DAI (days after imbibition).

Figure 4. Tocopherol content in dry seeds. Gamma, alpha and delta tocopherols were analysed by gas chromatography and are expressed as µg/g seed dry weight. Results are the average of four determinations from different biological replicates seed extracts.

Error bars indicate the standard error and the asterisks indicate significant differences ($P < 0.05$) as comparted to the Tocopherol content in the Piquillo samples (Student's t test).

Figure 5. Analysis of seed coat sections and determination polyphenolic compounds.

(A) Representative thin sections (4 µm) of seeds using Leica RM2125RTS and focused on the seed coat. Sections were stained with 0.1% toluidine blue (10 replicates). Left panel: Piquillo, middle panel: Bol-144 and right panel: Ecu-994. Red arrows indicate the outer integument. Bars, 50 µm. (B) Representative seed sections (16 µm) focused on the seed coat and stained with phloroglucinol from 10 biological replicates. Left panel: Piquillo, middle panel: Bol-144 and right panel: Ecu-994. Bars, 100 µm. (C) The absorbance (in units of the measured peak area) of polyphenolic compounds was measured at 280 nm after the extraction using the acetyl bromide method. The results are the average of 10 samples. Error bars indicate the standard error and the asterisks indicate significant differences *, $P < 0.05$, **, $P < 0.01$ compared to the Piquillo samples (Student's t test). EP (epidermis), PL (palisade layer), CL (cuticle), EN (endosperm), EM (embryo).

Seed coat lignification ~~levels and storage protein mobilization are~~level is crucial in *Capsicum spp* seed longevity

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Abstract

Capsicum (pepper) is known for its poor seed germination, particularly in terms of seed longevity, which is usually much shorter than other Solanaceae. However, the molecular mechanisms involved are mostly unknown in these species. The present study examines the differences in seed longevity among *Capsicum* species and varietal types. Feral or less domesticated species, such as *C. chinense* and particularly *C. frutescens*, showed higher germination rates than the more domesticated *C. annuum* after accelerated seed aging treatments. In addition, variability was detected in the expression of genes involved ~~in the major conserved molecular mechanisms used~~the response to ~~cope with~~ seed deterioration. The differences observed in *ASPG1* expression led us to study the seed protein profile in dry and germinating seeds. Seed storage protein mobilization during germination was faster in seed aging-resistant genotypes. Similarly, the transcriptional change observed for the orthologous gene of the trans-species regulator *AtHB25*, prompted us to study the structure and molecular components of the seed coat in peppers. All the *Capsicum* pepper ~~materials~~accessions analyzed presented very lignified testa and we observed a positive correlation between the amount of this polyphenolic compound and seed viability. Our results provide essential information to explain the poor germination observed in pepper seeds and provides an experimental framework for future improvements in this important character.

Introduction

Seeds are necessary for survival of spermatophytes since they are part of the reproduction process of this clade. Germination will only occur when the environmental conditions are optimal and thus the seed must sometimes remain viable for long periods of time. However, this viability in not unlimited and the embryo ~~perishes~~may perish before ~~those~~optimum conditions ~~arrive~~occur. The maximum time that a seed can remain viable is variable among species and even between varieties. This parameter is also dependent on external biotic and abiotic factors and it is named seed longevity (Rajjou and Debeaujon 2008; Wang et al. 2018).

Seed longevity ~~on~~in the mostly widely employed and modern varieties of *Capsicum* peppers, i.e. blocky and fleshy types, is known for its short duration. In fact, seeds from this genus lose viability faster than other Solanaceae like tomatoes or eggplants, and seedbanks and breeders usually need to renew accessions every 4-5 years (Adebisi and Abdul-Rafiu, 2016; Priestly, 1986; Yildrim et al. 2020). Nevertheless, *Capsicum* peppers are very diverse genetically and differences ~~among varieties can be found for~~in germination parameters can be found among varieties (Bosland and Votava, 2012; Yildirim et al. 2020).

In this regard, ~~originating from America,~~ there are five cultivated species in the genus *Capsicum* and about 30 wild species originating from America (Pereira-Dias et al. 2019). Within the domesticated taxons, three species, namely *C. annuum* L. var. *annuum*, *C. chinense* Jacq. and *C. frutescens* L. ~~integrate~~are included in the *annuum* botanical complex and are the most important ~~ones~~species deriving from America (DeWitt and Bosland, 2009, Pereira-Dias et al. 2019). Furthermore, they represent a range of domestication levels and seed germination ~~vigour~~capacity. Thus, *C. annuum* is the most economically ~~the most~~ important species and an intense domestication and breeding effort has been made ~~for adaptation~~to adapt this species to a plethora of agroclimatic conditions and culinary uses, ~~based on their~~due to its taste and flavour (Moreno-Peris et al. 2020~~-~~ and Rodríguez-Burruezo et al. 2010). By contrast, several authors consider *C. frutescens* as a semi-domesticated taxon and, in fact, this genotype may grow in nature without assistance from growers (Bosland and Votava, 2012). Finally, *C. chinense* can be considered as an intermediate case, with many varietal types derived from domestication and breeding, but to a lesser extent than *C. annuum* ~~and~~. Moreover, this species displays reduced germination ~~vigour~~capacity (Bosland and Votava, 2012; Mavi, 2018; Pereira-Dias et al. 2019; Russo 2012). Thus, the knowledge about the seed viability of the different ~~species in~~ *Capsicum* species is limited and ~~the lack of~~ studies investigating the molecular mechanisms involved ~~is even scarcer~~are scarce.

Several mechanisms involved in seed longevity have been well-studied in model plants, such as *Arabidopsis thaliana*. Two main strategies have been identified: protection and repair. It has been demonstrated that DNA, lipid and protein repair systems are crucial. For instance, it is known that the prevention of lipid oxidation increases seed viability, as observed for the germination of seeds produced by mutant plants lacking the homogentisate phytyl transferase (*VTE2*) gene, involved in tocopherol biosynthesis. These plants display a reduction in the level of vitamin E and produce seeds with reduced viability (Sattler et al. 2004). Another example is the L-isoaspartyl methyltransferase (PIMT) protein, a repair enzyme that catalyses the conversion of L-isoaspartyl residues, detrimental for protein folding and activity, to L-aspartyl forms.

PIMT1 over-accumulation increases both seed longevity and germination ~~vigour~~capacity (Ogé et al. 2008).

