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Review

Weathering the Cold: Modifying Membrane and Storage Fatty Acid Composition of Seeds to Improve Cold Germination Ability in Upland Cotton (*Gossypium hirsutum* L.)

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Abstract: Cotton is widely cultivated in temperate regions across the world and is often constrained by a short planting window that is bookended by low, suboptimal temperatures. With the growing interest in early season planting, improvements in the cold germination ability of cotton will be necessary to ensure the production stability of early planted crops. The importance of saturation levels of membrane and storage lipids in enhancing cold tolerance in plants, as well as improving cold germination ability in seeds have been widely researched in a range of plant species. While studies have shown that higher levels of unsaturated lipids can enhance cold germination ability and reduce seedling injury in other crops, similar efforts have been fairly limited in cotton. This review looks at the functional properties of membrane and storage lipids, and their role in membrane stability and reorganization during the early stages of germination. Additionally, the importance of storage lipid composition as an energy source to the growing embryo is described in the context of cellular energetics (i.e., fatty acid catabolism). Finally, perspectives in improving the cold germination of upland cotton by manipulating the fatty acid composition of both membrane and storage lipid content of seeds are presented.

Keywords: *Gossypium hirsutum* L.; upland cotton; cold tolerance; seed germination; fatty acids; lipid unsaturation

1. Introduction

Upland cotton (*Gossypium hirsutum* L.) is the most important source of natural fiber and a significant source of seed oil and stock feeds [1]. It is grown in over 30 countries worldwide, although more than 80% is produced in India, China, United States, Brazil, and Pakistan. In 2013, the FAO estimates for cottonseed and cotton fiber production were 45.6 and 24.8 million tons, respectively [2]. In 2017, data from the International Cotton Advisory Committee estimated cotton cultivation to cover a total of 32.2 million ha of land worldwide, with total fiber production of 24.9 million tons [3].

Like most major agricultural crops, cotton productivity is largely defined by external factors, particularly by temperature [4]. Upland cotton is native to the tropics/sub-tropics and despite thousands of years of domestication that afforded the species adaptability to a range of environments, it still thrives best under long-season cultivation in warm climates [5]. The established minimum cardinal temperature for most cultivated cotton varieties is 15 °C. Temperatures below 15 °C but above 0 °C

constitute chilling or cold stress that negatively impacts both the growth and productivity of the plant and consequently, its fiber yield and quality [6,7].

In temperate regions where cotton farmers face shorter growing seasons, planting windows are often established based on the minimum temperature requirement (15 °C) for seed germination [8]. More often than not, however, the actual planting dates are delayed beyond what is theoretically acceptable to avoid cold snaps that may injure the plant during its early growth stages [9]. These planting delays effectively shift cultivation outside the limits of the optimum temperature range for cotton during the later stages of growth, consequently impairing physiological processes necessary for normal development [10]. The combination of cooler fall temperatures and shorter day lengths during boll maturation causes poor seed quality [11] and significantly reduces fiber cellulose synthesis that effectively lowers both yield potential and fiber quality of cotton [12–14]. Moreover, the spread of late-season pests including whiteflies, boll weevils, and armyworms (*Helicoverpa armigera* Hübner) that thrive in the cool fall temperatures add to the negative impacts of delayed planting.

In contrast, shifts toward early season planting risk the exposure of the crop to low temperature stress during the early stages of growth. Colds snaps below the minimum requirement for cotton germination can cause irreversible chilling injury to seeds, resulting in poor seedling emergence and vigor, slow development and lower biomass accumulation that ultimately leads to poor stand establishment and/or complete crop failure [15,16]. Early season planting also exposes crops to higher chances of infection by soil-borne pathogens that thrive in the damp spring soil, as well as to injuries from pre-emergent herbicide applications [5,9].

Despite these disadvantages, farmers in some cotton growing regions have shown interest in planting earlier than recommended to take advantage of any residual moisture from winter precipitation and to capitalize on the reported benefits of this cultural practice on both cotton yield and quality [17]. In the mid-southern United States, early planting has been shown to increase the number of flowers per plant, boll weight, and lint yield of cotton [18]. Separate studies associated early season planting with higher leaf area index that contributed to a 10% yield improvement over the normally planted cotton [9,19]. Crop simulations to determine the effects of planting date, elevation, and irrigation on the economics of cotton production demonstrated a significant improvement in lint yield with a mid-March planting, particularly in areas that are highly elevated [20].

Given that cotton is highly sensitive to chilling temperatures, the reported advantages of early season planting as described above implies the availability of germplasm that meets both physiological and biochemical requirements for cold germination. Building on this premise, screening of genetic resources with the potential to withstand chilling stress during germination and identification of highly heritable traits or components that can be utilized to improve cold germination ability in the crop will be integral in establishing the long-term production stability of early planted cotton, as well as in the widespread adoption of the practice among farmers.

