UNDERSTANDING THE IMPACT OF LOW TEMPERATURES IN MAIZE SEED GERMINATION

Submitted to the University of Hertfordshire in partial fulfillment of the requirement of the degree of Master by Research, December 2020.

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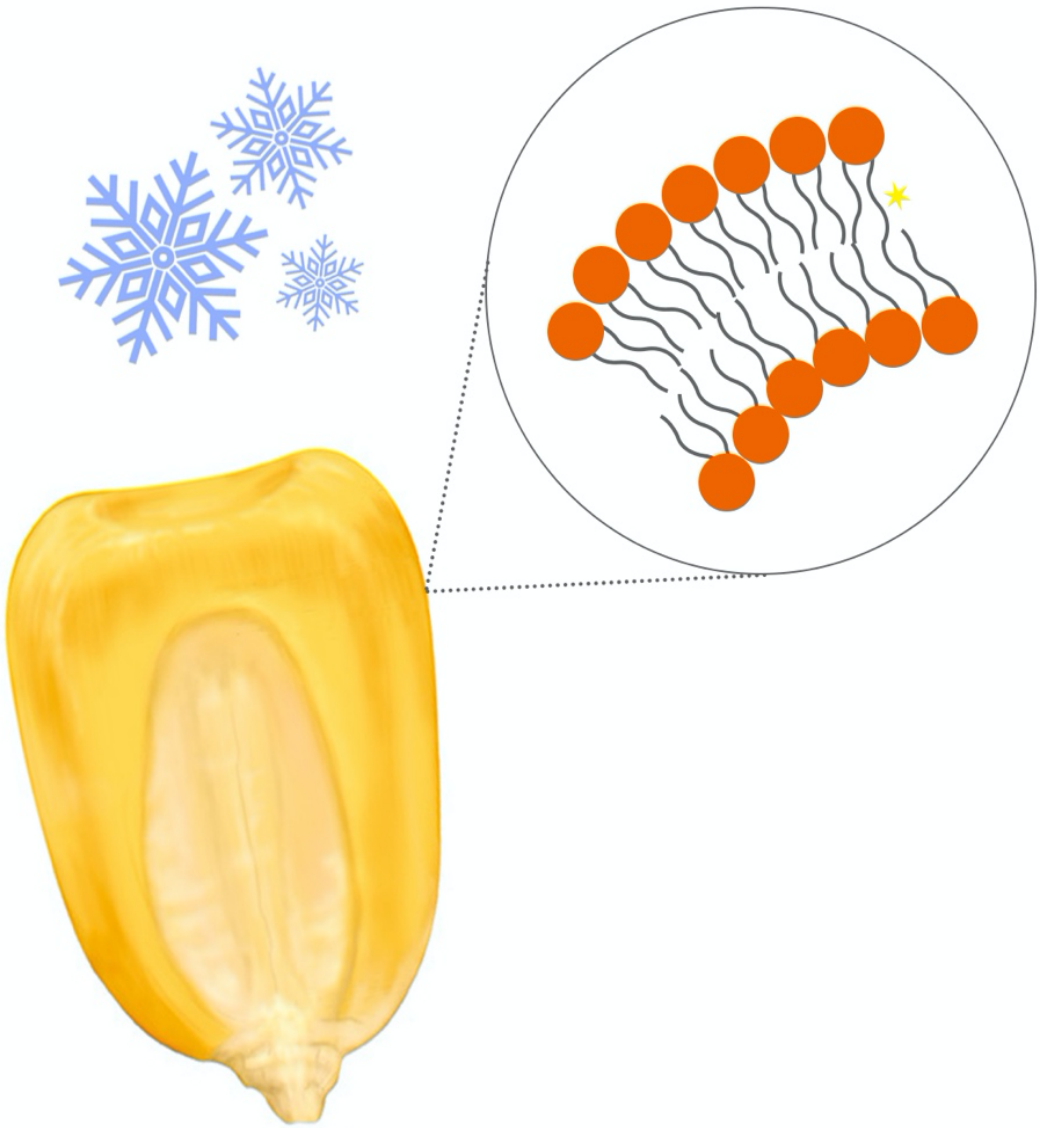

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SUMMARY

Maize (*Zea mays*) is one of the most widely cultivated crops worldwide; however, it is also considered as a chilling sensitive plant. Plants subjected to suboptimal temperatures suffer changes in numerous chemicals, metabolic and physiological responses. Thus, chilling results in decreased field productivity and quality and causes significant crop losses. Low temperatures are an important limiting factor that affects every plant developmental stage, especially germination. Germination is one of the most important plant phases, which has a huge impact on its future performance. Chilling temperatures cause modifications in the seed membrane structure and fluidity originating in the solid-gel phase. The lipids, which constitute this solid-gel phase, are more tightly packed, creating a more rigid and less fluid membrane, which obstructs water uptake and germination. The aim of this project is to study the role of desaturase enzymes during seed germination in two different maize cultivars (American cultivar, W2080 and European cultivar, Oxxgoode). Fatty Acid Desaturases (FAD) are enzymes responsible for regulating seed membrane fluidity by inserting double bonds in the fatty acid chain of the membrane bilayer. A total of 30 fatty acid desaturase genes have been identified in maize, which are distributed on the maize chromosomes. Based on the phylogenetic analysis, desaturase genes are classified in five different subgroups, regarding their mode of action and gene structures. Expression analysis reveals that *FAB2.3*, *FAB2.8, DES, FAD2.1, FAD6, FAB2.11, FAD7, SLD3* and *FAD7* genes play an important role during seed germination in both cultivars. However, under chilling conditions the gene upregulation varies; the American cultivar, W2080, shows a significantly higher level of gene expression of *FAD2.1, SLD3, FAB2.4* and *FAB2.8* genes while *FAD2.1* and *FAD2.2* desaturase genes are upregulated in the European cultivar, Oxxgoode. In conclusion, *FAD* genes play an important role in chilling stress response during maize seed germination.

1. INTRODUCTION

1.1. Maize germination under suboptimal temperatures.

Maize is one of the most widely cultivated temperate cereal crops worldwide (Sandhu & Singh, 2007). The Food and Agriculture Organization (FAO) estimated that the total world production of maize was 1046 million tonnes in 2018 (*USDA - National Agricultural Statistics Service - Data Visualization*, 2020.). The demand for and consumption of maize is increasing annually; however, cold temperatures threaten crop productivity and food security. This grass is considered a chilling sensitive crop as its optimum temperature for germination is between 20-28°C (Greaves, 1996; Zheng, 1991). When maize is exposed to low temperatures (suboptimal), direct damage and physiological defects occur, reducing the maize crop quality, production and utilization potential.

Temperature is an environmental factor that can alter plant development. Cold temperatures induce two different stresses: chilling stress and freezing stress. Plants suffer chilling stress when exposed to temperatures below 15 ºC and above the freezing point of the plant tissue. Freezing stress causes damage when plants are subjected to temperatures below the freezing point (Jackman *et al.*, 1988; Jan & Andrabi,2019). Chilling tolerant plants can with stand temperatures below 10ºC without injury or damage (Yadav, 2010). In particular, chilling temperatures cause a decline in seed germination (Wilson, 1985). Seeds germinated under chilling stress produce plants that show symptoms throughout their lifetime (Yadav, 2010). Low temperature leads to changes in numerous chemical, metabolic and physiological responses, which reduce the rate and length of the germination process.

Seed germination is one of the most important and complex stages of the plant life cycle governed by both internal and environmental factors (Bentsink & Koornneef, 2008). This process starts with the absorption of water by the dormant dry seed and terminates with the radicle protrusion through the seed surface (Figure 1). Germination is a triphasic process (Nonogaki *et al.*, 2010).

- Phase I: Firstly, seeds undergo a rapid imbibition phase, perturbing the membrane structure and allowing rapid leakage of solutes. Also, there are phospholipid (PL) composition changes within the membrane, maintaining and enhancing its integrity. Pre-existing mitochondria initiate the basic metabolism activity which provides adequate amounts of ATP to support initial germination steps. After imbibition, the repair in the DNA damaged during maturation drying and rehydration occur. They resume the transcriptional activity and newly synthesize proteins as germination proceeds. However, the synthesis of proteins occurs firstly using extant mRNAs in the ribosomes of the mature embryo.
- Phase II: The initial water uptake stops, and the water status remains constant. New polysomes are synthesized and protein synthesis from newly transcribed mRNA will occur until the germination concludes. These proteins are involved in cellular metabolism, cell elongation and enzymes are responsible for the mobilization of the storage reserves. DNA synthesis related with post-germinative cell division, occurs in the nuclei and new synthesized mitochondria. Radicle protrusion concludes the germination process "*sensu stricto*", and results from the turgor pressure growth of the radicle in the cell walls of the surrounding tissues as well as the weakening of the cell walls by hydrolysing enzymes.
- Phase III: A final increase in water uptake starts once the germination "*sensu stricto*" has concluded. This leads to an increase in the porosity of cell walls and intracellular spaces, which support cell division and embryo growth, mobilization of stored reserves. This occurs until seedlings become photosynthetically active (Bewley, 1997; Huang *et al.*, 2015).

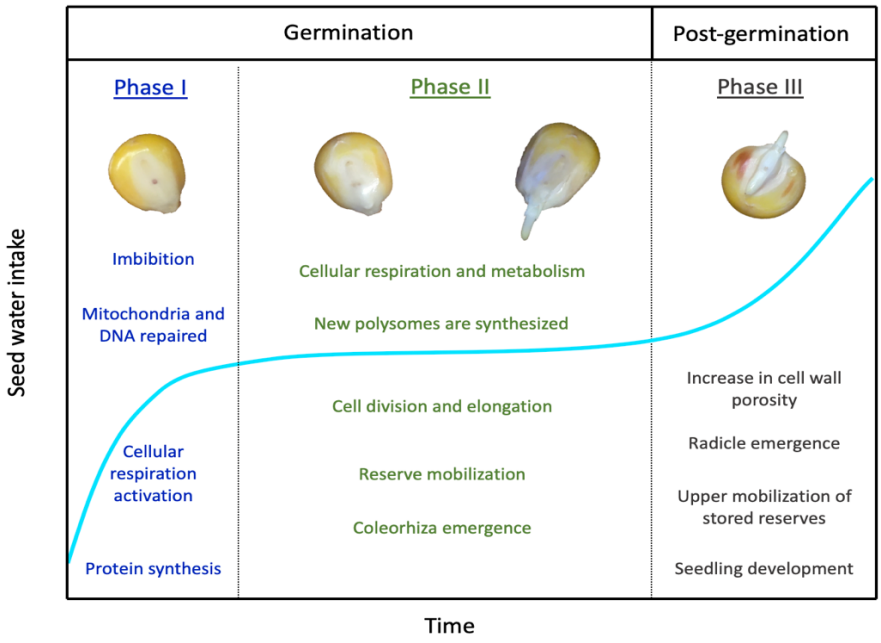


Figure 1: Time course of physical and metabolic events occurring during germination of the seed. The time for each phase can vary between species and influenced by environmental conditions. The curve shows the time course of the water uptake. Diagram adapted from Nonogaki *et al.* (2010) and Bewley *et al.* (1997)

The success and speed of the germination varies among species and even among cultivars (Saboya & Borghetti, 2012; Vallejo-Marín *et al.*, 2006). In maize, the coleorhiza is the first part to grow out, breaking the seed membrane (Wolny *et al.*, 2018). The remodelling function of the lipid membrane is an important event, which occurs upon seed germination (Yi xin Lin *et al.*, 2019). Before germination starts, dry seeds show low water content and poor membrane integrity (Simon, 1974). Imbibition can repair membrane lipid and dysfunction by changing the membrane lipid structure. The solid phase of the dry seed membranes turn to liquid phase which enhance the germination process (Yu, X., 2015).

However, abiotic stress such as chilling temperatures can impair cell membrane remodelling during seed germination, leading to delay or disruption of the process (Lin *et al.*, 2019). Low temperatures stimulate the opposite reaction, with membrane structure which is found in a flexible liquid-crystal phase changing into solid-gel phase, modifying the membrane fluidity (Figure 2) (Basra & Basra, 1997). Modification in the

seed membrane is one of the most adverse chilling effects (Basra & Basra, 1997). Membrane fluidity is determined by the number of FA that are unsaturated (double bond between two carbon atoms) and the FA chain length. A larger number of unsaturated fatty acids that have a longer FA chain provide plant resistance to chilling stress. On the one hand, longer FA arms have more extensive contact areas so extreme temperatures are required for destabilIzing the liquid-crystal phase. On the other hand, the kinks originated by the double bonds reduce packed membrane conformation and disrupt the order, giving elasticity. Thus, both factors give permeability to the membranes and improve the seed water uptake necessary for the germination process (Barrero-Sicilia *et al.*, 2017).

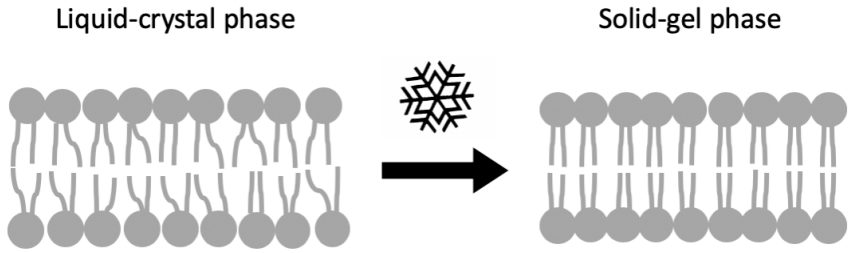


Figure 2: Lipid membrane modification by chilling temperatures. On the left side, the membrane is in the liquid-crystal phase. This phase is formed by unsaturated and/or long chain FA, which gives permeability to the membrane. Suboptimal temperatures cause a modification in the membrane structure, originating the solid-gel phase. The lipids that constitute the solid-gel phase, on the right side, are more tightly packed, creating a more rigid and less fluid membrane. Adapted from Los & Murata, 2004.