Regarding protection, the seed coat is the first barrier against the adversities of the environment (Mohamed-Yasseen et al. 1994). The testa isolates the embryo from many ~~stresses~~types of stress, such as UV radiation, temperature, moisture, oxygen, attack by pathogens and predators, among others. Therefore, its structure and composition are key factors in determining the longevity of the seed (Rajjou and Debeaujon, 2008). The seed coat begins to form by the differentiation of ovule integuments in the early stages of embryo development. At the end of seed maturation, the testa acquires the proper consistency by accumulating biopolymers. The most important polymers found in the testa are lignin, polysaccharides, suberin and cutin (Sano et al. 2016). Suberin is a lipid polyester located on the external part of the seed coat in some species. It confers impermeability and it has been demonstrated to play an important role in protecting the embryo in Arabidopsis (Renard et al. 2020, 2021). The mucilage, missing in pepper, is a viscous polysaccharide whose hygroscopic properties allow it to absorb and maintain water around the seed, providing favorable conditions for germination. The ~~euticule~~cuticle is associated ~~to~~with the endosperm in numerous species, where it regulates permeability to external compounds and therefore affects seed viability (De Giorgi et al. 2015). Finally, lignin is formed in the spaces between the cellulose microfibrils by the oxidative coupling of free lignin monomers secreted directly into the plant cell wall. The canonical monolignols are the non-methoxylated p-coumaryl alcohol, the monomethoxylated coniferyl alcohol and the dimethoxylated sinapyl alcohol that form H- (hydroxyphenyl), G- (guaicyl) and S- (syringyl) units in the lignin polymer, respectively. However, seed coat lignins also include the non-canonical C- (caffeyl) units which derive from an unusual monomer, caffeyl alcohol. Seed coat cells develop heavily lignified secondary cell walls to reinforce the outer surface of the seed mechanically and to make it impermeable to liquids and gasses (Barros et al. 2015). Thus, a loss-of-function mutant in the Arabidopsis *AtLAC15* gene, encoding a laccase involved in seed lignin synthesis, presents higher seed coat permeability (Liang et al. 2006).

On the other hand, many transcription factors have been described to participate in the regulation of the different developmental stages and layers of the testa (Haughn and Chaudhury, 2005), but not many of them have been found to be involved in seed longevity. AtHB25 is a member of the homeobox family and it is considered as a trans-species regulator of seed viability through the regulation of the expression of both gibberellin and lipid polyester biosynthesis enzymes (Bueso et al. 2014 and Renard et al. 2021).

Finally, in addition to the protection and repair mechanisms, it has been reported that the efficiency in seed protein storage mobilization is crucial for seed germination over time. For instance, a positive correlation between the expression of the aspartic protease *ASPG1* gene and seed viability has been demonstrated (Shen et al. 2018).

This work explores the variability of the seed longevity among *Capsicum* peppers and, to our knowledge, this is the first ~~work~~study aimed at ~~elucidating~~analysing the ~~basis of seed longevity~~genes and mechanisms involved in this trait in *Capsicum* peppers~~ on the molecular level~~, which is of paramount interest for plant physiologists, geneticists, breeders and seedbank managers.

Materials and methods

Plant material and growth conditions

Nine accessions of different species from *Capsicum* genus were obtained from the *Capsicum* breeding team of the Instituto Universitario de Conservación y Mejora de la Agrodiversidad Valenciana (Valencia, Spain). The *C. annuum* accessions were Bola, Pasilla, Piquillo, Chile Serrano and ~~california~~California Wonder breeding line 286.12.1. The *C. chinense* accessions were Habanero and Ecu-994. The *C. baccatum* accession was Bol-58 and the *C. frutescens* accession was Bol-144. This collection encompassed a range of different geographical origins and morphological traits. In order to work with new and standardized lots of seeds, plants from all the accessions were grown in greenhouses ~~along~~throughout the experiment. To achieve ~~that~~this, seedlings were transplanted ~~to~~in the COMAV greenhouses, located at the Campus de Vera of the Universitat Politècnica de València, 50-60 days after sowing. The cultivation was carried out in 10 L pots with coconut fibre in the spring-summer season, since this period is the best weather for pepper growth and reproduction ~~under~~for the Mediterranean climate (Nuez et al. 2003). In the greenhouse, the temperature was about 18-30˚C and plants grew under natural photoperiod. Plants were pruned in four stems and supported with strings to ~~make easier~~facilitate the vertical growth. Drip irrigation was applied every 8 hours for 3 min (4L/h) with 1 g/L of a commercial fertiliser 15N-2,2P-24,9K (BASF, Barcelona, Spain) diluted in the water.

Accelerated aging treatment and germination studies

Seeds were harvested when the fruit was at the fully ripe stage, so the seeds of all accessions were harvested at the same physiological maturity. The accelerated aging treatment was performed with hydrated seeds and 100% relative humidity (RH) atmosphere at 41ºC during 48h. Seeds contained 0.05–0.8 g H_2O/g dry weight before treatment and 0.13-0.16 g H_2O/g dry weight after treatment. After the treatment, seeds were grown and germinated on Murashige and Skoog (MS) plates with 1% sucrose ~~1%, MES,~~(w/v), 10 mM MES and 1% agar~~ and~~. The pH was adjusted ~~pH~~ to 5~~,~~.7 with Tris buffer (Renard et al. 2020). All germination analyses were performed after 7 days of sowing with ~~four replicates~~ using around 100 seeds per biological replicate. The experiments were repeated four times. The results are the average of these four experiments with 100 seeds per line. A Student's t test was used to analyse the significant differences ($P < 0.05$).

Orthologous gene identification and primer design

To identify orthologous ~~genes in~~ pepper genes corresponding to Arabidopsis genes implicated in seed longevity, we utilized Plaza by Ghent University~~,~~. More specifically, we used the Integrative Orthology Viewer (https://bioinformatics.psb.ugent.be/plaza). This software selects the orthologous gene of a species after performing four tests, "Tree-based ortholog", "Orthologous gene family", "Anchor point" and "Best-Hits-and-Inparalogs" (Van Bel et al. 2018). As shown in Table 1, we identified 13 unique candidate orthologs in *Capsicum*. However, *ABI3*, a gene essential for seed maturation, showed two potential candidates in *Capsicum*. ~~Those genes were: *ABI3*, *ABI5*, *HB25*, *PIMT1*, *SnRK2.6*, *OGG1*, *VTE1*, *VTE2*, *LEC1*, *LEA14*, *ASPG1*, *GOLS2* and *FUS3*.~~The genes analysed were: *ABSCISIC ACID INSENSITIVE 3 (ABI3)*, *ABA INSENSITIVE 5 (ABI5)*, *HOMEOBOX PROTEIN 25 (HB25)*, *PROTEIN-L-ISOASPARTATE METHYLTRANSFERASE (PIMT1)*, *SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 2-6 (SnRK2.6)*, *8-OXOGUANINE-DNA GLYCOSYLASE 1 (OGG1)*, *VITAMIN E DEFICIENT 1 (VTE1)*, *VITAMIN E 2 (VTE2)*, *LEAFY COTYLEDON 1*

(LEC1), LATE EMBRYOGENESIS ABUNDANT 14 (LEA14), ASPARTIC PROTEASE IN GUARD CELL 1 (ASPG1), GALACTINOL SYNTHASE 2 (GOLS2) and *FUSCA3 (FUS3).* To design primers to amplify the cDNA, we identified the sequences from orthologous genes in *Capsicum annuum, Solanum tuberosum and Solanum lycopersicum* in Plaza as well. The genomic information of *CapasicumCapsicum chinense* was found in Sol Genomics (https://solgenomics.net/) doing a BLAST (Basic Local Alignment Tool). A BLAST search was done for each exon using the exons of the *C. annuum* orthologous *C. annuum* genes previously found in Plaza. We performed the multiple alignment of the coding sequences (CDS) using ClustalW (gap penalty=8) (https://www.genome.jp/tools-bin/clustalw) to find the most conserved regions where primers were designed. As shown in Figure S1, primers were designed in the most conserved sequence of the gene with no nucleotide changes, and when this was not possible, degenerate primers were designed. Table S1 shows the direct and reverse primers designed for each gene. Before carrying out a differential gene expression analysis, we checked the primer efficiency in the three accessions and all were comprised between 96% and 120%. The R^2 value ranged from 0.99 to 1.00.