Research efforts directed toward elucidating the mechanisms underlying the ability of plants to sustain physiological functions under cold conditions identified oil composition as an important evolutionary adaptation of seeds to varying germination temperatures [21–23]. In this review, a summary of membrane and storage lipid biosynthesis and composition is presented in brief to provide a backdrop for the thesis of the paper. The biological functions of lipids during seed germination are described, particularly at the early stages of water imbibition and lipid reserve catabolism. Building on the defined roles of lipids in seeds, the effects of changes in seed oil composition on the ability of seeds to adapt and germinate under cold conditions are demonstrated based on reports from various oilseed crops. Finally, perspectives on advancing breeding efforts for cold germination ability in upland cotton by modifying the fatty acid composition of membrane and storage lipids in the seeds are presented.

2. Membrane and Storage Lipids Share Similar Biochemical Reactions and Intermediaries

Lipids are fundamental to the very existence of plant life. They serve as structural components of biological membranes, as energy stores in developing seeds, and as signaling molecules that are necessary for plant growth and development, abiotic stress response, and pathogen defense [24].

As major components of plant membranes, lipids create hydrophobic barriers that separate cells from their environment and prevent diffusion of organelle contents in and out of a cell. Lipids are composed of a hydrophilic, polar head attached to a glycerol backbone and a hydrophobic tail composed of two fatty acids [25,26]. Membrane lipids in plants are synthesized via two distinct pathways, i.e., the prokaryotic and the eukaryotic pathways. The prokaryotic pathway occurs in the chloroplasts and produces the major plastidic lipids such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), trigalactosyldiacylglycerol (TGDG), and sulfoquinovosyldiacylglycerol (SQDG), otherwise known as sulfolipids. In contrast, the eukaryotic pathway occurs in the endoplasmic reticulum (ER) and synthesizes phospholipids including phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS) [27,28]. In both the prokaryotic and eukaryotic pathways, membrane lipid biosynthesis is initiated by the formation of PAs, which are then utilized to produce plastidic lipids or phospholipids, depending on the site of biosynthesis. PAs produced in the chloroplasts can also be converted to diacylglycerol (DAG) which then serves as a precursor for plastidic lipid synthesis. During the life cycle of plants, an active lipid exchange between the chloroplast and ER occurs via the import of the DAG moiety of PCs from the ER to the chloroplast envelope where it contributes to the DAG pool used to synthesize plastidic lipids [27–30]. Besides their function in membrane lipid synthesis, PAs and PCs also serve as intermediaries in storage lipid metabolism.

The biosynthesis of fatty acids that make up storage lipids follows the same pathway as those that comprise membrane lipids using many similar precursors [30–33]. They are packaged in the seeds primarily as triacylglycerols (TAGs) which are composed of a glycerol backbone esterified with three fatty acids in saturated and/or unsaturated forms [34,35]. A general overview of the membrane and lipid biosynthesis is illustrated in Figure 1.

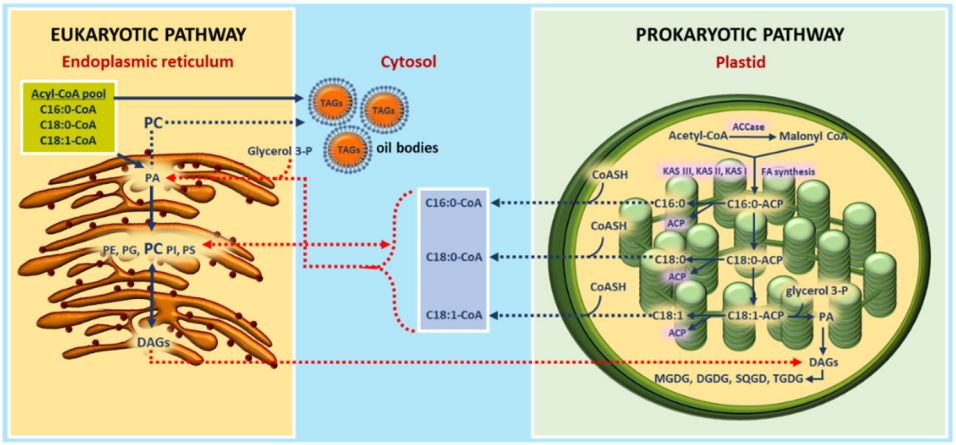

Figure 1. General overview of lipid biosynthesis in plants. Membrane lipid biosynthesis occurs via the eukaryotic (left panel) or prokaryotic pathway (right panel) in the endoplasmic reticulum and plastid, respectively. In both pathways, the synthesis of phosphatidic acid (PA) initiates the first step in lipid synthesis. Fatty acid synthesis occurs in the plastid through a series of reaction that incorporates acyl moieties of acetyl-Coenzyme A (-CoA) into acyl group of 16 or 18 carbon atoms. Fatty acids from acyl-acyl carrier protein (ACP) or acyl-CoA are transferred to a glycerol-3-phosphate (glycerol 3-P) to synthesize PAs. The PAs are converted to diacylglycerols (DAGs) which then serve as precursors for