1.2. Plant membrane composition.

The plant membrane is mainly composed of phospholipids (PLs), which can constitute approximately 30% of the lipid membrane. PLs are molecules constituted of two hydrophobic fatty acids (FAs) linked by hydrophilic glycerol and a phosphate group. The synthesis of FAs in plants occurs exclusively in plastids. Plant membrane PLs include six different classes of phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS),

phosphatidic acid (PA), and phosphatidylglycerol (PG), and two classes of galactolipids: monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), which are plastidic lipids (Yu *et al.*, 2015). Specifically, in seed membranes, high levels of PA are found due to the seed desiccation process inducing its formation. In contrast, low concentrations of MGDG and DGDG are found in seeds due to the lack of plastids (Yu *et al.*, 2015).

Among the PLs bilayer can be found the sphingolipids (SL) and their phosphorylated derivates (Cacas *et al.*, 2016). They are composed of a carbon amino-alcohol backbone, sphingosine, to which a fatty acid may be attached through an amide bond and a head group at the primary hydroxyl (Merrill, 2008). They constitute a significant part of the lipids present in higher plants (up to 10% plant lipids) (Dunn *et al.*, 2004). SL are considered a diverse group with a wide range of physical properties. Thus, they are involved as metabolites mediating cellular processes in response to plant stress (Coursol *et al.*, 2003), membrane trafficking (Moreau *et al.*, 1998) and modifying of the membrane structural integrity (Borner *et al.*, 2005; Michaelson *et al.*, 2016). The essential role of sphingolipids in the membrane organization and fluidity underline their importance in plant chilling responses (Ali *et al.*, 2018).

1.3. Fatty acid synthesis.

Fatty acid synthesis is regulated in consonance with the supply and demand of acyl chains. This process mostly takes place in the plastid, where pyruvate is synthesized from the carbon flux of the photosynthesis. Then, fatty acyl chains are channelled for the production of more complex lipid molecules in the plastid or transported to the cytosol and endoplasmic reticulum (ER) (Ohlrogge & Jaworski, 1997). Firstly, the plastid enzyme, acetyl-CoA carboxylase, catalyses the formation of malonyl-CoA from acetyl-CoA by an ATP-dependent reaction. Malonyl-CoA is made in the cytosolic pool by the homomeric ACCase and used for elongation of fatty acids into long- and very-long- chain fatty acids (VLCFA) required by phospholipids, surface waxes, or sphingolipids (Roesler *et al.*, 1994). Fatty Acid Synthase (FAS) transfers the malonyl group from the malonyl-CoA to the -SH group from Acyl Carrier Protein (ACP). Condensation, reduction and elongation reactions generate the end product, which is usually a 16:0 to 18:0-ACP

utilized by a variety of enzymes in the plastid, ER or cytosol (Fatland *et al.*, 2005; Guschina & Harwood, 2007; Li-Beisson *et al.*, 2013; Ohlrogge & Jaworski, 1997; Wada *et al.*, 1997).

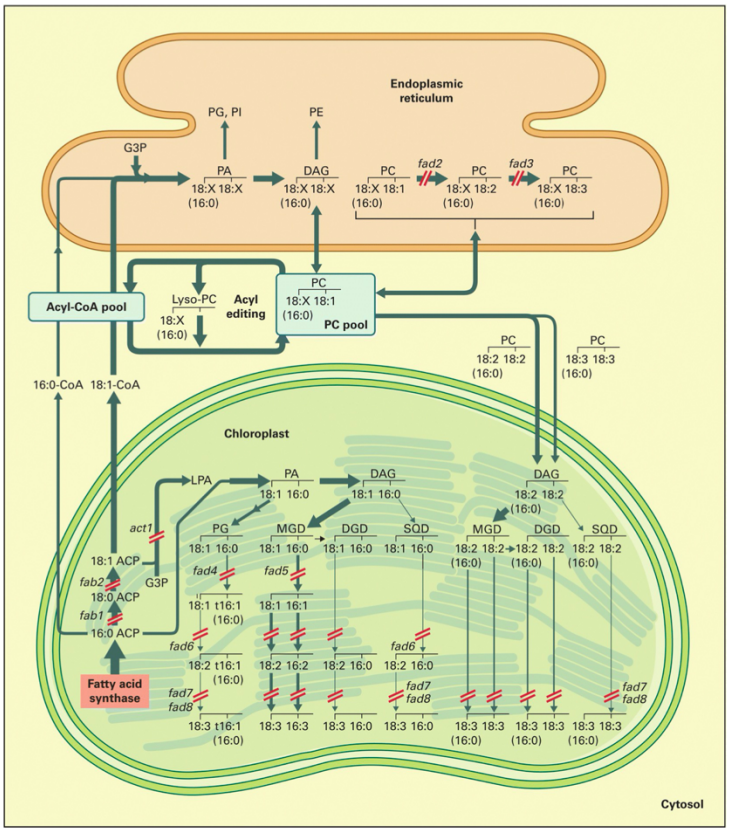


Figure 3: Abbreviated scheme for lipid synthesis in leaves of *Arabidopsis*. Reactions happening within the chloroplast represent the prokaryotic pathway while the eukaryotic pathway is constituted by reactions in the ER and successive transfer to the chloroplast. The red breaks in the pathway indicate the gene mutations obtained in *Arabidopsis*. The width of the arrows represents the relative flow between the route stages. ACP, acyl carrier protein; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; DGD, digalactosyldiacylglycerol; G3P, glycerol 3- phosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; MGD, monogalactosyldiacylglycerol; SQD, sulfoquinovosyldiacylglycerol. Figure 3 from Buchanan & Gruissem, (2015).

Modifications in the ER involve elongation of the chain-length by the Fatty Acid Elongase (FAE) and membrane bound desaturation (Shanklin & Cahoon, 1998) (Figure 3). Fatty acid desaturation is regulated by the desaturase enzymes. The C16 to C18 acyl chains produced are subsequently desaturated by chloroplast and ER membrane-bound desaturases, which regulate the membrane fluidity. Extra-chloroplast lipid desaturation is catalysed by FAD2 and FAD3, whereas FAD4, FAD5, FAD6, FAD7, and FAD8 desaturations take place in the chloroplast (Somerville & Browse, 1991; Upchurch, 2008; Wallis & Browse, 2002). Finally, fatty acyl chains can also be remodelled to produce complex lipids such as glycolipids, galactolipids, phospholipids, sphingolipids monogalactosyldiacylglycerol (MDGD) and digalactosyldiacylglycerol (DGDG) (Benning, 2008).

1.4. Fatty acid desaturase family

Fatty acid desaturases are enzymes responsible for the removal of two hydrogen atoms from a fatty acid, creating a double bond. The double bond can be cis or trans depending on the side of the carbon chain; cis-double bonds are found on the same side of the carbon chain while trans are on opposing sides. Desaturase enzymes regulate the membrane fluidity, synthesize molecules involved in the signalling pathways, and determine the nutritional value of vegetable fats (Los & Murata, 2004; Shanklin *et al.*, 2009). These enzymes act in different cell locations, use different electron donors and catalyse different substrates (Shanklin *et al.*, 2009).

Classification based in the cell location and electron donors:

- Membrane-bound FABs: Most plant desaturases are membrane-bound proteins located in the ER and chloroplast membrane. Desaturases located in the endoplasmic reticulum act on phosphatidylcholine, and possibly on other phospholipids using cytochrome b5 as an electron donor (Gargallo, 2010).
- Soluble FADs: they are situated freely in chloroplasts and are specific for saturated acyl-ACP fatty acids esterified to galactolipids, sphingolipid,

sulpholipids and phosphatidylglycerol (Shanklin & Cahoon, 1998). These enzymes catalyse the introduction of double bond saturated FA. The active site of the enzyme is constituted by a diiron centre (Fe-O-Fe) and use ferredoxin as an electron donor (Gargallo, 2010).

Classification based in the desaturation insertion (Tocher *et al.*, 1998b):

- Acyl-desaturase (FAB2) are enzymes responsible for the desaturation of 18:0-ACP at the Δ9 position.
- Fatty acid ω-6 desaturase (FAD2 and FAD6) both carry out double bond insertion in the ω-6/Δ-12 position. Differing from each other in the cell location, FAD6 acts in the plastid while as FAD2 acts in the endoplasmic reticulum.
- Fatty acid ω-3 desaturase (FAD3, FAD7 and FAD8) converts linoleate (18:2) substrates esterified to plasma cell and plastid lipids to linolenate (18:3).
- Fatty acid Δ3 desaturase (FAD4), introduction of trans double bond phosphatidylglycerol Δ16 position.
- Sphingolipid Δ4 desaturase (DES), insert of *trans* double bond between the Sphingolipid Δ4 and the Δ5 position.
- Sphingolipid Δ8 desaturase (SLD), catalyses the desaturation of sphingolipid at the Δ8 position.

Desaturases carry out the enzymatic reaction in which a double bond is inserted into the acyl chain and a molecule of dioxygen is reduced to water (Shanklin & Cahoon, 1998a). In addition, two electrons from the desaturase electron transport system are also necessary to create the double bond. Depending on the cellular location, desaturases have different electron transport systems. On the one hand, desaturases located in the chloroplast use the ferredoxin system, where ferredoxin is reduced, and supply desaturase electron needs for catalysis. On the other hand, desaturases located in the endoplasmic reticulum utilize the cytochrome b5 electron transport system. The catalytic mechanism of these enzymes has not been characterized. The hypothesis for the fatty acid desaturation mechanism by the ferredoxin system is illustrated in Figure 4.

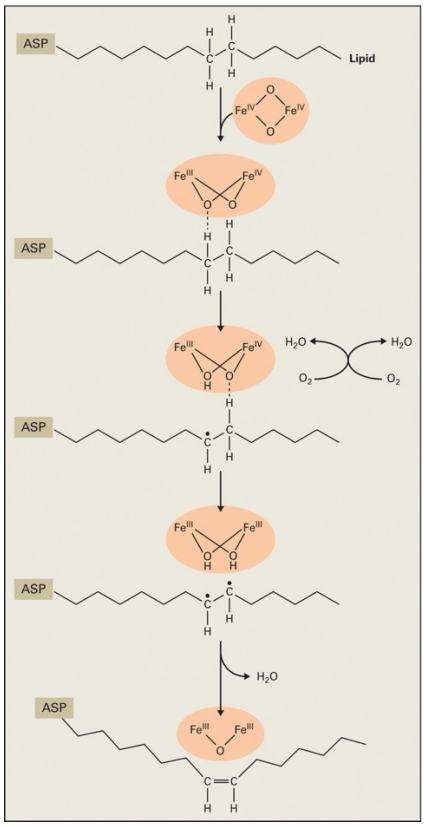


Figure 4: Scheme for the proposed catalytic mechanism for fatty acid desaturation. At the top, the desaturase diiron centre is oxidized (diferric, or FeIV–FeIV) with a μ-oxo bridge. Then, an iron ion is suffering a reduction reaction which results in the reduced (FeIII–FeIV) form. Secondly, the scheme represents the hydrogen abstraction from the methylene group of the inactivated fatty acid to yield a radical intermediate. Then, loss of the second hydrogen happens. The last reaction is the formation of the double bond along with the loss of H^2O and regeneration of the oxidized active site and the μ-oxo bridge (Buchanan & Gruissem, 2015).

Previous studies in a range of PLs report their key role in response to chilling stress (Dong *et al.*, 2016; Zhang *et al.*, 2018; Zhao *et al.*, 2019). Their actions desaturating FA provide more fluidity to the membranes and better chilling resistance (Barrero-Sicilia *et al.*, 2017). Silenced-*FAD8* rice mutants show a higher degree of sensitivity to chilling conditions (Tovuu *et al.*, 2016). In *Arabidopsis,* mutation of the *SLD1* and *SLD2* genes that lack Δ8 unsaturation reveal the significance of the sphingolipid structural diversity in the membrane for improving the plant adaptation to suboptimal temperatures (Chen *et al.*, 2012). Also, a SLD-silenced mutant of tomato plants had reduced chilling resistance (Zhou *et al.*, 2016).

1.5. Regulation of the genes involved in chilling response.

Gene expression induced by chilling stress can be classified as transient or long-term and is regulated in a complex regulatory gene network mediated by transcription factors (TFs) (Yamaguchi-Shinozaki & Shinozaki, 2006). TFs are responsible for activating the transcriptional cascade and regulate chilling responses by gene expression. *Cis*-regulatory elements associated with some TFs can be predicted in the promoter regions of the genes and provide primary information about the regulatory process in response to chilling stress (Yamaguchi-Shinozaki & Shinozaki, 2005). Recent transcriptomic analyses have revealed that a 1000 TFs show differential expression when maize plants experience low temperatures (Zhao *et al.*, 2019). The main TF families responsible for modulating the activity of the promoters are AP2/ERF, WRKY, C2H2, bHLH, bZIP, MYB and MYC and Zinc-finger proteins (Lenka & Bansal, 2019; Zhao *et al.*, 2019). Hormone signalling is an important pathway which modulates the chilling-stress regulation network. Specifically, ABA hormone plays an important role and its ABA-inducible promoters have identified cis-acting elements that confer the ABA response, ABREs ABA-independent regulon (ABRE) (Mishra *et al.*, 2014). A dehydration responsive element (DRE) is another cis-regulatory element which induces the ABA-independent regulation pathway to dehydration and chilling response. Both, ABRE and DRE are major cis-regulatory elements in abiotic stress-inducible gene expression (Yamaguchi-Shinozaki & Shinozaki, 2005).