Seed RNA extraction and RT-qPCR

The extraction of seed RNA was performed at a specific seed development stage: advanced cotyledonary (Manzur et al. 2015). This stage was at 30 DAF (days after fertilization) in the Piquillo accession and 25 DAF in Bol-144 and Ecu-994 accessions. The method utilized is described in Oñate-Sánchez and Vicente-Carbajosa (2008). Subsequently, RNA was purified with the Nucleo spin kit (Macherey-Nagel). About 1000 ng RNA were reverse transcribed using the Maxima first-strand cDNA synthesis kit for RT-qPCRqRT-PCR (Thermo Fisher Scientific) according to the manufacturer's instructions. Each reaction was performed in triplicate in a total volume of 20 µl. qRT-PCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific) with the 5 PyroTaq EvaGreen qPCR Mix Plus (ROX; Cultek S.L.U., Madrid, Spain) according to the manufacturer's protocol. Data are the mean of three biological samples and relative mRNA abundance was calculated using the comparative ΔCt method described in Pfaffl (2001). The housekeeping gene utilized was the pepper orthologousorthologue of the tomato Ef-1alphaEf1-alpha (López-Gresa et al. 2017). Standard curves were performed to calculate the efficiency of the reaction from a series of primer dilutions and a mixture of cDNA from each accession. Four-point standard curves of a dilution series (1:1, 1:5, 1:25 and 1:125) were utilized to calculate the R^2 value and primer efficiency.

Protein extraction and SDS-PAGE

Proteins were extracted from dry seeds and seeds 6 days after imbibition (50 mg). Seeds were frozen in liquid nitrogen in a mortar and were ground into powder with a pestle. Quickly, theThe powder was well quickly and thoroughly suspended in 400 µL of PBS andwith the protease inhibitor cocktail (cOmplete, Roche Ref. 04693159001). Following centrifugation for 15 min, the supernatant was pipetted transferred to a new tube and the protein content was quantified using the Bradford method. Extracted proteins (20 µg) were resolved on a 12% SDS-PAGE gel. The gel was stained using Coomassie Blue R-250.

Histological staining

Seeds were hydrated and fixed in a solution of 2.5% paraformaldehyde ~~2.5%~~ and 0.5% glutaraldehyde ~~0.5% solution, protocol~~, as described by Karnovsky (1965). Before the infiltration in LR White, the seeds were washed in phosphate buffer, post-fixed with 2% osmium ~~2%,~~, washed with water and dehydrated ~~through~~using an ethanol series (30%, 50%, 70%, 90%). The infiltration was carried out with mixes of 100% resin ~~100%~~ and ethanol (2 parts EtOH 90% + 1 part resin, 1 part EtOH 90% + 2 parts resin, 1 part EtOH + 2 parts resin). After complete polymerization in capsules in the absence of oxygen at 55/60°C, LR White embedded materials ~~are~~were sectioned with a Leica RM2125RTS microtome to obtain ~~thin~~4 µm sections ~~of 4 µm of thickness. Thin sections~~. Sections were stained with 0.1% ~~(w/v)~~ toluidine blue O (w/v) in distilled water. Sections (16 µm of thickness) were generated with Microm HM 520 Cryostat and stained with phloroglucinol (3% in ethanol) and ~~mix~~mixed with one volume of HCl (37N).

Lignin quantification

Polyphenolic compounds were extracted from seeds of each accession (8 replicates) with acetyl bromide, as described in Moreira-Vilar et al. (2014). Briefly, a protein-free cell wall sample (20 mg) was digested with 25% acetyl bromide (v/v in glacial acetic acid) and incubated at 70ºC for 30 min. Then, the sample was quickly cooled in an ice bath~~,~~ and mixed with 0.9 ml of 2 M NaOH, 0.1 ml of 5 M hydroxylamine-HCl, and 1 volume of glacial acetic acid. After centrifugation, the absorbance of the supernatant was measured at 280 nm.

Tocopherol analysis

The quantification of endogenous levels of tocopherols in pepper seeds was performed at the metabolomics facility of the Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC, Valencia, Spain) as previously described by Stahl et al. (2019) with ~~the~~ slight modifications. Briefly, 50 mg of seeds per sample were homogenized with liquid nitrogen and extracted in 1.4 mL 100% methanol supplemented with 1.4 µL of internal standard (1 mg/ml nonadecanoic acid methylester in $CHCl_3$) for 15 min at 70 °C. The extract was centrifuged for 10 minutes at 14000 rpm. The supernatant was transferred to a glass vial and 1 ml of $CHCl_3$ and 1ml of water were added. The mixture was vortexed for 15 seconds and centrifuged for 15 minutes at 14000 rpm. 600 µl of the lower organic phase were dried in vacuum for 6–16 h. For derivatisation, dry residues were redissolved in 70 µL MSTFA (N-methyl-N-[trimethylsilyl]trifluoroacetamide) with 6 µL of a retention time standard mixture (3.7% [w/v] mix of fatty acid methyl esters ranging from 8 to 24C) and incubated for 30 minutes at 37 ºC. Sample volumes of 2 µL were injected in splitless mode in a 6890 N gas chromatograph (Agilent Technologies Inc. Santa Clara, CA) coupled to a Pegasus 4D TOF mass spectrometer (LECO, St. Joseph, MI). Gas chromatography was performed on a BPX35 (30 m × 0.32 mm × 0.25 µm) column (SGE Analytical Science Pty Ltd., Australia) with helium as the carrier gas at a constant flow of 2 ml/min. The liner was set at 230 °C. The oven program was 85 °C for 2 min, 8 °C/min ramp until 360 °C. Mass spectra were collected at 6.25 spectra s^{-1} in the *m/z* range 35–900 and ionization energy of 70 eV. Chromatograms and mass spectra were evaluated using the CHROMATOF program

(LECO, St. Joseph, MI). The absolute contents of the fourthree tocopherols were calculated using a commercially available tocopherol mix (Sigma ref. W530066).

Statistical analysis of the results

The mean and the standard error (SE) were calculated and differences of the means were considered to be statistically significant when P<0.05 using a Student's t test. All the experiments were repeated at least three times.