plastidic lipids such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), trigalactosyldiacylglycerol (TGDG), and sulfoquinovosyldiacylglycerol (SQDG). In the eukaryotic pathway, PAs that are synthesized from the acyl-CoA pool give rise to eukaryotic lipids including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), or phosphatidylserine (PS). DAG moieties of PCs are returned to the chloroplast envelope and adds to the DAG pool that is used to synthesize MGDG, DGDG, TGDG, and SQDG. Triacylglycerols (TAGs) are synthesized by esterification of three fatty acids to a glycerol molecule using the acyl-CoA pool. The fatty acid substrates of TAGs are synthesized in the plastids. PC also serve as a substrate for the sequential unsaturation of C18:1 to C18:2 and C18:3. Abbreviations: ACCase, acetyl-CoA carboxylase; KAS, 3-ketoacyl-ACP synthase; FA, fatty acid; CoASH, coenzyme A that is not attached to an acyl group.

Fatty acids are the major components of both membrane and storage lipids. They are made up of carboxylic acids with highly reduced hydrocarbon chains. The saturated fatty acids contain carbon molecules linked by single bonds, whereas the unsaturated fatty acids consist of carbon chains that are linked by one or multiple cis double bonds [36]. The most common fatty acids in both membrane and storage lipids contain 16 or 18 carbons that are either saturated or unsaturated. All unsaturated fatty acid species are derived by the sequential unsaturation of the fully saturated species i.e., palmitic acid (C16:0) and stearic acid (C18:0) (the values preceding the colon represent the number of carbons in the fatty acid, whereas the numbers following it are the number of double bonds) (Table 1). This chemical reaction is catalyzed by the activity of various fatty acid desaturase enzymes. Desaturases convert single bonds at specific positions in the fatty acyl chains to double bonds by the removal of two hydrogen atoms [29–32,37,38].

Table 1. Common saturated and unsaturated fatty acids found in membrane and storage lipids of plants.

Common Name	Chemical Structure	Abbreviation	Melting Point
SATURATED FATTY ACID			
Lauric acid	$CH_3H_3(CH_2)_{10}COOH$	12:0	45.00
Palmitic acid	$CH_3(CH_2)_{12}CH_2CH_2COOH$	16:0	62.90
Stearic acid	$CH_3(CH_2)_{12}CH_2CH_2CH_2CH_2COOH$	18:0	69.60
Arachidic acid	$CH_3(CH_2)_{12}CH_2CH_2CH_2CH_2CH_2CH_2COOH$	20:0	76.00
Behenic acid	$CH_3(CH_2)_{12}CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH$	22:0	80.00
Lignoceric acid	$CH_3(CH_2)_{12}CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2COOH$	24:0	84.20
UNSATURATED FATTY ACID			
Palmitoleic acid	$CH_3(CH_2)_5C{=}C(CH_2)_7COOH$	$16{:}1^{\Delta 9}$	0.00
Roughanic acid	$CH_3CH_2C{=}C\text{-}CH_2\text{-}C{=}C\text{-}CH_2\text{-}C{=}C(CH_2)_5COOH$	$16{:}3^{\Delta 7,\,10,\,13}$	54.40
Oleic acid	$CH_3(CH_2)_7C{=}C(CH_2)_7COOH$	$18{:}1^{\Delta 9}$	13.40
Linoleic acid	$CH_3(CH_2)_4C{=}C\text{-}CH_2\text{-}C{=}C(CH_2)_7COOH$	$18{:}2^{\Delta 9,12}$	−5.00
α-Linolenic acid	$CH_3CH_2C{=}C\text{-}CH_2\text{-}C{=}C\text{-}CH_2\text{-}C\text{-}C(CH_2)_7COOH$	$18{:}3^{\Delta 9,\,12,\,15}$	−11.00
γ-Linolenic acid	$CH_3(CH_2)_4C{=}C\text{-}CH_2\text{-}C{=}C\text{-}CH_2\text{-}C{=}C(CH_2)_4COOH$	$18{:}3^{\Delta 6,\,9,\,12}$	−10.60
Erucic acid	$CH_3(CH_2)_7C{=}C(CH_2)_{11}COOH$	$22{:}1^{\Delta 13}$	33.50

Δ indicates the location of the double bond from the carboxyl end (–COOH).