The aim of this project is to evaluate the impact of chilling temperatures on two different maize cultivars. Maize cultivars have been chosen based on the climate conditions of the locations where the cultivars are usually planted (Figure 5). Oxxgoode maize cultivar is from North Europe where the oceanic climate provides a narrow range of temperatures (between 0ºC and 22ºC) and there is no dry season. This cultivar is planted from February to May and harvested from June to September. The second maize cultivar, W2080, is from California (USA). W2080 cultivar are sown in month of April and May and harvested during August and September. This cultivar is grown under Mediterranean climate conditions which are characterised by dry summers and mild, wet winters (Lionello *et al.*, 2006). Moreover, we have identified the desaturase enzymes, responsible for the double bond FA insertion and membrane fluidity, in maize.

In order to explore the gene expression of the desaturase genes when maize seeds are germinated under chilling temperatures.

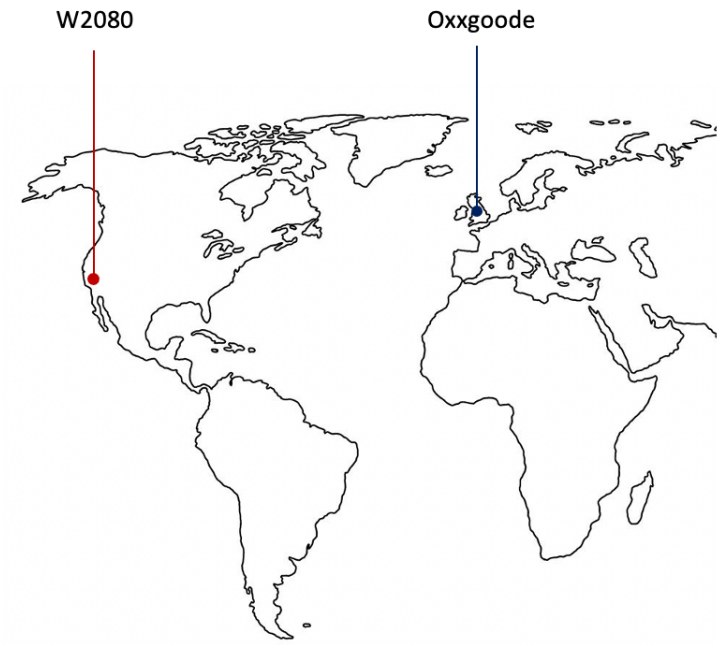


Figure 5: Map of the maize cultivars' origin. Red spot represents the original location of the W2080 cultivar, while as Oxxgoode cultivar origin is represented with a blue spot.

2. OBJECTIVES

- To study the germination kinetics of two different maize cultivars germinated under control and chilling temperatures.
- To identify and characterise the fatty acid desaturase genes present in maize and analyse their phylogenetic relationships.
- To analyse gene expression of desaturase genes expressed in seed during maize germination under chilling conditions.
- To predict cis-regulatory elements in the promoter of desaturase genes showing differential expression under chilling conditions.

3. MATERIALS AND METHODS

3.1. Plant material

Two different maize (*Zea mays*) cultivars, Oxxgoode (European cultivar) from RAGT company and hybrids W2080 (American cultivar) from Wyffels seed company were used for this study.

3.2. Germination kinetics assay

For germination assays, two different maize (*Zea mays*) cultivars, Oxxgoode (European cultivar) from RAGT company and hybrid W2080 (American cultivar) from Wyffels seed company, were bought and used for this study. Three replicate sets of 25 non-stratified seeds were surface sterilized with 1% NaOCl for 10 min and washed several times with sterile water. Then, seeds were placed on Petri dishes containing two filter paper discs each, moistened with 10 ml of sterile water. Germination was carried out at 5ºC, 10ºC, 15ºC and 20ºC in Controlled Environment Cabinets (Sanyo MLR-352-PE) in darkness. Seeds were scored as germinated when the coleorhiza had emerged beyond the husk. Germination rate was measured every 12 hours for each temperature treatment for 12 days. The results were presented as means of the germination percentages obtained from three replicates. The germination curve and T_{50} value is represented in Figure 6A-B. Besides, the germination curve fits to a logistic equation.

$$y = \frac{a}{1 + \left(\frac{t}{T50}\right)^{-b}}$$

In the equation," y" is the percentage of seed that germinated," t" is the days since the start of the experiment, "a" is the asymptote parameter that is the maximum percentage of seed germination, "b" is the curvature parameter related to the speed at which percentage of seed germination reached the asymptote, and "T_{50}" is the parameter, which is the time at which the percentage of seed germination reaches half of the asymptote. Figure 6C shows the logistic equation parameters for each treatment.

For the statistical analysis, Sigmaplot Software was used to obtain the different graphs and parameters.

3.3. Characterization, chromosome mapping and phylogenetic study of the maize desaturase genes

All desaturase genes were compiled from the Gene database of the National Biotechnology Information Center (NCBI), specifying *Zea mays* as organism and 'desaturases' as the title. The desaturase gene family of *Zea mays* have been identified previously (Zhao 2019). To verify the putative desaturase genes of maize, the gene sequences of *Arabidopsis* were used as a template. A Basic Local Alignment Search for gene sequences (BLAST) was run against the maize genome in the Reference Gene database. The thresholds established to select candidate genes were: Query cover ≥ 25%, E-value ≥ e-25, Identity ≥ 25%. Finally, an R software function was run to avoid the accession repetition enclosing the candidate desaturase genes of maize.

Phylogenetic analyses were done using Mega X (Kumar, 2020). A multiple alignment of nucleotide sequences (including UTR sections) of the desaturase genes was done using the MUSCLE program with the default parameters (Robert, 2004). Phylogenetic trees were created using a Neighbor-Joining method with the nucleotide sequences (Saitou & Nei, 1987). To identify the different protein domains in maize desaturase, we analysed the protein sequences using a free-online bioinformatic tool, SMART (Simple Modular Architecture Research Tool) (http://smart.embl.de).

All desaturase genes were mapped in maize chromosomes based on the location and chromosome number information obtained from the NCBI website. The image was done using phenogram web page (http://visualization.ritchielab.org/phenograms/plot) (Wolfe *et al.*, 2013).

3.4. RNA extraction and quantitative PCR analysis

Embryos of two different maize (*Zea mays*) cultivars, Oxxgoode (European cultivar) and hybrid W2080 (American cultivar) were used for this study. Maize seeds were placed

on filter paper in Petri dishes with 10 mL of sterile water and germinated in darkness at 10ºC, in Controlled Environment Cabinets (Sanyo MLR-352-PE), and at 20ºC in darkness at the glasshouse. When the germination of the seed concluded (the coleorhiza emerged), maize germinated embryos were isolated under the microscope. Three replicates with three embryos were collected for gene expression analysis.

Embryo collected samples were ground using a mixer mill (Retsch MM400) at 300/s frequency for 1 min. RNA was isolated following the Oñate-Sánchez and Vicente-Carbajosa (2008) seeds and siliques protocol. Extraction buffer (0.4 M LiCl, 0.2 M Tris pH:8, 25 mM EDTA, 1% SDS) and chloroform was added to the ground samples. The supernatant was washed with phenol and subsequently with chloroform, and RNA was precipitated with 8M LiCl at 4ºC for 16h. Samples were treated with DNase at 37ºC for 30 min. RNA was quantified with a Nanodrop ND-1000 Spectrophotometer (Labtech International, UK) and RNA integrity was tested by running an electrophoresis 1% agarose gel.

To evaluate the gene expression of the eleven desaturase genes involved in germination under optimal (20ºC) and chilling temperatures (10ºC), quantitative PCR (qPCR) analysis was done. cDNA was then synthesized using SuperScript IV First-Strand Synthesis System from Invitrogen and SYBR Select Master Mix RT-PCR system was used to do the qPCR reaction. The qPCR programme was set up as follows: pre-incubation 95ºC for 10 min, 35 cycles of two step amplification at 95ºC for 15 sec, 65ºC for 1 min and a final melting step at 95ºC for 1 min, 60ºC for 30 sec and 95ºC for 30 sec. A total of 13 primers were designed using Primer3Plus software (Untergasser *et al.*, 2007) and used to amplify the coding regions of interest spanning an exon-exon junction (Table 1). ZmActin was used as an internal reference gene (Lin et al., 2014; Zhao et al., 2019) and the efficiency of primers was calculated using cDNA 10^{-1} dilution series. The qPCR results were analysed using the MxPro qPCR Software. The expression levels were normalised respecting the reference gene using the 2-ΔΔCt method (Livak & Schmittgen, 2001). Three biological replicates were each used in two technical replicates per plate.

Table 1: ***Primers for desaturase genes (FAB2s and FADs) table.*** The table illustrate the locus name; the primer's forward sequence (R seq) and reverse sequence (L seq); the amplicon size (bp); and the primer's efficiency, slope and temperature of dissociation (ºC) for each desaturase gene studied.

Gene	Locus	Primer R seq	Primer L seq	Amplicon size	Eficiency	Slope	Tª dissociation
ZmACT	GRMZM2G094988	TGGCTGGGTGGTGCGATATTG	CAACCCGTGCTAGTTCAAAGGC	108	98	-3.3	79.25
ZmFAB2.3	GRMZM2G026793	CATGGCGTTGAGGGTGTC	CCCAAGTACGGGTTGTTCTC	150	109.7	-3.1	74.45
ZmFAB2.4	GRMZM5G852502	TACCTTTGCACCCTTGCTTC	CAGTGTTCCCGTGTGAGATG	109	101.5	-3.2	74.45
ZmFAB2.8	GRMZM2G180399	TTGGGCTGTTTGGACGAG	TCAGTCCTAGGATCCATTCCA	117	99.8	-2.9	81.3
ZmFAB2.11	AC215690.3_FG002	GGAATGGATCCTGGAACTGA	CTTGGCATGCCTTGCAGTAT	124	113	-3.03	79.75
ZmDES	GRMZM2G078373	GGCCTTCCTCAAGATTGGTT	CGACCCGAAGAAGTAGGACA	109	109	-3.5	83.3
ZmSLD3	GRMZM2G129453	CACATTCTGGGGTGCAAAT	TCCCATTTATCCATGGGTGT	133	108.8	-3.12	83.25
ZmFAD2.1	GRMZM2G064701	AGAAGGGGAGAGACCGAGAG	ATTTTCGCTGGTTGCTGAGA	106	120	-2.88	74.45
ZmFAD2.2	GRMZM2G056252	TCGTGCCGTGATCTGTTTTA	GCACCCATCTGACTGACACA	161	112.4	-3.05	75.05
ZmFAD2.3	GRMZM2G161792	TCATCTGTATTTTGTGTCAGCAG	GGATGGCCTTCTTGATCTGA	120	113	-3.03	72.7
ZmFAD6	GRMZM2G078569	TGGATGAGCACTTTTACAATGG	GAACTGTGCCGTTTAATTGAG	104	107.2	-3.16	73.95
ZmFAD7	GRMZM2G128971	TGGAGGATTAGCCACAGGAC	TGAACCGCAGTTTCCTAGTCA	124	99.7	-3.3	82.2
ZmFAD8.1	GRMZM2G074401	CGGAGTCCCATACTTTGTATTTG	CTCCATTCCTGTCCACGGTA	105	93.1	-3.4	73.95

3.5. Gene regulation analysis of the maize desaturase genes upregulated under chilling stress.

The cis regulatory motifs potentially involved in the chilling stress response were screened from PLANTCARE database (Lescot *et al.*, 2002). The upstream sequences of the genes of the interest were obtained from the phytozome database. The upstream sequences comprise 1000 kb, preceding the 5' UTR section, which were represented with their corresponding cis regulatory motifs involved in the chilling stress and the TATA box. The image was performed using Adobe Illustrator.

3.6. Statistical analysis

Physiological experiment data obtained were summarized by means of at least three replicates and the measures of variability by standard error. To compare means between the different treatments and factors, one-way and two-way ANOVA were used with R software. Microsoft Excel and Sigmaplot software was used to create the germination curves and graphics.

4. RESULTS

4.1. Germination kinetics under chilling conditions

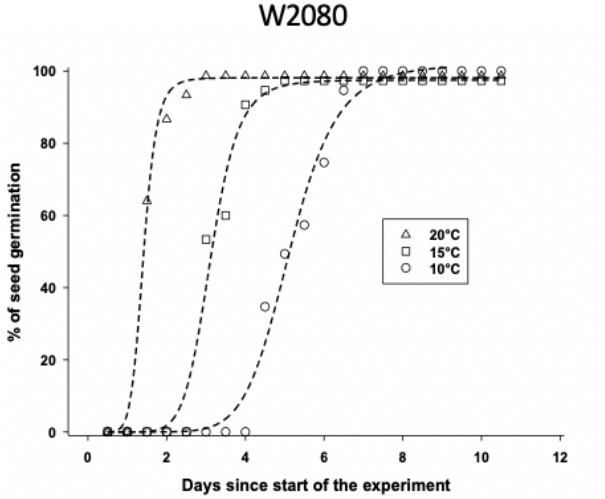
To evaluate the effect of low temperature on the germination kinetics, seed of two different maize cultivars, W2080 and Oxgoode, were analysed. We observed that seeds germinated at different temperatures, 5ºC, 10ºC, 15ºC and 20ºC, with 20ºC considered as the optimal germination temperature. The germination process was considered as completed when the coleorhiza had emerged. Figure 6 illustrates the kinetics of germination in both maize cultivars; graph A for W2080 seeds and graph B for Oxxgoode seeds. Germination curves are considered logistic curves, whose shapes depend on germination speed. Both graphs reveal the percentage of germinated seeds and the time taken to complete the process.