Results

The analysis of germination after accelerated aging treatments reveals variability in seed longevity between *Capsicum spp*

Natural seed aging is a complex trait that requires years to evaluate. To facilitate the study of this important trait, the scientific community has developed several artificial and accelerated, but validated methods to mimic seed aging (Zinsmeister et al. 2020). In the present study, we grew the *Capsicum annuum* accesionsaccessions, Bola, Pasilla, Piquillo, Chile Serrano and 286.12.1, the *Capsicum chinense* accesionsaccessions, Habanero and Ecu-994, the *Capsicum frutescens* Bol-144 and the *Capsicum baccatum* Bol-58 at the same time and under the same conditions. Then, we collected the ripe fruits and the seeds were dried following the standard procedures used at the COMAV-UPV seedbank for this crop: 1 week at room temperature, followed by 1 week within a sealed glass can together silica gel (to ensure the removal of any excess of moisture). These seeds were subjected to an accelerated ageingaging treatment (Renard et al. 2021) during 48 h and sown. As shown in figure 1, the percentage of germination without treatment was higher than 80% in all the accessions, which confirms that all the accessions were grown under optimal conditions and their seeds were well-dried and prepared, ensuring a reliable and reproducible experiment. By contrast, the rate of germination after the treatment revealed different responses to seed ageingaging among accessions, from very sensitive to very tolerant. Thus, the germination rate of most accessions after treatment was reduced to 50%. However, Piquillo, the most sensitive accession, showed seed germination rates of 15%, while the most tolerant were Ecu-994 (77% germination) and Bol-144, (72% germination), whose seed germination was not affected by the accelerated ageingaging treatment.

~~Identification of orthologous seed longevity genes in *Capsicum annuum* and primer design~~

~~Several genes involved in seed longevity have been characterized in Arabidopsis. In order to identify the closer orthologs of those genes in pepper, we performed an analysis using "Integrative Orhology Viewer" by *Plaza* (https://bioinformatics.psb.ugent.be/plaza). This software selects the orthologous gene of a species after performing four tests, "Tree-based ortholog", "Orthologous gene family", "Anchor point" and "Best-Hits-and-Inparalogs" (Van Bel et al. 2018). As shown in Table 1, we identified 13 unique candidate orthologs in *Capsicum*. However, *ABI3*, a gene essential for seed maturation, showed two potential candidates in *Capsicum*. Next, we designed primers to amplify the transcripts of selected genes in the two resistant accessions (Ecu-994 and Bol-144) and in Piquillo (*C. annuum*). Since the~~

three accessions are three different species, and one of them (*C. frutescens*) is not sequenced yet, we performed a multiple sequence alignment using "Clustaw" between different Solanaceae species. For this, we identified the orthologs in *Capsicum chinense*, *Solanum lycopersicum* and *Solanum tuberosum* and we aligned the genes of interest. As Figure S1 shows, primers were designed in the most conserved sequence of the gene that did not have any nucleotide change, and when this was not possible, degenerate primers were designed. Table S1 shows the direct and reverse primers designed for each gene. Before carrying out a differential gene expression analysis, we checked the primer efficiency in the three accessions and all were comprised between 96% and 120%. The R^2 value ranged from 0.99 to 1.00.

RT-qPCR analysis shows differential expression in seed longevity genes betweenamong *Capsicum* spp

We carried out a transcriptional analysis of genes putatively involved in seed longevity in the three pepper accessions. Ecu-994 and Bol-144, since they were the most tolerant accessions and in Piquillo, which is the most sensitive accession. RNA was extracted from seeds in the advanced cotyledonary stage, when seeds begin maturation, and longevity is acquired (Verdier et al. 2013). The most interesting results were In order to study the differences between the three accessions regarding seed viability, we focused on those obtained from genes that are more highly expressed in both tolerant accessions (Bol-144 and Ecu-994), as compared to the more sensitive accession Piquillo, which belongs to the more domesticated *C. annuum* (Figure 2). This differential pattern was observed for *VTE2*, *HB25*, *ASPG1, PIMT* and *LEC1*, a transcriptional activator of genes required for both embryo maturation and cellular differentiation (Braybrook and Harada, 2008).. Other genes that could specifically explaincontribute the resistance phenotype of one of the accessions are *GOLS2*, a galactinol synthase (2-fold increase in Ecu-994) and *ABI3* (*CAN.G771.56*), more highly expressed in Bol-144. However, for the other *ABI3* ortholog (*CAN.G771.55*), we could not detect the expression in any of the accessions.

Seed storage protein mobilization is less efficient in the Piquillo accession.

The *ASPG1* gene encodes an aspartic protease that plays an important role in seed dormancy, viability and germination. These proteases are responsible for the mobilization of SSPs (seed storage proteins) to provide energy during the germination process (Shen et al. 2018). According to the expression analysis, the *ASPG1* gene was 7.9 and 3.4 times more expressed in Bol-144 and Ecu-994, respectively, as compared to Piquillo (Figure 2). These results prompted us to investigate the protein profiles of the selected accessions before and during germination. We extracted the proteins from dry seeds and from 6 seeds 6 days after imbibition (during the germination process). The dry seed protein profile did not differ substantially between the accessions. However, after 6 days of imbibition, the Piquillo seeds, despite having started the process of germination, did not efficiently degrade putative globulins (≈35 kDa) or albumins (≈20 kDa) (Tan-Wilson and Wilson, 2012), in contrast to Bol-144 and Ecu-994, which degraded both kinds of proteins (Figure 3).

These results suggest that the *C. frutescens* Bol-144 and *C. chinense* Ecu-994 accessions mobilize seed storage proteins more efficiently at the end of dormancy and germination, which would provide them with an advantage during germination, as they

~~would be better able to cope with molecular deterioration that they could undergo during storage.~~

Tocopherol ~~accumulation~~accumulates in Bol-144 ~~could be important for longer seed viability~~

Tocopherols (vitamin E) are part of the battery of antioxidant molecules that plants have available. Specifically, tocopherols are responsible for preventing oxidative damage to lipids and are crucial for seed longevity in several species (Sattler et al. 2004, Hwang et al. 2014 and Chen et al. 2016).

The amount of α, γ and δ tocopherols was analyzed in dry seeds in order to determine whether the levels of this molecule are correlated with seed longevity among the accessions. The results indicate that, despite the transcriptomic results, where we observed that *VTE2* is more highly expressed in varieties with longer seed longevity (Figure 2), Ecu-994 does not contain more tocopherols than Piquillo~~.~~ (Figure 4). However, Bol-144 seeds accumulated more total tocopherols, accruing almost 700 µg/g dw seed~~.~~ (Figure 4). Gamma tocopherol was the more abundant form in seeds in the three accessions and specifically Bol-144 accumulated higher levels than Piquillo and Ecu-994 (+200 µg/g dw and +300 µg/g dw, respectively~~). Such~~) (Figure 4). These differences could explain the longer viability of these seeds ~~(Figure 4).~~ .

Histological analysis of seed coats shows differences in the levels and structure of lignification among the accessions

The seed coat is a structure specialized in embryo protection and seed dispersal. The accumulation of different biopolymers and their locations are specific for each species and occasionally differences can be found even between cultivars, ecotypes or accessions within the same species.