In the subsequent sections, the role of membrane and storage lipid composition in the normal biological function of seeds, particularly during the early stages of germination, i.e., imbibition and lipid reserve metabolism, is discussed.

3. Lipid Composition Is Consequential in Restoring Membrane Equilibrium in Seeds during Water Imbibition

Among the different phospholipid classes produced in the ER, PAs are reported to be the most abundant in mature seeds [33]. PAs accumulate in membrane phospholipids as the seed undergoes a controlled process of desiccation during maturation. As the water content of the membranes goes below 20%, the increase in PA levels promotes a shift in membrane lipid configuration from a lamellar phase into a hexagonal phase [28,39]. Membranes in lamellar organization are characterized by two lipid layers (bilayer) with the polar heads in contact with water and the hydrophobic tails directed inwards. The hexagonal phase on the other hand, is an ill-configured, inverted configuration that is both porous and highly permeable (Figure 2). During the early stages of germination, the rapid influx of water as the seeds begin to rehydrate triggers the reorganization of the membrane lipids from the hexagonal back to the lamellar phase. These phase transitions cause temporary, structural perturbations in the membrane, resulting in the leakage of solutes and low molecular weight metabolites out of the cell [39–41]. Hydration of the membranes by up to 20–30% restores the lamellar conformation, re-establishes the selective permeability of the membrane, and stops the leakage of solutes [39].

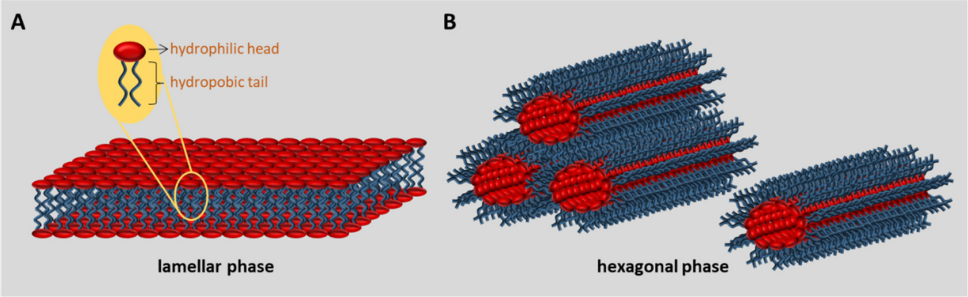

Figure 2. Lipid structure polymorphism. (**A**) Membrane lipids at the lamellar phase composed of a phospholipid bilayer. (**B**) Membrane lipids at the hexagonal phase. Red circles represent the hydrophilic head and the blue line represent the hydrophobic tails.

In soybeans, a significant decrease in PA, PI, and PS contents was observed during the initial phase of water imbibition. The reduction in PA levels accounted for 11% of the total loss in membrane lipid content before water imbibition by the seeds. Incidentally, decrease in the levels of PA was accompanied by the reversion of the membrane phospholipids into a more ordered and fluid, lamellar configuration, indicating the utilization of PA in membrane re-organization [28].

Additionally, the dry soybean seeds recorded negligible MGDG and DGDG contents. This was expected as plastids rarely develop in mature seeds. With continuous water uptake past the initial imbibition stage and up to the root elongation stage, the levels of both plastidic lipids increased, suggesting the initiation of plastid development in the cotyledons. The biosynthesis of plastids during the third phase of water uptake was confirmed by the detection of photosynthetic activity in the cotyledons of the seeds, as measured by the potential photochemical quantum efficiency of photosystem II [28].

The dynamic changes in the balance of membrane phospholipid composition during water imbibition indicate a specific function of each phospholipid in supporting the very early stages of membrane stability and therefore, seed germination.

4. Storage Lipids Are a Substantial Source of Energy for a Growing Embryo

Lipid reserves in seeds are commonly stored as triacylglycerols in oil bodies. Compared to carbohydrates which are highly hydrated, lipids are anhydrous and highly reduced, hence yield more energy on a mass basis when catabolized into water and carbon dioxide [42,43].