These maize cultivars show a different chilling tolerance, they were strongly inhibited at 5ºC, no germination occurred. 5ºC is considered as the critical chilling temperature, which interrupts the germination process.

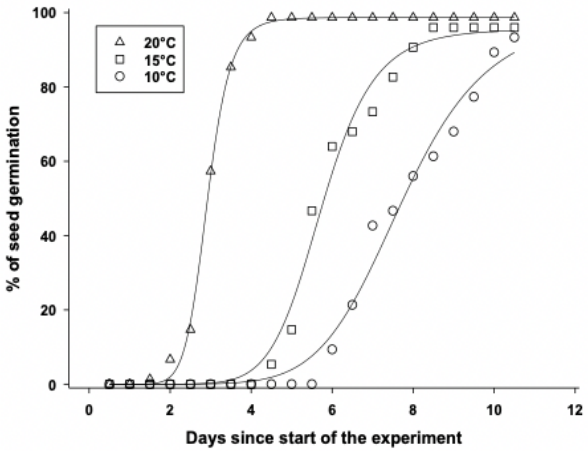
Seeds of W2080 and Oxxgoode variety have a similar percentage germination (98%) at 20ºC within 60 h and 84 h, respectively (Figure 6A and Figure 6B). The maximum percentage of germination reduced to 97% for W2080 seeds and 96% for Oxxgoode seeds at 15ºC. On one hand, Oxxgoode germination percentage remained the same, 98% at 10ºC. On another hand, W2080 germination increased to 100%. However, no significant difference was observed between cultivars or between temperatures in the maximum germination percentage.

Furthermore, in graph A, the W2080 germination curve is divided in two phases: a rapid increase, followed by a gentle increase until seeds reached the final range. However, the Oxxgoode germination curve (in graph B) also displayed a steady increase phase at the beginning of the germination process. Oxxgoode cultivar required 5.9 days to fully germinate, when seeds of W2080 germinated after 2.8 days at 20ºC. To confirm our observation, T_{50} values (Temperature at which 50% germination was achieved) were calculated with T_{50} values for the four experimental temperatures (Figure 6C). The T_{50} value was a larger for Oxxgoode when compared to W2080 seeds at chilling temperatures (7.74 days and 5.14 days at 10ºC and 5.75 days and 3.12 days at 15ºC, respectively) and optimal temperature (2.92 and 1.41 days at 20ºC, respectively).

A



B



C

Variety	Temperature	a	b	To	R^2(%)
USA	20°C	98.13	8.39	1.41	99.4
	15°C	97.48	8.88	3.12	98.5
	10°C	100.75	8.55	5.14	99.0
EU	20°C	98.71	10.28	2.92	99.9
	15°C	95.63	8.86	5.75	99.1
	10°C	98.96	7.28	7.74	98.5

Figure 6. **Germination curve and T_{50} for both maize cultivars.** The relationship between the percentage of seed germination and the days since the start of the experiment at three temperatures for American cultivar, W2080 (A) and European cultivar, Oxxgoode (B). Table (C) displayed the logistic equation values: a (maximum percentage of seed germination), b (seed germination speed) and T_{50} (time to reach 50% of final/maximum germination); and the variance accounted for (R^2) for each tª treatment and cultivar.

Ultimately, we compared seed germination speeds of the germinating seeds (b). Where W2080 shows no difference among the temperature treatments, Oxgoode cultivar decreased germination speed parallel with decreasing temperature. Different germination kinetics patterns are seen between cultivars at any temperature.

To study the linear effect of chilling temperature on the germination stage, a broken linear model was constituted. Figure 7 represents the germination rate ($1/T_{50}$) at a range of temperatures (from 5ºC to 20ºC) for both maize cultivars. The trendline was used to calculate the critical temperature (CT) at which both maize cultivars were not able to germinate. Their trendline equations and the coefficient of determination were y = 0.0011x – 0.0055 and R^2 = 0.97 for Oxxgoode and y = 0.0021x – 0.0123 and R^2 = 0,98 for W2080. From these data, we obtained the CT value for Oxxgoode (5ºC) which was one degree more chilling tolerant than W2080 (6ºC). However, the increase in the germination rate with temperature was less rapid for Oxxgoode than for W2080.

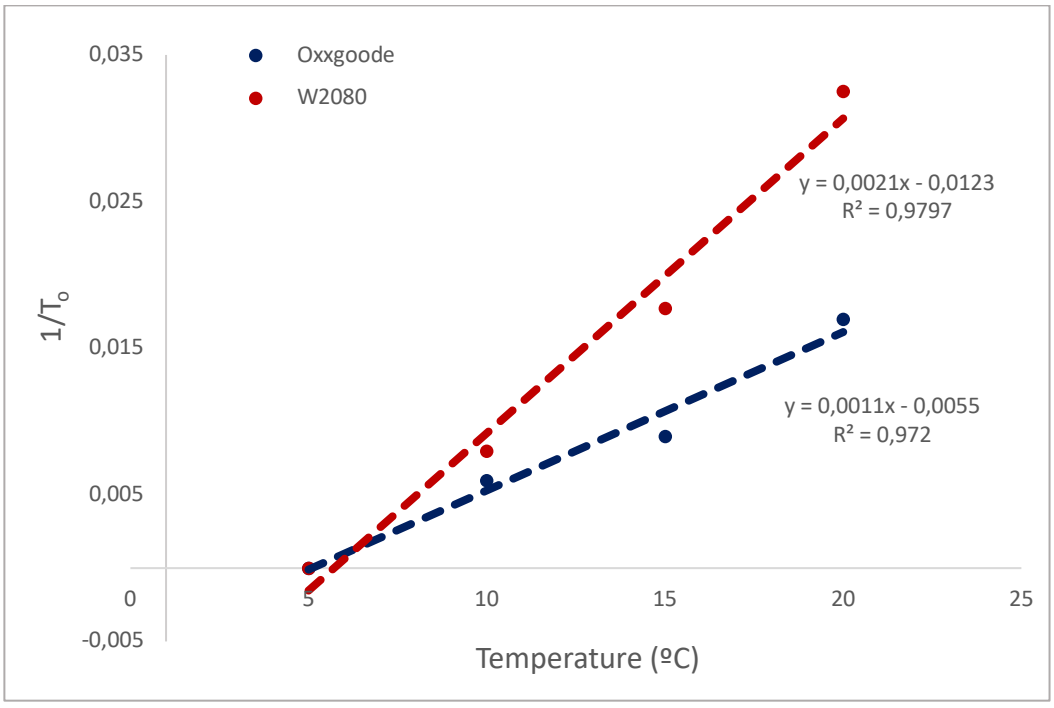


Figure7. **Relationship between temperature and germination speed of two maize cultivars.** Temperature (20ºC, 15ºC, 10ºC and 5ºC) against $1/T_{50}$ germination is represented for Oxxgoode (blue) and W2080 (red) by average values.

4.2. Characterization, chromosome mapping and phylogenetic study of the maize desaturase genes.

Resilience to chilling temperature during maize germination can be a consequence of the plasticity of seed membranes. Desaturases are enzymes responsible for lipid desaturation, which enhance membrane fluidity, hence chilling tolerance. We have characterized the maize desaturase genes to study their role during maize germination under chilling temperatures. A total of 30 genes were characterised, encoding different desaturase genes in maize (Zhao *et al.*, 2019). Among them, 17 genes codify membrane-bound fatty acid desaturases (FADs) and 13 genes of soluble stearoyl-ACP (Acyl-carrier protein) desaturases (FABs). Table 2 shows the fatty acid desaturase classes present in maize.

To confirm Zhao *et al.* (2019) results, a blast against the maize genome was done using NCBI tools and database to obtain the full gene sequences of FAD and FAB2 desaturase genes. Then, its phylogenetic tree was also estimated with desaturase sequence alignment to study the evolutionary history and relationships of this gene family in maize (Figure 8).

The phylogenetic tree contains two predominant branches which separate membrane-bound FADs and soluble FAB2s. Fewer *FAD6* and *DES* genes were found in an independent section; the rest of the desaturase genes were clustered in different groups.

Soluble FAD desaturases are divided into three subgroups; subgroup I contains the Sphingolipid Δ8 desaturase (*ZmSLD1, ZmSLD2, ZmSLD3*) and fatty acid Δ3 desaturase genes (*ZmFAD4.1 and ZmFAD4.2*). Then, subgroup II comprises the fatty acid ω-6 desaturase genes (*ZmFAD2.1, ZmFAD2.2, ZmFAD2.3, ZmFAD2.4 ZmFAD2.5 and ZmFAD2.6s*). Finally, subgroup III contains the fatty acid ω-3 desaturase genes (*ZmFAD8.1 ZmFAD8.2, ZmFAD7 and ZmFAD3*).

Membrane FABs are divided into; one group formed from *ZmFAB2.1, ZmFAB2.2, ZmFAB2.5, ZmFAB2.6, ZmFAB2.7, ZmFAB2.9, ZmFAB2.10, ZmFAB2.12* and *ZmFAB2.13*; and the second group of acyl desaturases including the *ZmFAB2.3, ZmFAB2.4, ZmFAB2.8* and *ZmFAB2.11* genes

Table 2: ***Classification of the desaturase genes in* Zea mays (reviewed by Tocher *et al.*, 1998).**

Fatty acid desaturases		**Genes**	**Function**
FADs	Sphingolipid Δ4 desaturase	*ZmDES*	Insertion of *trans* double bond between the Sphingolipid Δ4 and the Δ5 position
	Sphingolipid Δ8 desaturase	*ZmSLD1, ZmSLD2, ZmSLD3*	Desaturation of sphingolipid at the Δ8 position
	Fatty acid ω-6 desaturase	*ZmFAD2.1, ZmFAD2.2, ZmFAD2.3, ZmFAD2.4 ZmFAD2.5 and ZmFAD2.6s, and ZmFAD6*	Double bond insertion in the ω-6/Δ-12 position. FAD6 is localized in the plastid while as FAD2 in the endoplasmic reticulum
	Fatty acid ω-3 desaturase	*ZmFAD8.1 ZmFAD8.2, ZmFAD7 and ZmFAD3*	Conversion of linoleate (18:2) substrates esterified to PC or plastid lipids to linolenate (18:3)
	Fatty acid Δ3 desaturase	*ZmFAD4.1 and ZmFAD4.2*	Introduction of trans double bond in phosphatidylglycerol Δ16 position.
FABs	Acyl-desaturase	*ZmFAB2.1, ZmFAB2.2, ZmFAB2.3, ZmFAB2.4, ZmFAB2.5, ZmFAB2.6, ZmFAB2.7, ZmFAB2.8, ZmFAB2.9, ZmFAB2.10, ZmFAB2.11, ZmFAB2.12 and ZmFAB2.13*	Desaturation of 18:0-ACP at the Δ9 position

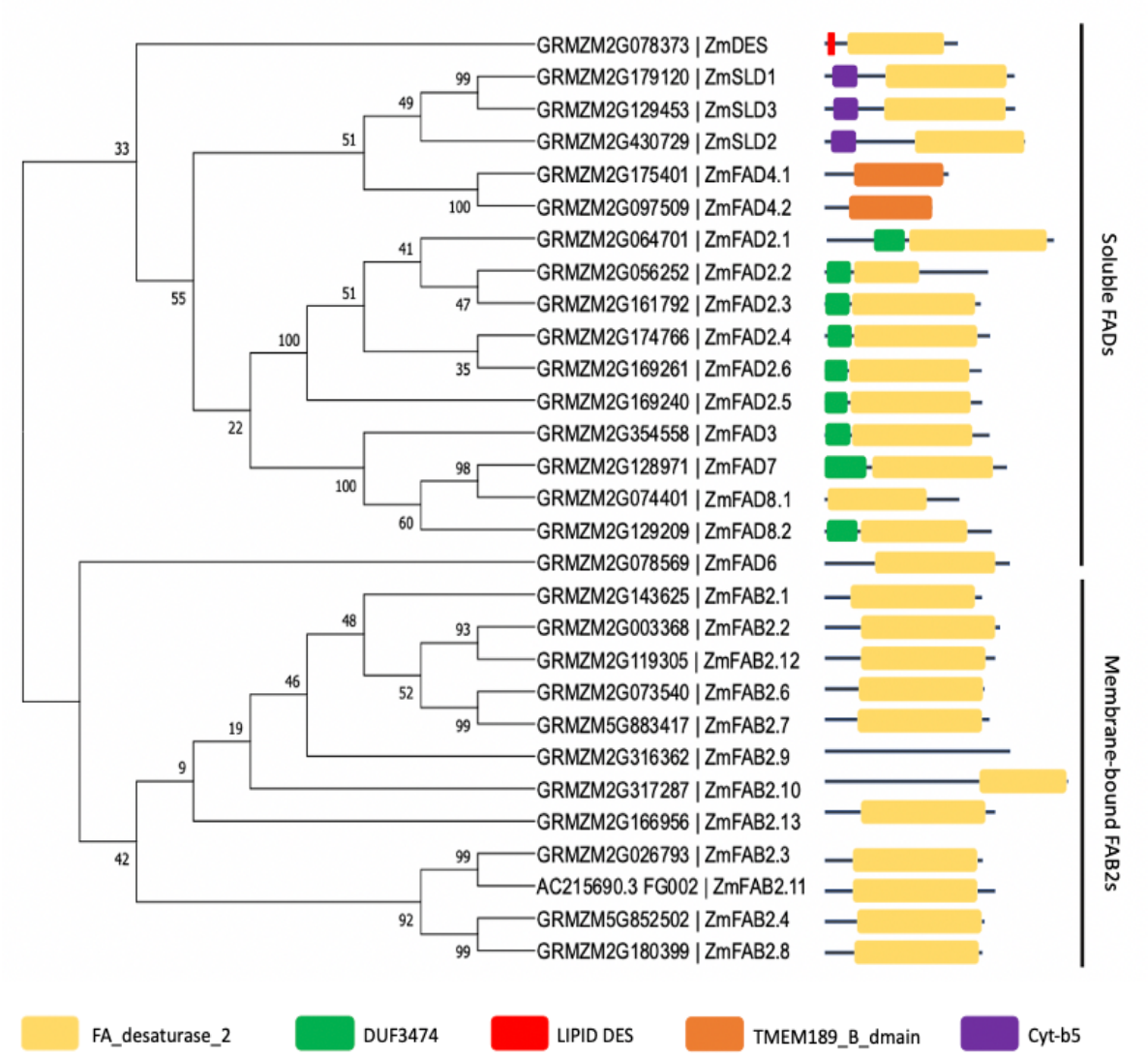


Figure 8: **Phylogenetic relationships between fatty acid desaturase (FADs) genes in maize.** Black lines indicate the different desaturases subgroups: Soluble FADs and Membrane-bound FAB2s. Protein domains of desaturase genes are represented by coloured squares. The phylogenetic tree was done in MEGA X.