There are no reports in the literature characterizing the composition of the pepper seed coat. ~~In this regard, our study on seed sections stained with phloroglucinol uncovered that lignin is the most abundant component in all the accessions (Figure 5A). However, the determination of polyphenolics extracted from dry seeds using the acetyl bromide method suggested that the amount of lignin of the domesticated species *C.annuum* is lower than in *C. frutescens* and *C. chinense* (Figure 5B). Finally, to better visualize seed coat structures~~Therefore, we performed thin histological sections that we stained with toluidine blue~~.~~ to better visualize seed coat structures (Figure 5A). We could recognize a cuticle surrounding a thin endosperm. This cuticle was quite similar in the three accessions. The outer integument includes an inner palisade layer. In the Piquillo ~~presented a seed coat~~ accession, this integument was much less lignified than in Bol-144 and Ecu-994 ~~and a~~ . The Piquillo accession also presented a subepidermal layer very characteristic of a well-organized palisade. By contrast, in the less domesticated *C. frutescens*, the palisade layer was thinner and was formed by only one row of cells. In addition, another lignified layer was found between the palisade layer and the endosperm-surrounded cuticle in this species~~. This extra layer could hinder the diffusion between the environment and the embryo (Figure 5C).~~ (Figure 5A). Compared to *C. annuum* and *C. frutescens*, *C. chinense* Ecu-994 showed an intermediate structure and distribution of layers ~~on~~within its coat. The presence of lignin was detected by staining with phloroglucinol (Figure 5B). In addition, the determination of polyphenolics extracted from dry seeds using the acetyl bromide method suggested that the amount of lignin in the domesticated species *C.annuum* is lower than in *C. frutescens* and *C. chinense* (Figure 5C).

Discussion

Both interspecific and intraspecific variability of seed longevity in crops is widespread, but poorly studied. Species with seeds lacking mechanisms to tolerate desiccation and storage at low temperatures are named recalcitrant and their seed longevity is very short. In comparison, the longevity in orthodox seeds is longer in general. However, there are important differences between some species, such as sunflower, chives and lettuce with a P50 (half-viability period) lower than five years, whereas species like maize ~~in which after 12 years of storage~~ germination is still over 80% after 12 years of storage (Nagel and Börner, 2009). *Capsicum* peppers, especially *C. annuum*, is an economically important crop, although its poor seed longevity is one of the drawbacks, in comparison to tomato plants. The reason for this short viability is unknown, but it is likely a consequence of the domestication syndrome (Ensslin et al. 2017) that may have fostered this negative trait, since seed longevity was not the criteria for selection. Thus, in this particular case, although domestication has improved traits in cultivated species, such as seed germination and uniformity rates and the decrease of seed dormancy, it could be detrimental for seed longevity. The results of our study support this hypothesis, since accessions from species with historically less intensive breeding efforts~~;~~, such as *C. frutescens* and *C. chinense*, generate seeds with longer viability.
~~The next question we wanted~~In order to ~~address was~~generate a list of candidates for further functional analysis of the molecular mechanism underlying these differences in seed longevity, we carried out gene expression analyses in seeds. Very few molecular mechanisms controlling seed longevity have been established in crops, thus we focused ~~in~~on those described in Arabidopsis. We began with studies of the expression of homologs of Arabidopsis genes known to play a role in seed longevity. ~~Open reading frames~~Gene sequences are widely conserved among the species in the *Capsicum* genus and, therefore, primers to amplify genes from different species can be designed, as reported by Pereira-Dias et al. (2019). In this regard, the final stage of the maturation process is crucial for seeds to acquire longevity and, thus this stage was selected for the transcriptomic study. This stage is marked by the accumulation of protective molecules, such as sugars and LEA proteins. Sugars maintain the integrity of membranes and proteins during the formation of the glassy state (Sano et al. 2016). For instance, galactinol is one of the few molecules whose role in seed longevity has been demonstrated in multiple species like tomato, cabbage and Arabidopsis (De Souza Vidigal et al. 2016). We observed a two-fold increase in the expression of *GOLS2* during seed maturation in Ecu-994, suggesting that this accession could accumulate more galactinol, thus providing one of the possible mechanisms to preserve seed longevity in *C. chinense*. However, this may not be a general mechanism in pepper species since, in *C. frutescens* Bol-144, the expression of this galactinol synthase was similar to *C. annuum* Piquillo.
Vitamin E is another of the few molecules with a described function in seed longevity in different species. One of the pioneering studies using molecular biology technics to investigate seed longevity demonstrated that vitamin E is essential for this trait due to

its ability to reduce the oxidation of reserve lipids in Arabidopsis (Sattler et al. 2004). In addition, three different studies concluded that tocopherols play an important role in extending seed viability in rice (Chen et al. 2015, Hwang et al. 2014, Lee et al. 2020). In our transcriptomic study, the expression of *VTE1* expression was similar in the three accessions analysed. Although the *VTE2* expression was two-fold higher in feral species, compared to Piquillo (Figure 2), only Bol-144 accumulated more γ-tocopherol during seed maturation (Figure 4), suggesting that this antioxidant could be a specific mechanism of *C. frutescens* to avoid lipid oxidation over time~~.~~ and such differences could explain the longer viability of this genotype.

As mentioned, a new mechanism to conserve seed viability has been recently described. The mobilization of seed storage proteins is a crucial process for germination over time and plants ensure their prompt availability by accumulating active proteases or their mRNAs to ensure the availability of amino acids for fast protein synthesis (Bai et al. 2020 and Sano et al. 2015). These kinds of proteases that function by degrading seed storage proteins are commonly cysteine proteases, but serine or aspartic metalloproteases are also involved (Tan-Wilson and Wilson, 2012). ASPG1 was first described to function in guard cells, but recently it has been demonstrated that this aspartic protease is important for seed longevity (Shen et al. 2018). Thus, we checked its expression in the selected accessions and we found that ~~this gene~~ the expression was ~~repressed~~lower during seed maturation in the sensitive accession in response to the accelerated aging treatment when compared with the resistant accessions (Figure 2). In addition, we observed a positive correlation between the expression of this protease and the degradation of putative globulins and albumins during the germination of the selected accessions (Figure 3). These results suggest that the *C. frutescens* Bol-144 and *C. chinense* Ecu-994 accessions mobilize seed storage proteins more efficiently at the end of dormancy and germination, which would provide them with an advantage during germination, as they would be better able to cope with molecular deterioration that they could undergo during storage. An open question to address is if this is a specific event and whether it could be used as a marker to screen the viability of the seeds of other species and genotypes of the *Capsicum* genus.

The role of the seed coat in preserving seed viability as a primary defense against adverse environmental conditions has been described in multiple studies over the years. These ~~articles~~studies, however, addressed mainly basic aspects, such as color, thickness or embryo position with respect to the seed coat. ~~Nowadays~~Currently, there is a lack of knowledge regarding the relationship between the longevity of crop seeds, the compounds that form the seed coat and the molecular ~~mechanism~~mechanisms that are involved in their biosynthesis. In one of the few studies reported to date, it was demonstrated that the transcription factor AtHB25, a trans-species regulator of seed longevity, stimulates the accumulation of different lipid polyester monomers in the seed cuticles of tomato and wheat, providing seed coat impermeability (Renard et al. 2021).