The mobilization of lipid reserves as an energy source for the growing embryo is initiated by the hydrolysis of TAG into its glycerol and fatty acid components through the activities of the lipase

enzyme. Once released from the oil body, the glycerol is converted into triosephosphate in the cytosol, which is then used to synthesize sucrose via gluconeogenesis. On the other hand, the fatty acids are imported into the peroxisome and are broken down via the cyclic process of β-oxidation (Figure 3). This process requires the oxidation of the second carbon (or β-carbon) of the fatty acid chain, hence the term β-oxidation. Before the initiation of the oxidation reaction, fatty acids are activated by the addition of coenzyme A. Two carbon units of fatty acids are removed in one cycle and the remaining compound is again fed into the β-oxidation cycle for further breaking down [42]. During the recurring β-oxidation cycles, nicotinamide adenine dinucleotide (NAD) hydride is produced from NAD^+ and fed into the electron transport chain to produce energy in the form of adenosine triphosphate (ATP) [44]. As has been demonstrated in *Arabidopsis thaliana* (L.) Heynh., acetyl-CoA, the end product of β-oxidation, may be used in respiration through the mitochondrial tricarboxylic acid cycle or converted to succinate and used as a substrate for the production of different carbohydrates necessary to feed a growing embryo [45]. The *Arabidopsis thaliana* mutant *ped1*, which is deficient in the production of the thiolase enzyme required for β-oxidation cycling, exhibits arrest in seedling development, indicating the importance of storage lipids as an energy source for seedling growth [46].

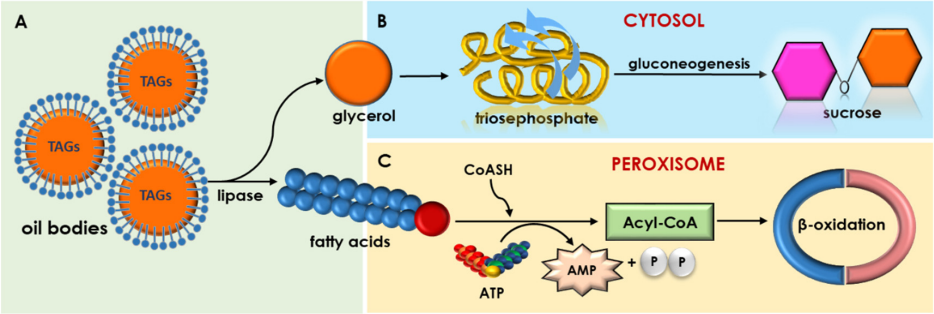

Figure 3. General pathway for lipid degradation in seeds. (**A**) Triacylglycerols are hydrolyzed into its glycerol and fatty acid components through the activity of lipases. (**B**) Glycerol is transported to the cytosol after being released from triacylglycerols (TAGs) where it is converted first to triosephosphate and then to sucrose via gluconeogenesis. (**C**) Fatty acids are imported to the peroxisome where they are broken down via β-oxidation.

5. Lipid Unsaturation Ensures the Integrity of Cell Membranes during Cold Water Imbibition

Dynamic changes in the membrane lipid composition of imbibing seeds are required to maintain the structural equilibrium of membranes necessary for proper seed germination. At low temperatures, imbibition of cold water by the seeds causes lipids with high melting temperature (because of high saturation in the fatty acid chains) to solidify and become separated within the membrane. This results in the loss of fluidity and selective permeability that disrupts membrane reorganization, thereby exacerbating cytoplasmic leakage [28,47–50]. Unless the structural integrity of the membranes is re-established, the excessive solute leakage can lead to chilling injuries resulting in metabolic dysfunction, abnormal root tip development, and even embryo death.

Re-modelling cell membrane fluidity is an adaptive response of plants to cold stress and is achieved by increasing the unsaturated fatty acid composition of membrane lipids [50,51]. These modifications are mediated in part by the activities of fatty acid desaturases [52–54]. The cis-double bonds created by the desaturases prevent the fatty acid molecules from packing tightly together, thereby keeping the membranes fluid and allowing membrane-bound proteins to move through [55]. Under cold stress, membrane flexibility through increased lipid unsaturation allows the maintenance of a homeostatic environment that is integral to the functioning of the cell [51].

The importance of increasing the levels of polyunsaturated fatty acids, via the desaturation of fatty acids such as palmitic, stearic, and oleic acids, toward enhancing the ability of higher plants to adapt to decreases in temperature has been illustrated in several studies. For example, transgenic

tobacco plants overexpressing an *Arabidopsis thaliana ω-fatty acid desaturase* gene (*FAD7*) exhibited lesser chilling injuries compared to the wild-type when subjected to 15 °C. The enhanced chilling tolerance of the transformed plants was attributed to the increased production of linolenic acid (18:3) in the leaves in response to cold [56]. In rice, overexpression of the Δ-*12 fatty acid desaturase* that catalyzes the conversion of oleic acid to linoleic acid resulted in the accumulation of the latter in the leaf tissues. The higher production of linoleic acid in the plant tissues enhanced the chilling tolerance of the transgenic plants at the vegetative and reproductive stages upon exposure to 10 °C for 8 days and to 20 °C for 2 days, respectively [57]. Recent studies in olives also showed that exposure of fruits to a suboptimal temperature of 15 °C increased the expression of *stearoyl-acyl carrier protein desaturase* (*SAD*), concomitantly increasing the unsaturated fatty acid contents in the microsomal membrane lipids of the mesocarp of a cold-tolerant cultivar. Increase in both *SAD* transcripts and unsaturated fatty acid levels in the cold-tolerant but not the cold-sensitive cultivar underscores the role of membrane unsaturation in the ability of the plants to adjust to low temperatures [54].