The conserved and functional protein domains were analysed, to study their similarity and protein structures. Most desaturase proteins contain a unique domain "FA_desaturase_2" in a major part of their sequence, except for FAB2.9 that is characterised by no domains. In addition, FAD4.1-2 presents a single "TMEM189_B_dmain" instead. Besides a "FA_desaturase_2" domain, a second domain is present in the rest of the FAD proteins. At the N-terminal part of the SLDs protein is found the "cyt-b5" domain. Also, ZmDES presents a "LIPID DES" domain in the N-

terminus. Finally, Most of FAD protein sequences (FAD2.1-6, FAD7 and FAD8.2) harbour a “DUF3474” domain.

Chromosomal ideograms help to obtain additional data on the evolution and relationships among the desaturase gene family. Desaturase gene sequences and maize chromosome information were obtained from the NCBI database and used to construt the chromosomal map (Figure 9). FAD and FAB genes are distributed among the maize chromosomes except chromosome 6, which does not contain any desaturase genes. In addition, several gene clusters were found on chromosome 3 (*SLD2 and FAD3*), chromosome 5 (*FAD2.2* and *FAD2.3*), chromosome 8 (*FAB2.8 and FAB2.9*) and three clusters on chromosome 10 (*FAD2.5 and FAD2.6; FAD4.2, FAD4.1 and FAB2.11; and FAB2.11 and FAB2.13*). The remaining desaturase genes are distributed unevenly in the rest of chromosomes. Chromosome 4 and chromosome 9 contain just one desaturase gene (*FAD2.1* and *FAD7*, respectively); two FABs genes (*FAB2.8* and *FAB2.9*) in chromosome 9; three chromosomes (3, 5 and 7) with three genes; two chromosomes (1 and 2) with four genes and chromosome 10 carrying seven desaturases genes.

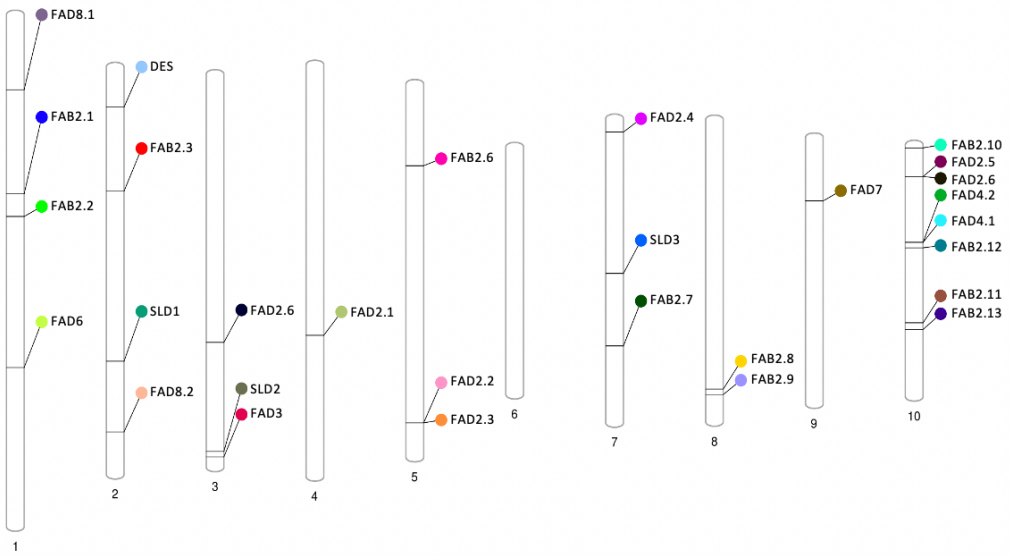


Figure 9: **Chromosome localization of the maize desaturase genes.** The chromosome numbers are indicated below each vertical bar, and the scale of the chromosomes is in megabases (Mb).

Most of desaturases are randomly dispersed in the maize chromosomes. Nevertheless, we have found that chromosomes 3, 4 and 9 specifically embody FAD genes and chromosome 8 displays only FAB duplicated gene pairs (*FAB2.8* and *FAB2.9*). Duplication events also occur in chromosomes 3 (*SLD2* and *FAD3*), 5 (*FAD2.2* and *FAD2.3*) and 10 with 3 duplicated gene association (*FAD2.5* and *FAD2.6; FAD4.1* and *FAD4.2; and FAB2.11, FAB2.12* and *FAB2.13*).

4.3. Expression patterns of maize desaturase genes germinated at optimal and suboptimal temperatures in two different maize cultivars.

Previous studies reveal that desaturase genes play an important role in response to chilling stress (Cossins *et al.*, 2002). To evaluate the role of the desaturase genes during germination in our two different maize cultivars, a gene expression analysis was done. We targeted 12 desaturase genes identified by Zhao *et al.* (2019) as germination-related genes in maize (*FAD2.1, FAD2.2, FAD2.3, FAD6, FAD7, FAD8.1, DES, SLD3, FAB2.3, FAB2.4, FAB2.8* and *FAB2.11*). A total of 13 primers were designed and used to amplify the coding regions of interest of the selected genes, including ZmActin, the internal reference gene. The heatmap (Figure 10) represents the relative gene expression values of 12 desaturase genes of interest (relative to ZmActin) Embryos from seeds germinated at 20ºC (optimal germination temperature) and 10ºC (chilling temperature) in both cultivars, W2080 and Oxxgoode were used.

The results show that most of the *FAB2s* genes studied (*FAB2.3, FAB2.8* and *FAB2.11)* were highly expressed, along with *FAD2.1, FAD6, FAD7, DES* and *SLD3*, in both cultivars and treatments during germination. Specifically, *FAB2.3* is the most expressed gene in both treatments, though at a chilling temperature (10ºC) it shows a higher level of expression. Together with FAB2.8 and FAD2.1, which also show relative higher expression levels at 10ºC. Besides, FAD2.3 and SLD3 display higher expression levels at the optimal temperature, 20ºC. The rest of the desaturase genes have no significant differences in the gene expression between cultivars. In addition, significant differences between cultivars were seen in *FAD2.2* gene which shows twice the relative expression in the W2080 cultivar at the optimal temperatures, 20ºC. In chilling conditions, FAD2.3,

FAD7 and FAB2.4 expression levels were significantly higher in the American cultivar W2080.

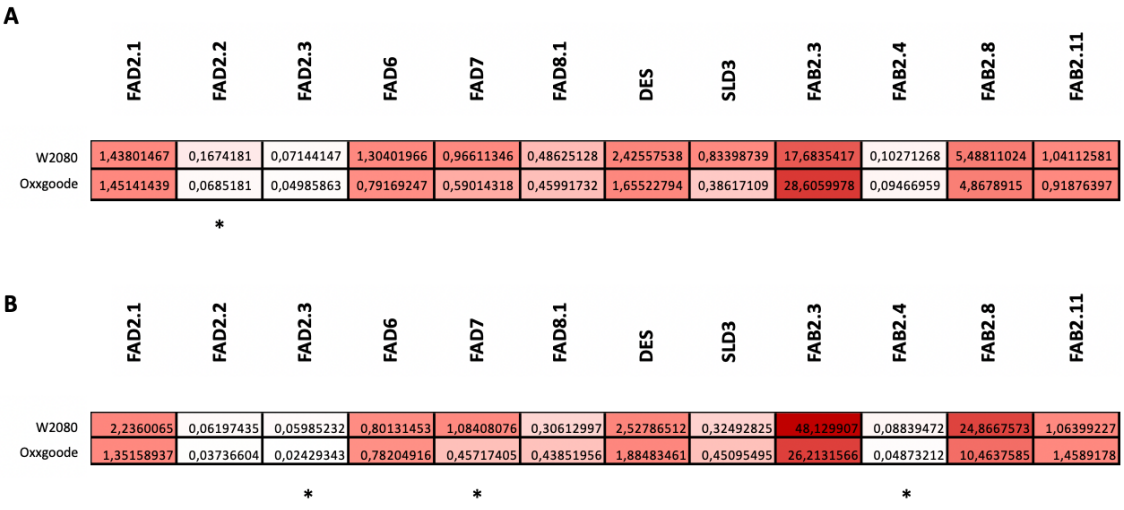


Figure 10: **Expression profiles of 12 desaturase genes involved in germination.** Relative expression levels of desaturase genes in American (W2080) and European (Oxxgoode) maize cultivars under optimal temperature, 20ºC (A) and chilling temperature, 10ºC (B). The bright red-off white colour scale represents high and low relative values of the desaturase genes (respectively). The asterisk indicates a significant difference between treatments statistically analysed by the Student's T test.

To explore the response of our 12 selected-desaturase genes to chilling stress during maize germination, we studied their expression in embryos from seed germinated at 10 ºC and compared it with those germinated at 20 ºC (Figure 11). The ratio of the relative expression of the desaturase genes during germination at optimal (20 ºC) and suboptimal (10 ºC) temperatures is represented in Figure 11. Among the genes studied which codify desaturases, W2080 cultivar shows significant differences between treatments in *SLD3, FAB2.4* and *FAB2.8* relative gene expression. Under chilling stress, *FAB2.4* and *FAB2.8* present a 2-fold increase in gene expression while *SLD3* peaked with more than 6 times gene upregulation. Furthermore, *FAD2.3* is doubly expressed in the Oxxgoode cultivar, together with *FAD2.2* which manifests a significant gene induction in both cultivars. We conclude that *FAD2.2, FAD2.3, SLD3, FAB2.4* and *FAB2.8* genes show significant difference when plants were germinated under suboptimal temperatures.

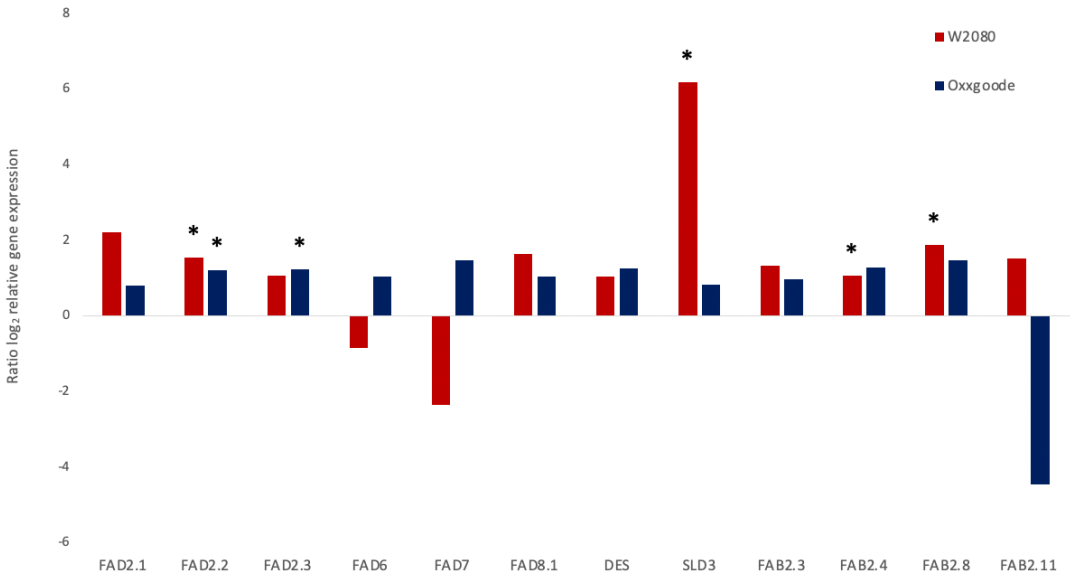


Figure 11: **Expression profile of maize desaturase genes during germination under chilling stress.** The bars represent the ratio of desaturase gene expression during germination at optimal and suboptimal temperatures.

4.4. Gene regulation analysis of the maize desaturase genes upregulated under chilling stress.

To explore the regulation of the five significantly expressed desaturase genes during unfavourable germination conditions, we have mapped the cis regulatory motifs of each gene of interest. The upstream sequences of each desaturase gene were obtained from the phytozome database and the cis-regulatory elements of the sequences were identified by PLANTCARE. Figure 12 shows where the different cis regulatory motifs are localized, involved in the chilling response, in the 1000 pb upstream sequence of the FAD2.2, FAD2.3, SLD3, FAB2.4 and FAB2.8 genes. A total of 6 cis-regulatory motifs were shown to be involved in the regulation of desaturase genes when germinating under chilling conditions: ABRE, CGTCA-motif, LRT motif, P-box, DRE and CCAAT-box.