Histological analysis of the seed coats in the pepper accessions did not display differences in the endosperm-associated cuticle. However, *C. annum* presented ~~a thinner layer and~~ lower amount of lignin in the outer integument of the seed coat, as compared to feral species (Figure 5). The role of lignin in seed longevity has been demonstrated in Arabidopsis (Renard et al. 2020) and ~~this~~our study suggests that it could be an important factor in many species, since this biopolymer is predominant in seed coats. This lignified layer could hinder the diffusion between the environment and the embryo. These results could be decisive, since they would establish ~~the bases~~that lignin accumulation in the pepper seed coat as an important parameter to ~~solve the germination~~

~~problems presented by the~~ extend seed vigor and longevity in commercialized pepper seeds.

Author contributions

EB and ARB conceived the project, supervised the experiments and wrote the article.

RS, AF, MAN and LY advised on the experiments.

GB, MB, JR and IMA performed most of the experiments.

EMP grew the plants and checked the maturation stage of the seeds and embryos.

AE performed tocopherol determinations.

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Figure legends

Figure 1. Pepper accessions present ~~high~~variability in germination~~ variability~~ after accelerated aging treatment. Habanero, Pasilla, Bola, Ecu-994, 286.12.1, Piquillo, Chile Serrano Bol-58 and Bol-144 seeds were subjected to an accelerated aging treatment (100% relative humidity (RH) atmosphere at 41ºC during 48h) and sown on MS plates. The percentage of germination was recorded after 7 days in both control (dark grey) and aged seeds (light grey). The results are the average of ~~three~~four experiments with 100 seeds per line. Error bars ~~denote SE. *, ~~indicate the standard error and the asterisks indicate significant differences ($P < 0.05$~~.~~) compared to control germination from fresh seeds (Student's t test).

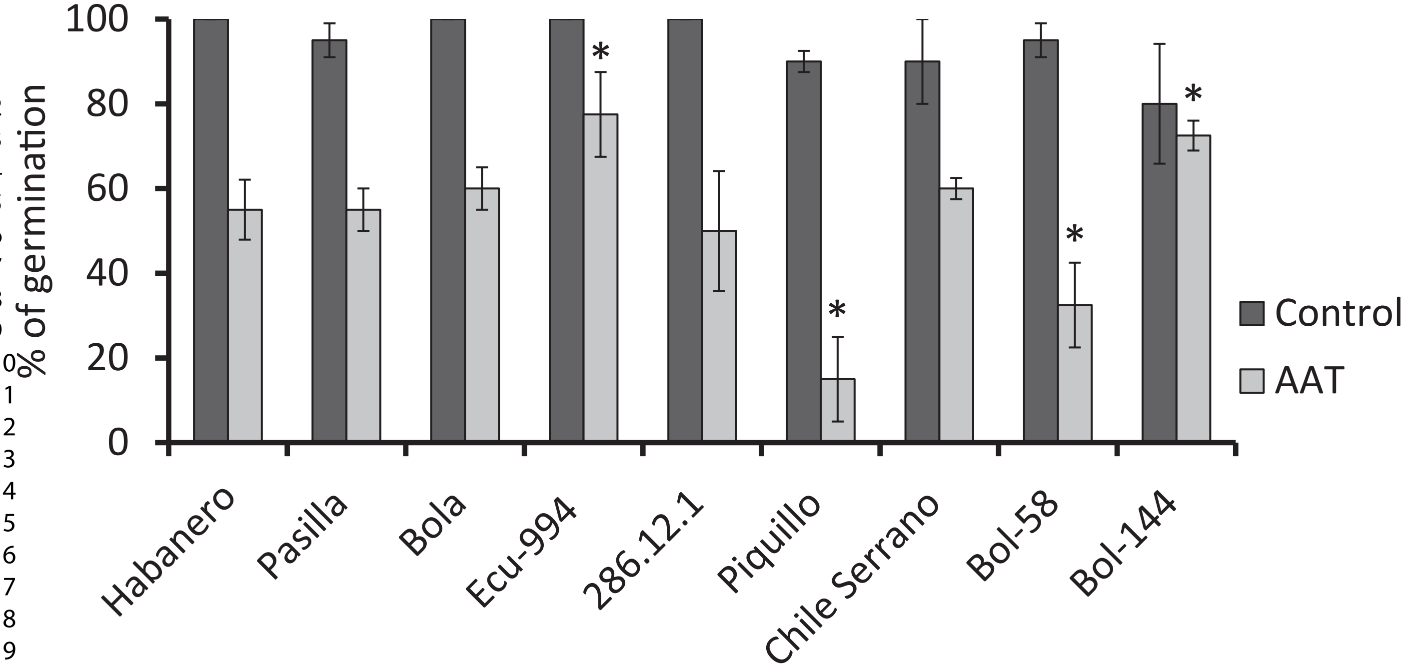
Figure 2. Gene expression analysis ~~of seed longevity-involved orthologs in *C. Annuum*~~ during seed maturation ~~stage~~stages of *C. Annuum* orthologs involved in seed longevity . Gene expression analysis of *GOLS2, ABI3* (*CAN.G771.56*), *VTE1, VTE2, PIMT1, OGG1, LEC1, LEA14, ASPG1, SnRK2.6* and *FUS3* in maturing seeds. Expression values are relative to the housekeeping gene EF1α and normalized to Piquillo. Results are the average of ~~three determinations. The error bars denote SE. *, P < 0.05.~~the analysis of three biological samples. Error bars indicate the standard error and the asterisks indicate significant differences ($P < 0.05$) compared to control germination from fresh seeds (Student's t test).