Aside from enhancing cold tolerance of plants in vivo, modulation of membrane lipid composition also provides an adaptive mechanism for seeds to germinate at low temperatures [49,57]. In corn, cold germination screening of two hybrids at 10 °C resulted in higher electrolyte leakage in the cold-sensitive than in the tolerant hybrid. Phospholipidomic assays to profile changes in the lipid membrane composition of the two hybrids during seed imbibition at suboptimal temperature revealed a significant accumulation in the di-unsaturated linoleic acid (C18:2) fraction, accompanied by a substantial decrease in the mono-unsaturated oleic acid (C18:1) fraction in the cold-tolerant hybrid. Conversely, the proportion of the saturated stearic acid (C18:0) and oleic acid (C18:1) fractions increased, whereas linoleic acid (C18:2) content decreased in the seeds of the cold-sensitive hybrid [49]. Transgenic rice seeds engineered to overproduce linoleic acid also showed higher germination rate at 4 °C compared to the wild type seeds. Biochemical assays revealed a significant reduction in the electrolyte leakage of the transgenic seeds germinated at 4 °C compared to the seeds of the untransformed plants germinated at the same suboptimal temperature [57].

Transgenic canola seeds producing three times more stearic acid compared to conventional varieties because of the knocked-down expression of *stearoyl-acyl desaturase* demonstrate in reverse the importance of lipid unsaturation in the ability of seeds to germinate under low temperature. A 30% increase in the saturated stearic acid levels and a 40% increase in the proportion of the total saturated fatty acids of the transgenic seeds relative to the non-transgenic seeds resulted in poor germination even at an ambient temperature of 25 °C. The incorporation of higher levels of saturates in the membrane lipids is presumed to have contributed to the rigidity of the cell membrane during imbibition, ultimately resulting in deficient germination [58].

Extensive studies on cell membrane re-modelling show that the incorporation of unsaturated fatty acids in the membrane lipids during cold water imbibition effectively increases cell membrane fluidity. During cold water imbibition, the more flexible membranes are expected to reorganize faster from the rigid, gel state (hexagonal configuration) to a crystalline-liquid state (lamellar configuration). This significantly minimizes the electrolyte leakage during imbibition and rapidly restores cellular function, thereby facilitating faster and higher seed germination even under cold conditions.

6. Faster Catabolism of Unsaturated Fatty Acids Provides Energy for the Growing Embryo at Low Temperature

Aside from conferring membrane flexibility, lipids also serve as a major energy resource for growing embryos during the early stages of germination. A brief overview of lipid catabolism was given previously. Examination of the seed oil composition of more than 700 species of flowering plants indicates that the balance between saturated and unsaturated fatty acid contents in seeds evolved as an adaptive mechanism in response to selection pressures imposed by germination temperature i.e., cooler mean daily germination temperature favors lower saturated fatty acid content in seeds and vice versa. This supposition is supported by studies showing tropical species that are acclimated to

warmer temperatures producing higher amounts of saturated fatty acids in their oils compared to temperate species [21]. Species of *Helianthus* and *Arabidopsis* that were grown at higher altitudes, where the temperature is expected to be lower, exhibited reduced proportions of saturated fatty acids in their seeds [21–23]. Similarly, *Plukenetia volubilis* L. plants growing at higher altitudes demonstrated higher proportions of unsaturated fatty acids compared to similar species growing at lower altitudes [59].

The evolutionary changes in seed oil composition in response to selective temperature pressure are functions of both the energetics and melting point/temperature of seed oils [21]. The energy required to synthesize saturated and unsaturated fatty acids, and the energy that can be produced by each upon catabolism via the ß-oxidation pathway are distinct from each other. Saturated fatty acids cost less to synthesize but yield more energy upon oxidation compared to unsaturated fatty acids on a per carbon basis. The penalty in producing unsaturated fatty acid comes from the additional energy costs required to produce desaturase enzymes responsible for the unsaturation of fatty acids and to form double bonds. During catabolism, the lower energy yield of unsaturated fatty acids is attributed to the additional steps and enzymes required to process them so they can be oxidized via the same pathway as saturated fatty acids [21].