Table 3: **Cis- regulatory elements table.** Abbreviation and name of the cis regulatory elements presented in the desaturase genes upregulated under chilling stress.

ABRE	Abscisic-acid response element
LTR	Low temperature regulon
P-box	The prolamin box
DRE	Drought regulatory elements

Among them, ABRE and CGTCA-motif are present in all the genes studied. DRE is also found in most of the genes, except FAB2.4 which displays the LTR motif instead. In addition, P-box and CCAAT-box motifs are the fourth cis regulatory motifs class present in SLD3 and FAD2.2, respectively. The desaturase gene with the largest number of cis regulatory elements (11) in its upstream sequence is FAB2.4 followed by SLD3 (9), FAD2.2 and FAB2.8 (8 cis-regulatory motifs) and lastly, the FAD2.3 gene presents the smallest number of cis-regulatory motifs (5).

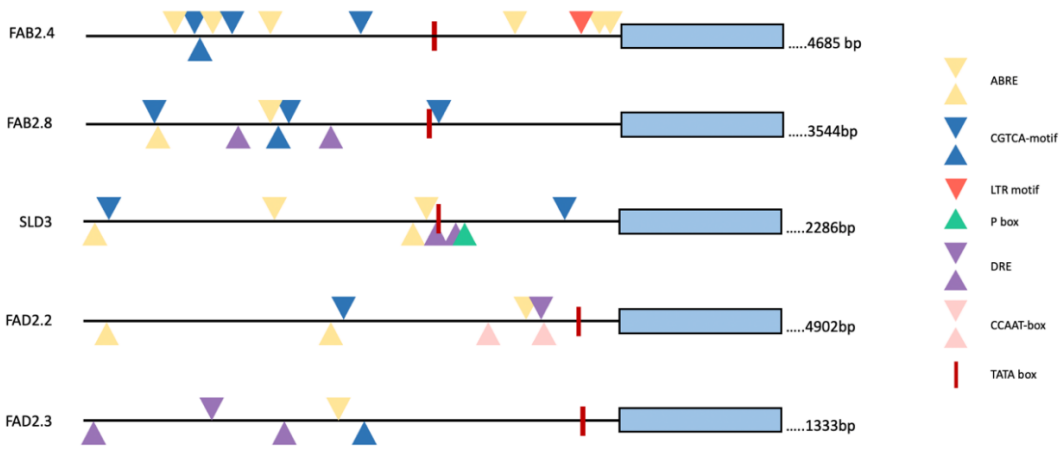


Figure 12: **Analysis of cis regulatory elements involved in the gene regulation under chilling stress.** The figure represents the upstream regions (1000 bp) of the five desaturase genes upregulated during germination under suboptimal conditions. Genes are represented by a blue box and their length is indicated. Six different cis-regulatory motifs are represented by colours: ABRE (yellow), CGTCA-motif (blue), LRT motif (orange), P-box (green), DRE (purple) and CCAAT-box (pink); and the TATA box as red line.

5. DISCUSSION

The challenge of increasing food production in the face of a growing population and changing climate needs multiple approaches (Hannah *et al.*, 2020). Maize is one of most important crops to ensure global food security but is also a chilling sensitive cereal (Marocco & Lorenzoni, 2005). Low temperatures can negatively influence the germination process, a crucial stage that influences plant development (Thakur *et al.*, 2010). This work reveals the impact of chilling temperatures during germination and how desaturase enzymes could mitigate the chilling damages in the seed membrane and encourage its germination.

5.1. Differences in maize germination patterns under chilling stress.

In the first assay, the kinetics of germination of two different maize cultivar seeds under chilling conditions were studied. This cultivar choice was based in the original location of the cultivars where the weather conditions differed from each other in temperature and humidity (Figure 5). On one hand, both cultivars suffered deceleration in the germination process when the temperature decreased. This problem can be caused by different possible reasons:

i) Disruption in the water intake at the imbibition stage due to changes in membranes conformation; low temperatures solidify the membrane structure blocking the water flux into the seed (El-Maarouf-Bouteau & Bailly, 2008).

ii) In addition, seed metabolism is altered by denaturalization of proteins and enzymes (Woodstock & Grabe, 1967).

iii) Ultimately, chilling stress also originates lipid from peroxidation which leads to increase in free radicals in the cells (Dreyer & Dietz, 2018).

Moreover, lack of seedling emergence has been showed in both maize cultivars germinated at 5ºC. This results in an irreversible metabolic imbalance that stops the germination process (Lukatkin et al, 2012).

On the other hand, germination timing differs between both maize cultivars. The European cultivar (Oxxgoode) takes longer to germinate than the American one (W2080). Germination success and speed can vary between cultivars of the same

species, influenced by different endogenous and exogenous factors (Eskandari & Kazemi, 2011). The seed morphology can influence the germination process as well as aging, which can cause reduction of vigour and loss of viability in some maize species (Revilla *et al.*, 2006; Saboya & Borghetti, 2012). Also, this difference in germination kinetics between cultivars can be caused by seed priming treatments. Pre-sowing treatment which partially hydrates seeds with a certain solution which could address this problem. Seed priming activates metabolic activities, preparing the radicle for protrusion without initiating the germination process (Tian *et al.*, 2014).

Lastly, the germination kinetic experiment also reveals the difference in behaviour and patterns of germination under chilling conditions between cultivars. Among the possible physiological and biochemical responses to chilling stress that can be involved in chilling tolerance, we have studied the role of desaturase genes during seed germination in maize.

5.2. Phylogenetic and evolutionary study of maize desaturase family.

The maize genome possesses 30 fatty acid desaturase genes which are classified in different subgroups (sphingolipid Δ4 desaturase, sphingolipid Δ8 desaturase, fatty acid ω-6 desaturase, fatty acid ω-3 desaturase, fatty acid Δ3 desaturase and acyl-desaturase) depending on the catalysed substrate and desaturation location in the lipid (Table 3) (Berestovoy *et al.*, 2020) . The number of desaturase genes in maize (30) is larger than that in *Arabidopsis thaliana* (16), *Oryza sativa* (19), *Cucumis sativus* (23) , *Sorghum bicolour* (20) or *Medicago truncatula* (20) (Dong *et al.*, 2016; Z. Zhang *et al.*, 2018; Zhao *et al.*, 2019). The expansion of the desaturase family is different in each species, which is a result of the number of duplication events. In addition, maize has a larger genome compared to other species due to its domestication (Shi & Lai, 2015). Studying the phylogenetic relationship in the desaturase family gives us an insight into the gene evolution.

The desaturase family is divided in two major groups: soluble desaturases, (FABs) where FAB2s are found, and membrane-bound desaturases (FADs). Cell location, enzymatic reaction and metabolic pathways differ between both groups, which is reflected in our phylogenetic tree. FAB2s and FADs enzymes have different functions;

hence different gene and protein structures (Hajiahmadi *et al.*, 2020; López-Alonso *et al.*, 2003).

The first major cluster is constituted by *FAD* genes that are assembled in subgroups according to function (Li *et al.*, 2016). This differentiation corroborates that desaturase gene structure varies according to the enzyme function and cell location. *DES* is found in an independent branch within the *FAD* cluster. Although *DES* catalyses a sphingolipid desaturation as in the *SLD* subgroup, it is the only gene in maize that inserts a *trans*- double bond in the Δ4 position (Ternes *et al.*, 2002). DES protein contains a "LIPID DES" protein domain while SLDs have a "Cyt-b5" domain. Additionally, the *SLD* clade is formed by three sphingolipid Δ8-desaturase genes with a recent duplication between *SLD1* and *SLD3.* Also, *FAD4.1* and *FAD4.2* genes were duplicated recently, and their protein sequence lacks the most common domain, "Fa_desaturase_2". The 100% identity coefficient of FAD2 subgroup means that it is highly similar and conserved, even though it is constituted of two sister pairs (*FAD2.4* and *FAD2.3*, and *FAD2.4* and *FAD2.6*). The last clade is constituted by *FAD3, FAD7, FAD8.1* and *FAD8.2* with an identity coefficient of 100%. Although these genes codify the enzyme responsible for the linoleate conversion into linolenate, FAD3 acts in the ER while as FAD7 and FAD8s act in the plastid (Torres-Franklin *et al.*, 2009). This grouping may be the product of an ancient endosymbiosis (Sperling *et al.*, 2003).

In the second major classification, *FAD6* gene is enclosed with *FAB2s* genes. This may be because both FAB2s and FAD6 catalyse in the same cell location, plastids. However, they are also characterised by presenting a unique protein domain in their sequences, the "Fa_desaturase_2" domain. Among *FAB2s,* we found four sister-pairs which indicates recent duplications. Also, a larger number of *FAB2* genes (13) were found belonging to other desaturase subgroups; sphingolipid Δ4 desaturase (1), sphingolipid Δ8 desaturase (3), fatty acid ω-6 desaturase (7), fatty acid ω-3 desaturase (4) and fatty acid Δ3 desaturase (2). FAB2 is an important enzyme responsible for introducing the first double bond in 18:0 ACP to catalyse 18:1 ACP, the primary substrate necessary for following FADs desaturations (Berestovoy *et al.*, 2020).

To support the information obtained from the phylogenetic tree and study the desaturase gene family evolution, all genes were mapped to maize chromosomes.

Desaturase genes were distributed across all chromosomes except chromosome 6. Two sister pairs cladded in the phylogenetic tree (*FAD4.1/FAD4.2* and *FAD2.2/FAD2.3*) were also found in the chromosome map. This duplication can be caused by tandem replication events. The rest of the duplicated pairs may have originated by segmental duplication events. However, in the chromosome map were found more segmental (chromosome 10' FADs and FABs genes) and tandem events (*FAB2.8* and *FAB2.9*; *SLD2* and *FAD3*; and *FAD2.2* and *FAD2.3*) responsible for the expansion of the desaturase family. Also, these genes can be considered linked genes that are likely to be inherited together due to their proximity on the chromosome. The rest of the genes may be inherited independently as they are located far from each other (Cooper, 2000; Santoni *et al.*, 2013).

5.3. Differences in the expression patterns of maize desaturase genes at optimal and suboptimal temperatures.

Since kinetic of germination changed in response to chilling imbibition, we would expect significant changes in expression of desaturase genes. First, we have identified the expression level of desaturase genes during maize germination. We have studied the 13 desaturase genes identified by Zhao *et al.* (2019) (*FAD2.1, FAD2.2, FAD2.3, FAD6, FAD7, FAD8.1, DES, SLD3, FAB2.3, FAB2.4, FAB2.8* and *FAB2.11*) during germination in two maize cultivars. *FAB2.3, FAB2.8, FAB2.11, FAD2.1, DES* and *SLD3* transcripts were the most abundantly expressed genes during germination among the desaturase genes in both cultivars. These highly expressed genes may be responsible for the lipid synthesis in imbibed seeds. FAB2 genes participate in seed development and germination which generates precursors for embryo tissues, hence promoting embryo growth (Kazaz *et al.*, 2020). *Arabidopsis* FAB2 mutants accumulate stearate lipid in their membranes, which interacts with the lipid synthesis during germination (Lightner *et al.*, 1994). Also FAD2 and sphingolipid desaturases (*DES* and *SLD3)* are responsible for polyunsaturated lipid and sphingolipid synthesis in germinating seed, respectively (Ali *et al.*, 2018; J. Zhang *et al.*, 2012). A previous study in sunflower seeds revealed that higher content of linolenic acid was found in the germinating embryonic axes. High linolenic acid levels enhance rate of germination (Munshi *et al.*, 2007). Thus, the FAD2 enzyme is a key enzyme during germination as it is responsible for desaturating oleic acid into linoleic acid (Dar *et al.*,

2017). Between cultivars, only FAD2.2 showed higher levels of gene expression in the W2080 cultivar, which could be related with its differences in germination speed.

In this study, to explore the response of desaturase genes to chilling stress during germination, we carried out gene expression analysis. The results showed that the expression of *FAD2.2/2.3*, *SLD3* and *FAB2.4/2.8* was significantly upregulated in at least one of the two cultivars studied. Consequently, these genes may play an important role in chilling tolerance during maize germination by membrane modification (Dong *et al.*, 2016; Huby *et al.*, 2020; Los & Murata, 1998). High concentrations of unsaturated fatty acids reduce permeability of the cell membrane. Through membrane modifications, seeds prevent water uptake, leakage of solutes and metabolism imbalance which brings better tolerance to suboptimal temperatures. Previous studies with *Arabidopsis FAD2* deficient mutants showed reduction in polyunsaturated FA and high chilling sensitivity whereas wild-type *Arabidopsis* plants present higher polyunsaturated FA level and chilling tolerance (Miquel *et al.*, 1993). A positive relationship was observed between FAD2 enzyme action and chilling stress (Zhang *et al.*, 2012). Zhou *et al.* (2016) experiments also demonstrated the importance of SLD enzymes in chilling tolerance. *SLD*-silenced tomato plants showed higher MDA content, indicating that *SLD*-silenced mutants were chilling sensitive plants. Gene expression analysis also concluded that SLD is essential for chilling stress tolerance (Zhou *et al.*, 2016). Our analysis shows a significant maximum in SLD3 expression in the American cultivar (W2080) which suggests a role in W2080 chilling tolerance. Regarding FAB2 gene up-regulation, studies in cyanobacteria cell membranes reveal its significant role under low temperatures (Murata & Wadat, 1995). In addition, the *FAD2.2* gene also plays an important role in chilling tolerance. It is the only gene which shows significant upregulation in both cultivar when germinated at low temperatures.