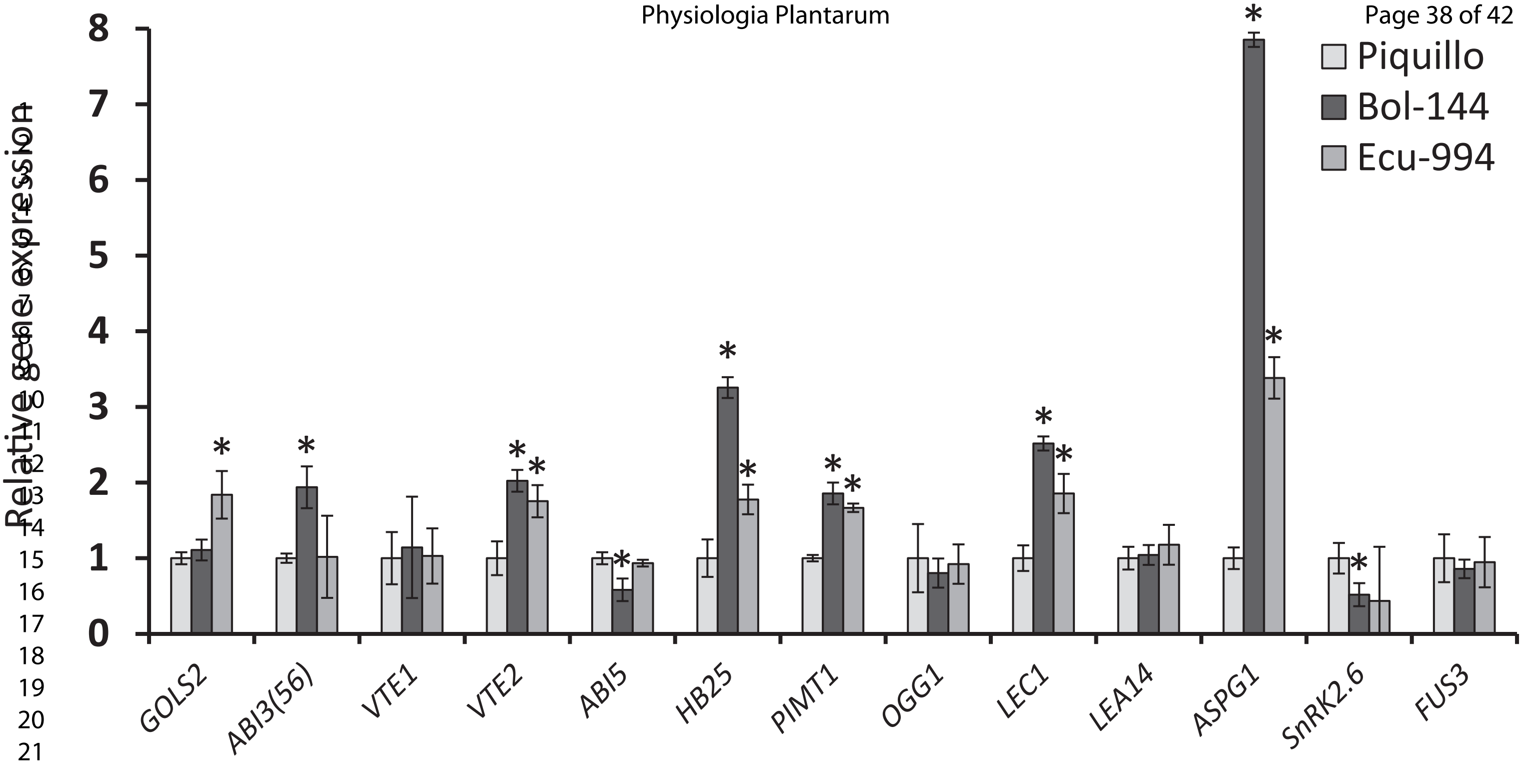
Figure 3. Seed storage protein degradation during germination. SDS-PAGE gel electrophoresis of ~~seed~~proteins extracted ~~proteins~~ from seeds of the different accessions and stained with Coomassie Blue. ~~On~~In the left panel, the protein profile from dry seeds~~. On~~ is shown. In the right panel, the protein profile from seeds after 6 days of imbibition is shown. Results are representative of 4 experiments. DAI (days after imbibition).

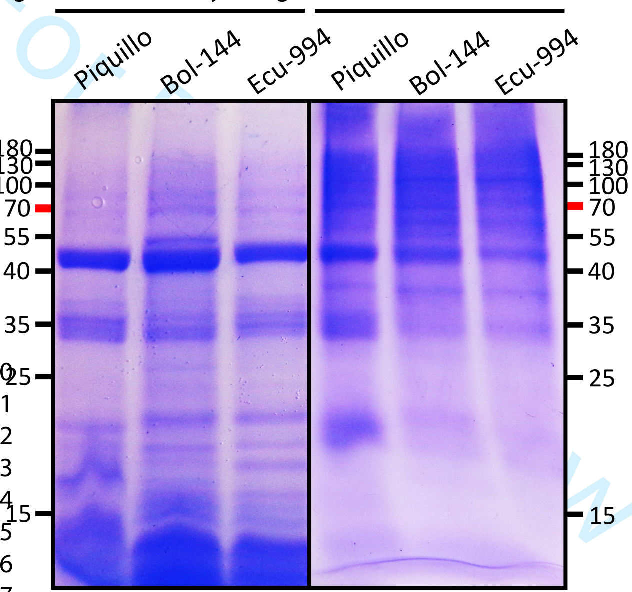
Figure 4. Tocopherol content in dry seeds. Gamma, alpha and delta tocopherols were analysed by gas chromatography and are expressed as µg/g seed dry weight ~~seed~~. Results are the average of four determinations~~. The~~ from different biological replicates seed extracts. Error bars indicate the standard error ~~bars denote SE. *,~~ and the asterisks indicate significant differences (P < 0.05~~.~~) as comparted to the Tocopherol content in the Piquillo samples (Student's t test).

Figure 5. ~~Stained~~Analysis of seed coat sections and determination polyphenolic compounds ~~determination.~~ .

(A) Representative thin sections (4 µm) of seeds using Leica RM2125RTS and focused on the seed coat. Sections were stained with 0.1% toluidine blue (10 replicates). Left panel: Piquillo, middle panel: Bol-144 and right panel: Ecu-994. ~~seed section~~Red arrows indicate the outer integument. Bars, 50 µm. (B) Representative seed sections (16 µm) focused ~~in~~on the seed coat and stained with phloroglucinol from 10 biological replicates. Left panel: Piquillo, middle panel: Bol-144 and right panel: Ecu-994. Bars, 100 µm. ~~(B) Polyphenolic compounds~~ (C) The absorbance (in units of the measured peak area) of polyphenolic compounds was measured at 280 nm after the extraction ~~with~~using the acetyl bromide method. The results are the average of 10 samples. ~~The~~Error bars indicate the standard error ~~bars denote SE.~~and the asterisks indicate significant differences *, P < 0.05, **, P < 0.01~~. (C) Representative seed thin section (4µm) using Leica RM2125RTS and focus in the seed coat. Sections were stained with 0.1% toluidine blue~~ compared to the Piquillo samples (Student's t test). EP (epidermis), PL (palisade layer), CL (cuticle), EN (endosperm), EM (embryo).~~(10 replicates). Left panel: Piquillo, middle panel: Bol-144 and right panel: Ecu-994. Red arrows indicate outer integument. Bars, 50 µm.~~


% of germination
100
80
60
40
20
0
Habanero
Pasilla
Bola
Ecu-994
286.12.1
Piquillo
Chile Serrano
Bol-58
Bol-144
Control
AAT


Relative gene expression
0
1
2
3
4
5
6
7
8
Piquillo
Bol-144
Ecu-994
GOLS2
ABI3(56)
VTE1
VTE2
ABI5
HB25
PIMT1
OGG1
LEC1
LEA14
ASPG1
SnRK2.6
FUS3