In terms of melting point, the tightly packed, saturated fatty acids register a higher melting temperature compared to unsaturated fatty acids of the same length. The bent acyl chains formed by the cis double bonds between two carbon molecules in unsaturated fatty acids prevents close packing of the molecules, thereby lowering the temperature at which the chain melts [60]. Because triacylglycerols are composed of different fatty acids, their melting point is estimated roughly equal to the cumulative melting point (CMP) of the fatty acids that compose them. This can be calculated using the following formula:

$$\mathrm{CMP} = \frac{\sum_{i=1}^{n}(f_i t_i)}{100} \quad (1)$$

where

f = percent composition of a fatty acid in the seed
t = melting temperature of the fatty acid

Under normal or warm temperature, seeds with higher proportions of saturated fatty acids benefit from the higher total chemical energy that can be generated to fuel the growth and eventually, the germination of the embryo. At lower temperatures, however, the saturated fatty acids become more viscous, thus slowing the chemical reactions required for the catabolism of the fatty acids and significantly delaying germination. In cases where germination is delayed and slowed, the higher energy potential produced by catabolization of saturated fatty acids is not only less advantageous to the seeds but also wasteful. In contrast, seeds with higher proportions of unsaturated fatty acids will have lower melting temperatures facilitating better and faster germination under cold conditions [21,23].

Germination studies on *Helianthus* species from Texas and Canada showed that accessions with lower proportions of saturated fatty acids had higher and/or faster germination at lower temperatures than those with higher proportions of the same [21]. Similarly, experiments conducted by Miquel [52] showed that *Arabidopsis thaliana* seeds having higher proportion of polyunsaturated fatty acids (linolenic acid) germinated faster than seeds with high monounsaturated fatty acid (oleic acid) content. The higher melting point of saturated fatty acid contents of transgenic canola described in the previous section also contributed to the poor germination of seeds even at 25 °C. Lipidomic studies showed that the amount of lipids in transgenic seeds that failed to germinate barely changed within 6 days of germination experiments compared to the wild-type, which consumed approximately 75% of its stored lipids. This implies the possible crystallization of the TAGs even at ambient temperature, making them less accessible to lipases [58]. Consequently, the energy required by the embryo to grow was not sufficient, resulting in poor germination. In cotton, genetic modifications to produce higher proportions of the saturated stearic acid in the seeds also significantly reduced germination rates at

lower temperatures [48]. Collectively, these studies suggest an adaptive mechanism in plants to have higher amount of unsaturated fatty acids for a better chance of survival under cooler temperatures.

7. Unsaturation of Membrane and Storage Lipids as Breeding Targets to Improve cold Germination in Upland Cotton

Increasing polyunsaturated fatty acid composition in seeds has been demonstrated to contribute in conferring cold or chilling tolerance in plants, particularly during seed germination. Scientific evidence that supports this contention can be found in literature on cold tolerance research on *Arabidopsis thaliana*, tobacco, corn, soybean, and peanuts [28,49,52,56,61]. In cotton, cold germination studies have been carried out as early as the 1960s, although the functional roles of fatty acid composition on the ability of cotton to germinate under low temperatures has not been fully explored [62,63].

Compared to other oilseed species, cottonseed oils are highly unsaturated, containing approximately 50% linoleic acid (C18:2), 21–26% palmitic acid (C16:0), 16–20% oleic (C18:1), and 2–3% stearic acid (C18:0) [64,65]. Despite the higher proportions of unsaturated fatty acids in cottonseed oils, the crop remains highly sensitive to cold or chilling temperatures [16]. During germination, imbibition of cold water by the seeds have been reported to cause irreversible chilling injuries that can adversely affect the subsequent growth and maturity of the plants [15]. Cotton seeds that imbibed water at 5 °C for 96 h before getting transferred to the greenhouse have been reported not to germinate. However, seed imbibition for 4 h at temperatures above 20 °C before the cold treatment did not reduce seed germination and subsequent plant growth [62]. The results of the study suggest that during germination, cotton is sensitive to chilling stress only at the very early stages of water imbibition by the dry, mature seed. In fact, germination of cottons seeds that imbibed 13% more water under normal temperatures was no longer affected by subsequent cold stress [63]. Based on these observations, the critical time window when the seeds are most sensitive to chilling injuries seem to coincide with the phase transition of membrane lipids from the hexagonal to the lamellar conformation. In cotton, as in soybeans or corn, low temperature during this critical phase transition forces membrane lipids into a more rigid, gel organization that significantly delays the re-establishment of a more fluid, lamellar conformation necessary for the normal physiological functions of the seed. The slower re-establishment of the lamellar phase of the membrane lipids makes the seeds more vulnerable to leaching, which can be detrimental to germination.