We also need to highlight the difference in desaturase gene upregulation between maize cultivars (W2080 upregulates *FAD2.2 SLD3* and *FAB2.4/2.8* genes and Oxxgoode upregulates *FAD2.2/2.3* genes). W2080 showed a larger number of upregulated genes than Oxxgoode, which suggests a better response to chilling temperatures. However, it has a greater variety of types of desaturase genes which act in different cell locations and desaturate different FA positions. Thus, it confers a larger number of substrates available for desaturation of the enzymes and chilling tolerance.

5.4. Gene regulation of maize desaturase genes expressed under chilling conditions.

In our last experiment, we have identified the *cis*-regulatory element to elucidate the molecular mechanisms of gene expression in response to chilling stress. Cis-regulatory elements identification is a great tool to identify the mechanism and control the gene expression of the genes of the study. Five desaturase genes (*FAD2.2-2.3*, *SLD3* and *FAB2.4/2.8*), were significantly expressed during germination under suboptimal conditions. We localized six different cis-regulatory motifs, ABRE, CGTCA-motif, LRT motif, P-box, DRE and CCAAT-box, in their 1000kb upstream sequences. Abscisic acid responsive element (ABRE) and methyl jasmonate (MeJa) responsive element (CGTCA-motif) are hormone-related regulatory elements present in all the genes studied. ABA and MeJa are hormones that regulate gene expression of many genes under chilling conditions (Battal *et al.*, 2008; Nakashima *et al.*, 2014). Previous experiments in maize, oilseed rape, rye, rice, tomato, wheat and *Arabidopsis* overexpressing or knocking out DRE regulon confirm its role in the regulation of chilling stress response (Mizoi *et al.*, 2012; Nakashima *et al.*, 2014). Furthermore, P-box is a hormone-related cis-element regulated by gibberellin (GA). Recently, it has been demonstrated that imbibed seeds enhance GA biosynthesis to promote germination under chilling condition (Lee *et al.*, 2005; Yamauchi *et al.*, 2004). Finally, CCAAT-box was identified in *Arabidopsis thaliana* and related with osmotic stress and LTR motif as specific low temperature regulon involved in chilling stress response (Edwards *et al.*, 1998).

To conclude, the differences in the germination kinetic curves between cultivars could be caused by previous adaptation to low temperatures. The European cultivar, Oxxgoode, requires a wider length of time for completing its germination which confers the ability of arresting the process. Consequently, Oxxgoode seeds can carry out germinate without suffering chilling damage when subjected to unfavourable conditions. This previous adaptation to chilling stress from the European cultivar (Oxxgoode) is also seen in the desaturase gene expression and chilling response which is less than that of the American cultivar (W2080). This suggests that Oxxgoode seeds could limit the desaturase action by concentrating a larger number of desaturated lipids in the membrane. Further investigation the phospholipidome profile in both cultivars under optimal and suboptimal temperatures is required to understand the membrane capacity to tolerate chilling in maize. Likewise, further analysis of desaturase gene expression throughout germination will enable identification of candidate targets for improving chilling tolerance in maize.

6. CONCLUSIONS

- The kinetics of germination experiment verified that the European cultivar (Oxxgoode) germinates slower than the American cultivar (W2080). Thus, different germination patterns have been seen between cultivars under optimal and suboptimal conditions.
- A total of 30 desaturase genes were identified in *Zea mays.* Among them, 13 desaturase genes were identified as membrane-bound fatty acid desaturases (FABs) and the rest of the desaturase genes (17) as soluble sterol-ACP (Acyl-carrier protein) desaturases (FADs). Maize contains a larger number of desaturase genes than other species such as *Arabidopsis thaliana* (16), rice (*Oryza sativa)* (19) and cucumber (*Cucumis sativus)* (23).
- On one hand, several upregulated desaturase genes during maize germination under optimal temperatures were identified: *FAB2.3* (which is highly expressed), *FAB2.8, DES, FAD2.1, FAD6, FAB2.11, FAD7, SLD3* and *FAD7,* named in descending order of relative expression, in both cultivars. In addition, desaturase gene expression does not show significant difference during germination between cultivars, except from FAD2.2. On the other hand, when seeds are subjected to chilling stress, W2080 cultivar shows significant upregulation in FAD2.1, SLD3, FAB2.4 and FAB2.8 gene expression level compared to optimal conditions. While as FAD2.1 and FAD2.2 desaturase genes are upregulated in Oxxgoode cultivar.
- 6 different *cis*-regulatory elements (ABRE, CGTCA-motif, LRT motif, P-box, DRE and CCAAT-box) could be involved in the gene regulation of maize desaturases when maize seeds germinate under chilling conditions.

7. FUTURE DIRECTIONS

This MSc thesis reveals new findings about the desaturase family and its mode of action when maize seeds are exposed to chilling temperatures during their germination. Consequently, the following approaches will help us to gain further insights:

- A comparative phylogenetic study with other grass or cereal species (as rice, wheat, *Brachypodium*…). It would open up the opportunity to examine the evolution of this gene family and the germination process.
- Functional assays using gene-silenced mutants with our five candidates' desaturase genes (FAD2.1, FAD2.2, SLD3, FAB2.4 and FAB2.8) to validate its role under chilling stress.
- Deep transcriptomics and lipidomic screening at different stages of the germination process to evaluate the changes of the lipidomic profile and the action of desaturases along the germination process, at optimal and chilling temperatures.

8. REFERENCES

Ali, U., Li, H., Wang, X., & Guo, L. (2018). Emerging Roles of Sphingolipid Signaling in Plant Response to Biotic and Abiotic Stresses. In *Molecular Plant 11* (11), 1328–1343.

Basra, A. S., & Basra, R. K. (1997). *Mechanisms of Environmental Stress Resistance in Plants* (1st ed.) *Amsterdam: Harwood academy publishers*.

Andreu Gargallo, V. (2010). Caracterización bioquímica, molecular y celular de las ω3 desaturasas plastidiales de plantas y análisis de su implicación en la síntesis de jasmonatos [Doctoral thesis, Consejo Superior de Investigaciones Cientificas].

Barrero-Sicilia, C., Silvestre, S., Haslam, R. P., & Michaelson, L. V. (2017). Lipid remodelling: Unravelling the response to cold stress in Arabidopsis and its extremophile relative Eutrema salsugineum. In *Plant Science, 263*, 194–200.

Battal, P., Erez, M. E., Turker, M., & Berber, I. (2008). Molecular and physiological changes in maize (Zea mays) induced by exogenous NAA, ABA and MeJa during cold stress. *Annales Botanici Fennici, 45* (3), 173-185.

Benning, C. (2008). A role for lipid trafficking in chloroplast biogenesis. In *Progress in Lipid Research, 47* (5), 381–389.

Bentsink, L., & Koornneef, M. (2008). Seed dormancy and germination. *The Arabidopsis Book, 6.*

Berestovoy, M. A., Pavlenko, O. S., & Goldenkova-Pavlova, I. V. (2020). Plant Fatty Acid Desaturases: Role in the Life of Plants and Biotechnological Potential. *Biology Bulletin Reviews, 10* (2), 127–139.

Bewleyl, J. D. (1997). Seed Germination and Dormancy. *The Plant Cell, 9,* 1055-1066.

Bob B. Buchanan, Wilhelm Gruissem, R. L. J. (2015). Biochemistry and molecular biology of plants. *In Biochemistry and molecular biology of plants* (2nd ed.). John Wiley & Sons, Ltd.

Borner, G. H. H., Sherrier, D. J., Weimar, T., Michaelson, L. V., Hawkins, N. D., MacAskill, A., Napier, J. A., Beale, M. H., Lilley, K. S., & Dupree, P. (2005). Analysis of detergent-resistant membranes in arabidopsis. Evidence for plasma membrane lipid rafts. *Plant Physiology, 137* (1), 104–116.

Cacas, J. L., Buré, C., Grosjean, K., Gerbeau-Pissot, P., Lherminier, J., Rombouts, Y., Maes, E., Bossard, C., Gronnier, J., Furt, F., Fouillen, L., Germain, V., Bayer, E., Cluzet, S., Robert, F., Schmitter, J. M., Deleu, M., Lins, L., Simon-Plas, F., & Mongrand, S. (2016). Revisiting plant plasma membrane lipids in tobacco: A focus on sphingolipids. *Plant Physiology, 170* (1), 367–384.

Chen, M., Markham, J. E., & Cahoon, E. B. (2012). Sphingolipid Δ8 unsaturation is important for glucosylceramide biosynthesis and low-temperature performance in Arabidopsis. *The Plant Journal, 69 (*5), 769–781.

Cooper, G. M. (2000). Heredity, Genes, and DNA. (2nd ed.) Sunderland (MA): Sinauer Associates.

Cossins, A. R., Murray, P. A., Gracey, A. Y., Logue, J., Polley, S., Caddick, M., Brooks, S., Postle, T., & Maclean, N. (2002). The role of desaturases in cold-induced lipid restructuring. *Biochemical Society Transactions, 30* (6), 1082–1086.

Coursol, S., Fan, L. M., Stunff, H. Le, Splegel, S., Gilroy, S., & Assman, S. M. (2003). Sphingolipid signalling in Arabidopsis guard cells involves heterotrimeric G proteins. *Nature, 423 (*6940), 651–654.

Dar, A. A., Choudhury, A. R., Kancharla, P. K., & Arumugam, N. (2017). The FAD2 gene in plants: Occurrence, regulation, and role. *Frontiers in Plant Science, 8.*

Dong, C. J., Cao, N., Zhang, Z. G., & Shang, Q. M. (2016). Characterization of the fatty acid desaturase genes in cucumber: Structure, phylogeny, and expression patterns. *PLoS ONE, 11* (3).

Dreyer, A., & Dietz, K. J. (2018). Reactive oxygen species and the redox-regulatory network in cold stress acclimation. *Antioxidants, 7 (*11).

Dunn, T. M., Lynch, D. V., Michaelson, L. V., & Napier, J. A. (2004). A post-genomic approach to understanding sphingolipid metabolism in Arabidopsis thaliana. *In Annals of Botany, 93* (5), 483–497.

Edwards, D., Murray, J. A. H., & Smith, A. G. (1998). Multiple genes encoding the conserved CCAAT-box transcription factor complex are expressed in arabidopsis. *Plant Physiology, 117* (3), 1015–1022.

Eskandari, H., & Kazemi, K. (2011). Germination and Seedling Properties of Different Wheat Cultivars under Salinity Conditions. *Notulae Scientia Biologicae, 3* (3), 130–134.

El-Maarouf-Bouteau, H., & Bailly, C. (2008). Oxidative signaling in seed germination and dormancy. In *Plant Signaling and Behavior*, *3* (3), 175–182.

Fatland, B. L., Nikolau, B. J., & Wurtele, E. S. (2005). Reverse genetic characterization of cytosolic acetyl-CoA generation by ATP-citrate lyase in Arabidopsis. *Plant Cell, 17* (1), 182–203.

Greaves, J. A. (1996). Improving suboptimal temperature tolerance in maize- the search for variation. *Journal of Experimental Botany, 47* (3), 307–323.

Guschina, I. A., & Harwood, J. L. (2007). Complex lipid biosynthesis and its manipulation in plants. In Improvement of Crop Plants for Industrial End Uses. *Springer Netherlands* (253–279).

Hajiahmadi, Z., Abedi, A., Wei, H., Sun, W., Ruan, H., Zhuge, Q., & Movahedi, A. (2020). Identification, Evolution, Expression, and Docking Studies of Fatty Acid Desaturase Genes in Wheat (Triticum aestivum L.). *BMC Genomics, 21* (778), 1–36.

Hannah, L., Roehrdanz, P. R., Bahadur, K. C., K., Fraser, E. D. G., Donatti, C. I., Saenz, L., Wright, T. M., Hijmans, R. J., Mulligan, M., Berg, A., & van Soesbergen, A. (2020). The environmental consequences of climate-driven agricultural frontiers. *PLoS ONE, 15* (2).

Huby, E., Napier, J. A., Baillieul, F., Michaelson, L. V., & Dhondt-Cordelier, S. (2020). Sphingolipids: towards an integrated view of metabolism during the plant stress response. N*ew Phytologist, 225*(2), 659–670.

Huang, X., Chen, M. H., Yang, L. T., Li, Y. R. & Wu, J. M. (2015). Effects of Exogenous Abscisic Acid on Cell Membrane and Endogenous Hormone Contents in Leaves of Sugarcane Seedlings under Cold Stress. *Sugar Tech, 17,* 59–64.

Jackman, R. L., Yada, R. Y., Marangoni, A., Parkin, K. L. & Stanley, D. W. (1988) Chilling injury. A review of quality spects. J. *Food Quality, 11,* 253–278.

Jan, N., ul-Hussain, M. & Andrabi, K. Cold resistance in plants: A mystery unresolved. *Electronic Journal of Biotechnology, 12*, 14–15 (2009).

Kazaz, S., Barthole, G., Domergue, F., Ettaki, H., To, A., Vasselon, D., De Vos, D., Belcram, K., Lepiniec, L., & Baud, S. (2020). Differential Activation of Partially Redundant Δ9 Stearoyl-ACP Desaturase Genes is Critical for Omega-9 Monounsaturated Fatty Acid Biosynthesis During Seed Development in Arabidopsis. *The Plant Cell, 8*.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, C. (2020). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology Evolution, 35* (6), 1547–1549.