0 DAI
6 DAI
Piquillo
Bol-144
Ecu-994
Piquillo
Bol-144
Ecu-994
180
130
100
70
55
40
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25
15
180
130
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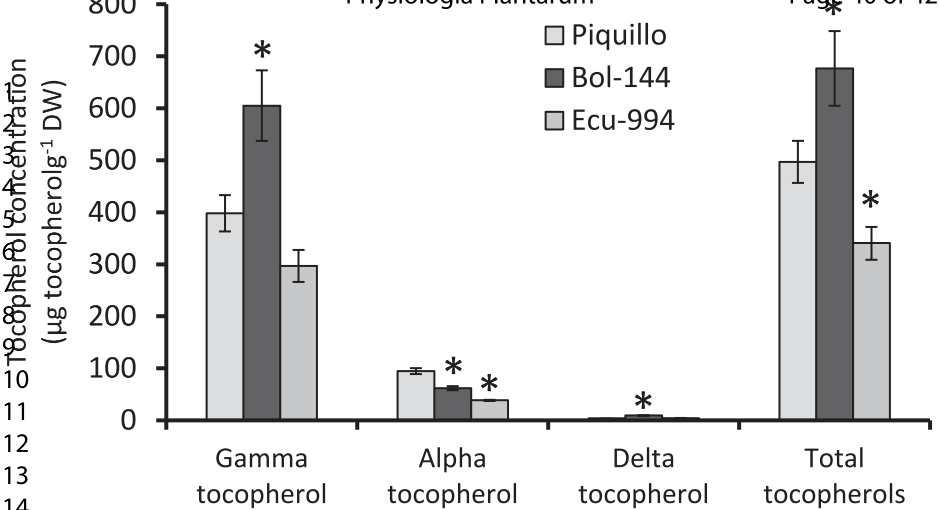
55
40
35
25
15


Tocopherol concentration
(µg tocopherolg-1 DW)
800
700
600
500
400
300
200
100
0
Piquillo
Bol-144
Ecu-994
*
Gamma tocopherol
Alpha tocopherol
Delta tocopherol
Total tocopherols

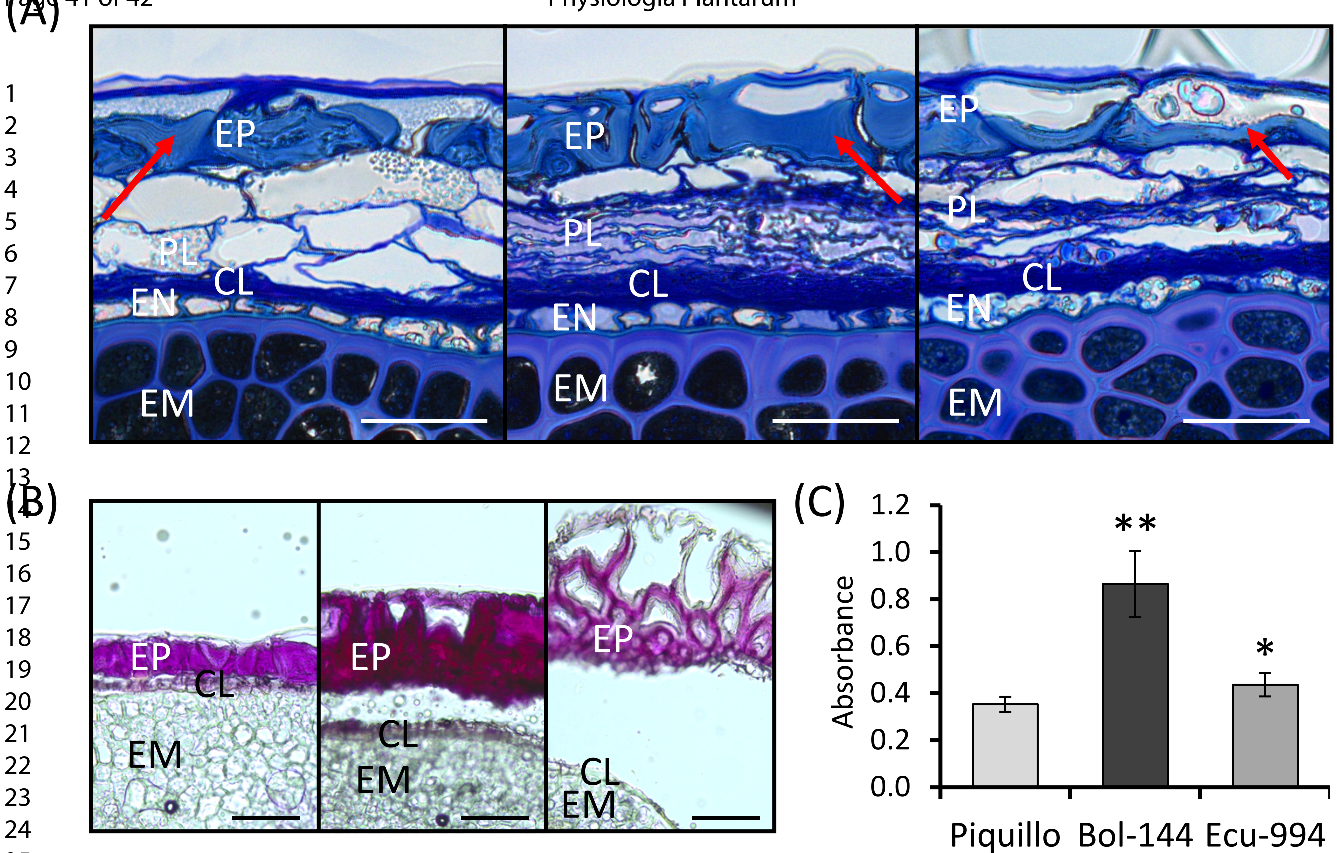

(A)
EP
PL
CL
EN
EM
(B)
EP
CL
EM
(C)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance
**
*
Piquillo
Bol-144
Ecu-994

Table 1. Seed longevity orthologous genes in *C. annuum*. To identify orthologous genes in pepper corresponding to Arabidopsis genes implicated in seed longevity, we utilized Plaza by Ghent University, specifically, the Integrative Orthology Viewer (https://bioinformatics.psb.ugent.be/plaza).

Arabidopsis thaliana	*Capsicum annuum*
AT2G36270 (ABI5)	*CAN.G397.17*
AT3G24650 (ABI3)	*CAN.G771.55 / CAN.G771.56*
AT5G65410 (AtHB25)	*CAN.G358.72*
AT3G48330 (PIMT1)	*CAN.G126.92*
AT4G33950 (*SnRK2.6*)	CAN.G836.53
AT1G21710 (OGG1)	CAN.G1391.3
AT4G32770 (VTE1)	CAN.G21.50
AT2G18950 (VTE2)	CAN.G33.20
AT1G01470 (LEA14)	CAN.G78.132
AT3G18490 (ASPG1)	CAN.G587.80
AT1G56600 (GOLS2)	CAN.G358.49
AT1G21970 (LEC1)	CAN.G1156.20
AT3G26790 (FUS3)	CAN.G473.153