By and large, enhancing the potential of cotton seeds to germinate under low temperatures will require the ability of membrane lipids to remain fluid under cold conditions. To this end, screening of genetically diverse germplasm for potential sources of higher lipid unsaturation in seeds is extremely important. Results of recent studies conducted in our laboratory showed the faster and more uniform germination of ethylmethanesulfonate (EMS)-induced mutants of cotton with higher linoleic acid (52–63%) and lower palmitic acid contents (17–20%) compared to natural (i.e., non-mutated) accessions from the *Gossypium* Diversity Reference Set (GDRS) [66,67]. The mean average germination rate for the fatty acid (FA) mutants at 15 °C was almost 95%, whereas that of the GDRS accessions was only 36%. Hydropriming of the GDRS seeds for 8 h at higher temperatures (28–30 °C) prior to cold exposure increased their overall germination to 92% at 15 °C, although the improvements were not at par with the germination rates of the non-hydroprimed seeds of the FA mutants germinated at the same suboptimal temperature. At an even lower temperature of 12 °C, the FA mutants recorded a mean germination rate of 33%, whereas the GDRS accessions showed only 14% germination.

Conversely, a separate study by Liu et al. [48] demonstrated a significant decrease in germination at low temperature of transgenic cotton engineered to produce higher levels of the saturated stearic acid in the seeds. Survey of the fatty acid composition showed that the transgenic seeds had 40% stearic acid and greatly reduced fractions of palmitic acid (15%) and linoleic acid (38.8%) compared to the non-transgenic seeds.

In both FA mutants with high unsaturated fatty acid content and transgenics with high-stearic acid content, the cold germination ability of seeds was associated with the degree of seed oil unsaturation.

The increase in the linoleic acid and decrease in the palmitic acid content in the FA mutants significantly increased the unsaturation levels in the seed oils. The enhanced degree of unsaturation likely contributed in maintaining the fluidity of the membrane lipids at 15 °C, allowing the rapid transition from the porous hexagonal to the lamellar phase, consequently leading to a high germination rate. At 12 °C, a 33% mean germination of the FA mutants compared to the 14% mean germination of the GDRS accessions implies the maintenance of some degree of fluidity in the membrane as a function of seed oil unsaturation. This proposition is reflected in reverse in the reduced germination rate of the transgenic seeds engineered to produce very high proportions of stearic acid in the seed oil [48]. The significant increase in the overall saturation level of the transgenic seeds would have concomitantly increased its melting temperature such that during cold germination, the lipids in the transgenic seeds would have been forced into a more rigid organization, delaying the recovery of the functional, lamellar configuration of the membrane lipids. Biochemical assays, such as electrolyte leakage and lipid peroxidation analysis of seeds of FA mutants and their wild-types, or GDRS accessions at several time points during cold imbibition can validate these assumptions.

In addition to the effects on lipid reconfiguration, membrane unsaturation would have also contributed in the mobilization of storage lipids toward providing energy to a growing embryo. In the FA mutants, the increase in the levels of linoleic acid effectively lowers the cumulative melting temperature of the storage lipids, facilitating faster lipid catabolism even at low temperatures and providing the seed with the required energy to germinate faster and at a higher rate. In the transgenic seeds, the higher melting temperature brought on by a significant increase in the stearic acid fractions may have caused the crystallization of storage lipids at lower temperature making it inaccessible to lipases for hydrolysis (Figure 2). The bound fatty acid would then be unavailable for β-oxidation and subsequent mobilization to fuel embryo growth.

More than 50% of cottonseed oil is composed of linoleic acid, making it highly unsaturated. Despite this fact, cotton remains sensitive to chilling temperatures, indicating a critical threshold for fatty acid unsaturation that will allow uniform germination of cotton seeds under low temperature. Recent studies showing the ability of FA mutants with increased fatty acid unsaturation to achieve more than 90% germination under cold conditions [67] supports this hypothesis. Results of phospholipidomic studies in corn also suggest that cold germination ability can be enhanced with modifications in fatty acid composition of seed oils toward higher levels of unsaturation [49].

Although much remains to be discovered about the theoretical underpinnings of the effects of lipid unsaturation in the ability of cotton to germinate in cold conditions, collective scientific evidence strongly indicates a potential in targeting this trait to improve cold or chilling tolerance in cotton especially during the critical stages of germination. To this end, the genetic resources (i.e., FA mutants, transgenics) that have been developed and selected for significant modifications in fatty acid saturation levels will be crucial in elucidating the molecular basis of the trait toward its purposeful utilization in breeding for cotton improvement.

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