Lee, B. H., Henderson, D. A., & Zhu, J. K. (2005). The Arabidopsis cold-responsive transcriptome and its regulation by ICE1. *Plant Cell, 17* (11), 3155–3175.

Lenka, S., & Bansal, K. (2019). Abiotic stress responsive cis-regulatory elements (CREs) in rice (Oryza sativa L.) and other plants.

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van De Peer, Y., Rouzé, P., & Rombauts, S. (2002). PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Research, 30* (1), 325–327.

Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M. X., Arondel, V., Bates, P. D., Baud, S., Bird, D., DeBono, A., Durrett, T. P., Franke, R. B., Graham, I. A., Katayama, K., Kelly, A. A., Larson, T., Markham, J. E., Miquel, M., Molina, I., Nishida, I., & Ohlrogge, J. (2013). Acyl-Lipid Metabolism. *The Arabidopsis Book, 11*.

Lightner, J., Wu Jingrui, & Browse, J. (1994). A mutant of Arabidopsis with increased levels of stearic acid. *Plant Physiology, 106* (4), 1443–1451.

Lin, Yi xin, Xin, X., Yin, G. kun, He, J. juan, Zhou, Y. chang, Chen, J. ying, & Lu, X. xiong. (2019). Membrane phospholipids remodeling upon imbibition in Brassica napus L. seeds. *Biochemical and Biophysical Research Communications, 515* (2), 289–295.

Lin, Yueai, Zhang, C., Lan, H., Gao, S., Liu, H., Liu, J., Cao, M., Pan, G., Rong, T., & Zhang, S. (2014). Validation of potential reference genes for qPCR in maize across abiotic stresses, hormone treatments, and tissue types. *PLoS ONE, 9* (5).

Lionello, P., Malanotte-Rizzoli, P., Boscolo, R., Alpert, P., Artale, V., Li, L., Luterbacher, J., May, W., Trigo, R., Tsimplis, M., Ulbrich, U., & Xoplaki, E. (2006). The Mediterranean climate: An overview of the main characteristics and issues. *In Developments in Earth and Environmental Sciences, 4*, 1–26.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods, 25* (4), 402–408.

López Alonso, D., García-Maroto, F., Rodríguez-Ruiz, J., Garrido, J. A., & Vilches, M. A. (2003). Evolution of the membrane-bound fatty acid desaturases. *Biochemical Systematics and Ecology, 31* (10), 1111–1124.

Los, D. A., & Murata, N. (1998). Structure and expression of fatty acid desaturases. In Biochimica et Biophysica Acta - *Lipids and Lipid Metabolism, 1394* (1), 3–15.

Los, D. A., & Murata, N. (2004). Membrane fluidity and its roles in the perception of environmental signals. In Biochimica et Biophysica Acta - Biomembranes- *Lipids and Lipid Metabolism,* 1666, (1–2), 142-157.

Lukatkin, A., Brazaitytė, A., Bobinas, C., & Duchovskis, P. (2012). Chilling injury in chilling sensitive plants: a review. *Žemdirbystė=Agriculture, 99*, 111–124.

Marocco, A & Lorenzoni, C & Fracheboud, Y F. (2005). Chilling stress in maize. *Maydica, 50*, 571–580.

Merrill, A. H. (2008). Sphingolipids. In Biochemistry of Lipids, Lipoproteins and Membranes. *Elsevier*, 363–397

Michaelson, L. V., Napier, J. A., Molino, D., & Faure, J. D. (2016). Plant sphingolipids: Their importance in cellular organization and adaption. Biochimica et Biophysica Acta - *Molecular and Cell Biology of Lipids, 1861* (9), 1329–1335.

Miquel, M., James, D., Dooner, H., & Browse, J. (1993). Arabidopsis requires polyunsaturated lipids for low-temperature survival. *Proceedings of the National Academy of Sciences of the United States of America, 90* (13), 6208–6212.

Mishra, S., Shukla, A., Upadhyay, S., Sanchita, Sharma, P., Singh, S., Phukan, U. J., Meena, A., Khan, F., Tripathi, V., Shukla, R. K., & Shrama, A. (2014). Identification, occurrence, and validation of DRE and ABRE Cis-regulatory motifs in the promoter regions of genes of arabidopsis thaliana. In Journal of Integrative *Plant Biology, 56* (4), 388–399.

Mizoi, J., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2012). AP2/ERF family transcription factors in plant abiotic stress responses. In Biochimica et Biophysica Acta - *Gene Regulatory Mechanisms, 1819* (2) 86–96. Elsevier.

Moreau, P., Bessoule, J. J., Mongrand, S., Testet, E., Vincent, P., & Cassagne, C. (1998). Lipid trafficking in plant cells*. In Progress in Lipid Research, 37* (6), 371–391. Elsevier Ltd.

Munshi, S. K., Sandhu, S., & Sharma, S. (2007). Lipid composition in germinating sunflower seeds*. Plant Physiology, 33* (4).

Murata, N., & Wadat, H. (1995). Acyl-lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria. *Biochemestry Journal, 308*.

Nakashima, K., Yamaguchi-Shinozaki, K., & Shinozaki, K. (2014). The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. *In Frontiers in Plant Science, 5* (May), 170.

Nonogaki, H., Bassel, G. W. & Bewley, J. D. (2010). Germination-still a mystery. *Plant Sci.* 179, 574–581.

Ohlrogge, J. B., & Jaworski, J. G. (1997). Regulation of fatty acid synthesis. *Annual Review of Plant Biology, 48*, 109–136.

Oñate-Sánchez, L., & Vicente-Carbajosa, J. (2008). DNA-free RNA isolation protocols for Arabidopsis thaliana, including seeds and siliques. *BMC Research, 1* (1), 93.

Revilla, P., Velasco, P., Malvar, R. A., Cartea, M. E., & Ordás, A. (2006). Variability among maize (Zea mays L.) inbred lines for seed longevity. *Genetic Resources and Crop Evolution, 53* (4), 771–777.

Robert C. E. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleid Acids Research, 32* (5), 1792–1797.

Roesler, K. R., Shorrosh, B. S., & Ohlrogge, J. B. (1994). Structure and expression of an Arabidopsis acetyl-coenzyme A carboxylase gene. *Plant Physiology,105* (2), 611–617.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution, 4 (*4), 406–425.

Sandhu, K., & Singh, N. (2007). Some properties of corn starches II: Physicochemical, gelatinization, retrogradation, pasting and gel textural properties. *Food Chemistry, 101* (4), 1499–1507.

Santoni, D., Castiglione, F., & Paci, P. (2013). Identifying Correlations between Chromosomal Proximity of Genes and Distance of Their Products in Protein-Protein Interaction Networks of Yeast. *PLoS ONE, 8 (*3).

Shanklin, J., & Cahoon, E. B. (1998a). DESATURATION AND RELATED MODIFICATIONS OF FATTY ACIDS. *Annual Review of Plant Physiology and Plant Molecular Biology, 49* (1), 611–641.

Shanklin, J., Guy, J. E., Mishra, G., & Lindqvist, Y. (2009). Desaturases: Emerging models for understanding functional diversification of diiron-containing enzymes. *In Journal of Biological Chemistry, 284* (28) 18559–18563.

Shi, J., & Lai, J. (2015). Patterns of genomic changes with crop domestication and breeding. *Current Opinion in Plant Biology, 24*, 47–53.

Simon, E. W. (1974). Phospholipids and plant membrane permeability. *New Phytologist, 73* (3), 377–420.

Somerville, C., & Browse, J. (1991). Plant lipids: Metabolism, mutants, and membranes. *Science, 252* (5002), 80–87.

Sperling, P., Ternes, P., Zank, T. K., & Heinz, E. (2003). The evolution of desaturases. *Prostaglandins Leukotrienes and Essential Fatty Acids, 68* (2), 73–95.

Ternes, P., Franke, S., Zähringer, U., Sperling, P., & Heinz, E. (2002). Identification and characterization of a sphingolipid Δ4-desaturase family. *Journal of Biological Chemistry, 277* (28), 25512–25518.

Thakur, P., Kumar, S., Malik, J. A., Berger, J. D., & Nayyar, H. (2010). Cold stress effects on reproductive development in grain crops: An overview. *In Environmental and Experimental Botany, 67* (3) 429–443.

Tian, Y., Guan, B., Zhou, D., Yu, J., Li, G., & Lou, Y. (2014). Responses of seed germination, seedling growth, and seed yield traits to seed pretreatment in maize (Zea mays L.). *Scientific World Journal, 2014*, 1-8.

Tocher, D. R., Leaver, M. J., & Hodgson, P. A. (1998a). Recent advances in the biochemistry and molecular biology of fatty acyl desaturases. *In Progress in Lipid Research, 37* (2–3) 73–117.

Torres-Franklin, M. L., Repellin, A., Huynh, V. B., d'Arcy-Lameta, A., Zuily-Fodil, Y., & Pham-Thi, A. T. (2009). Omega-3 fatty acid desaturase (FAD3, FAD7, FAD8) gene expression and linolenic acid content in cowpea leaves submitted to drought and after rehydration. *Environmental and Experimental Botany, 65* (2–3), 162–169.

Tovuu, A., Zulfugarov, I. S., Wu, G., Kang, I. S., Kim, C., Moon, B. Y., An, G., & Lee, C. H. (2016). Rice mutants deficient in ω-3 fatty acid desaturase (FAD8) fail to acclimate to cold temperatures. *Plant Physiology and Biochemistry, 109*, 525–535.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., & Leunissen, J. A. M. (2007). Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Research, 35* (2), 71–74.

Upchurch, R. G. (2008). Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *In Biotechnology Letters, 30* (6) 967–977.

USDA - National Agricultural Statistics Service - Data Visualization. (2020). https://www.nass.usda.gov/Data_Visualization/Commodity/index.php

Vallejo-Marín, M., Domínguez, C. A., & Dirzo, R. (2006). Simulated seed predation reveals a variety of germination responses of neotropical rain forest species. *American Journal of Botany, 93* (3), 369–376.

Wada, H., Shintani, D., & Ohlrogge, J. (1997). Why do mitochondria synthesize fatty acids? Evidence for involvement in lipoic acid production. *Proceedings of the National Academy of Sciences of the United States of America, 94*(4), 1591–1596.

Wallis, J. G., & Browse, J. (2002). Mutants of Arabidopsis reveal many roles for membrane lipids. *In Progress in Lipid Research, 41 (*3), 254–278.

Wilson, J. M. (1985). The economic importance of chilling injury. *Outlook on Agriculture, 14* (4), 197–203.

Wolfe, D., Dudek, S., Ritchie, M. D., & Pendergrass, S. A. (2013). Visualizing genomic information across chromosomes with PhenoGram. *BioData Mining, 6* (1), 18.

Wolny, E., Betekhtin, A., Rojek, M., Braszewska-Zalewska, A., Lusinska, J., & Hasterok, R. (2018). Germination and the early stages of seedling development in brachypodium distachyon. *International Journal of Molecular Sciences, 19* (10).

Woodstock, L. W., & Grabe, D. F. (1967). Relationships Between Seed Respiration During Imbibition and Subsequent Seedling Growth in Zea mays L. *Plant Physiology, 4*2 (8), 1071–1076.

Yadav, S. K. (2010). Cold stress tolerance mechanisms in plants. A review. *Agronomy for Sustainable Development, 30* (3), 515–527.

Yamaguchi-Shinozaki, K., & Shinozaki, K. (2005). Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. *In Trends in Plant Science, 10* (2), 88–94.

Yamaguchi-Shinozaki, K., & Shinozaki, K. (2006). Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *In Annual Review of Plant Biology, 57*, 781–803.

Yamauchi, Y., Ogawa, M., Kuwahara, A., Hanada, A., Kamiya, Y., & Yamaguchi, S. (2004). Activation of Gibberellin Biosynthesis and Response Pathways by Low Temperature during Imbibition of Arabidopsis thaliana Seeds. *Plant Cell, 16* (2), 367–378.

Yu, X., Li, A., & Li, W. (2015). How membranes organize during seed germination: three patterns of dynamic lipid remodelling define chilling resistance and affect plastid biogenesis HHS Public Access. *Plant Cell Environ, 38 (*7), 1391–1403.

Zhang, J., Liu, H., Sun, J., Li, B., Zhu, Q., Chen, S., & Zhang, H. (2012). Arabidopsis Fatty Acid Desaturase FAD2 Is Required for Salt Tolerance during Seed Germination and Early Seedling Growth. *PLoS ONE, 7* (1).

Zhang, Z., Wei, X., Liu, W., Min, X., Jin, X., Ndayambaza, B., & Wang, Y. (2018). Genome-wide identification and expression analysis of the fatty acid desaturase genes in Medicago truncatula. *Biochemical and Biophysical Research Communications, 499* (2), 361–367.

Zhao, X., Wei, J., He, L., Zhang, Y., Zhao, Y., Xu, X., Wei, Y., Ge, S., Ding, D., Liu, M., Gao, S., & Xu, J. (2019). Identification of Fatty Acid Desaturases in Maize and Their Differential Responses to Low and High Temperature. *Genes, 10* (6), 445.

Zheng, G. H. (1991). Physiological, biochemical and ultrastructural aspects of imbibitional chilling injury in seeds: a review of work carried out at the Beijing Botanical Garden. *Seed Science Research, 1* (2), 127–134.

Zhou, Y., Zeng, L., Fu, X., Mei, X., Cheng, S., Liao, Y., Deng, R., Xu, X., Jiang, Y., Duan, X., Baldermann, S., & Yang, Z. (2016). The sphingolipid biosynthetic enzyme Sphingolipid delta8 desaturase is important for chilling resistance of tomato. *Scientific Reports, 6*.