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大豆种子吸胀冷害抗性与其质体膜脂不饱和度正相关*

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摘要: 吸胀冷害是干种子在吸胀阶段遭受低温造成不萌发的现象, 结果可能造成农作物损失严重。虽然吸胀过程中细胞膜的修复是关键事件, 而且细胞膜在响应水分和温度胁迫中扮演重要角色, 但是种子吸胀过程中膜变化的过程, 特别是膜流动性变化过程研究较少。本文比较了吸胀冷害耐受型 (LX) 和敏感型 (R5) 两个大豆品种在吸胀冷害过程中膜脂不饱和度 (double-bond index, DBI) 的变化, 结果发现, LX 和 R5 在常温 (25 ℃) 吸胀时变化趋势一致, 质体膜脂 DBI 升高, 质体外膜脂中磷脂酰甘油 (phosphatidylglycerol, PG) 分子 DBI 下降。LX 和 R5 在低温 (4 ℃) 吸胀时 DBI 变化有很大差异, 低温吸胀仅仅延缓了耐受型 LX 中质体膜脂 DBI 的升高, 但是敏感性 R5 质体膜脂 DBI 不仅没有升高反而下降。用浓度 33%的聚乙二醇 (polyethylene glycol, PEG) 引发没有直接引起 DBI 变化, 但是所引起的细微而显著的变化可能为萌发做好准备。PEG 引发处理后的 R5 在吸胀冷害后第二和第三阶段质体膜脂 DBI 迅速增加, 这个增加模式与 LX 的 DBI 增加相似。结果表明, 吸胀冷害延缓或者阻滞了质体膜脂不饱和度的升高, 大豆种子的吸胀冷害抗性与质体膜脂不饱和度正相关, 提高质体膜质 DBI 可以提高吸胀冷害抗性。

关键词: 大豆种子; 吸胀冷害; 膜脂不饱和度; 渗透调控; 双键指数
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The Degree of Unsaturation of Plastidic Membrane Lipids is Positively Associated with Tolerance to Imbibitional Chilling in Soybean Seeds

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Abstract: Injury caused by imbibitional chilling is a common phenomenon during rehydration of desiccated seeds, and usually causes seriously compromises seedling emergence and crop yield. Restoration of the cell membranes is a critical event during imbibition, moreover it is very important in responses to water and temperature stress. However, the changes in membrane structure during seed imbibition, especially those related to membrane fluidity, have yet to be investigated. This study compared changes in the level of unsaturation of membrane lipids (double-bond index, DBI) between chilling-tolerant 'LX' and chilling-sensitive 'R5' soybean cultivars during imbibitional chilling. After imbibition at normal temperature (25 ℃), seeds of LX and R5 showed similar changes in the level of lipid unsaturation, with increased DBI values for plastidic lipids and reduced levels of extraplastidic phosphatidylglycerol (PG). In contrast, there were dramatic differences in the changes of DBI between LX and R5 following imbibition under chilling conditions (4 ℃). Chilling only delayed the increase of plastidic DBI in seeds of the tolerant cultivar

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LX, whereas plastidic DBI did not increase or even decreased in the sensitive cultivar R5 under these conditions. Priming of seeds by incubation in 33% polyethylene glycol (PEG) did not directly cause changes in DBI, however, the subtle but significant changes in DBI that it induced prepared the soybean strain for germination. Plastidic DBI increased during phases II and III of germination in PEG-osmoconditioned R5 seeds under imbibitional chilling stress; this increase was similar to the patterns of increase in DBI of LX. Our results suggest that chilled imbibition delays or prevents the increase of the degree of unsaturation in plastidic membrane lipids, that tolerance to imbibitional chilling in soybean is positively associated with an increase in the level of unsaturation of plastidic membrane lipids, and that increased plastidic DBI could improve tolerance of imbibitional chilling.

Key words: Soybean seeds; Imbibitional chilling injury; Degree of unsaturation of membrane lipids; Osmoconditioning; Double-bond index

Imbibition is a critical process during seed germination. The uptake of water by a mature dry seed is triphasic (Bewley, 1997; Yin *et al.*, 2009; Han *et al.*, 2013), with rapid initial uptake (phase I) followed by a plateau phase (phase II). A further increase in water uptake occurs only after germination has been completed, as the embryonic axes elongate (phase III). In dry seeds, cell membrane systems lose their integrity and adopt a wrinkled and disordered form, such as the hexagonal II or crystalline gel forms. Upon rehydration, these systems repair themselves into an orderly and stable configuration, which restores integrity and fluidity (Zheng, 1991). The restoration of the cell membrane occurs during the phase involving the physical uptake of water (phase I), and it is very important for seed germination (Bewley, 1997). It is generally recognised that injury caused by imbibitional chilling occurs when dry seeds imbibe at a low temperature at this stage (Zheng, 1988, 1991). The imbibitional temperature and rate are the two main factors that determine the success of membrane reorganization (Yin *et al.*, 2009; Lyons, 1973). Low temperature and rapid uptake of water can severely disturb phospholipids and induce a change in their arrangement from a hexagonal (dehydrated) to a lamellar (hydrated) architecture (Simon, 1974).

Severe injury caused by imbibitional chilling occurs when chilling-sensitive seeds absorb water at a lower temperature. This results in poor germination, reduced seedling emergence, decreased seedling vigour, and ultimately loss of yield (Pollock, 1969). Such injury is a common problem in agriculture and has been reported for a wide range of crops, including green bean and lima bean (Pollock, 1969), cotton (Christia, 1967), pea (Powell and Matthews, 1978), cucumber (Willing and Leopold, 1983), corn and soybean (Obendorf and Hobbs, 1970). On one occasion, a 25% decrease in soybean yield caused by chilling injury was reported, even though the field seedling density remained the same (Hobbs and Obendorf, 1972).

Osmoconditioning involves the incubation of seeds in an osmoticum, which is usually a solution of a salt or polyethylene glycol (PEG). Priming seeds for germination by incubating them in solutions of PEG was previously shown to reduce or eliminate damage caused by chilled imbibition, and it also clearly improved seed vigour (Woodstock and Tao, 1981; Posmyk *et al.*, 2001). Chilling injury and PEG priming are associated with various biochemical and metabolic alterations. In chilling-sensitive seeds, the greatest sensitivity to low temperatures occurs during the first hours of the imbibition phase (Pollock and Toole, 1966; Christia, 1967; Bramlage *et al.*, 1978; Leopold, 1980). The leakage of solutes from chilled imbibing seeds suggests that membrane reorganisation is impaired at low temperatures (Bramlage *et al.*, 1978), and thus that membrane systems are the cell components that are most vulnerable to injury by chilling; however, only a limited number of studies have addressed this issue.

Membranes, particularly plasma and chloroplast membranes are sensitive to environmental stimuli. The fluidity of cell membranes can influence their stability

during exposure to stress, it can be optimised by adjustment of the membrane lipid or fatty acid composition, structure, and level of unsaturation in response to stress, which are important determinants of the ability to adapt to stress (Navari-Izzo *et al.*, 1993). The degree of unsaturation of membrane glycerolipids (measured as the double-bond index, DBI) is the major factor that determines membrane fluidity. A high DBI indicates the presence of more unsaturated membrane lipids. At low temperatures, the DBI of fatty acids is increased. Tolerance of leaves to dehydration or desiccation is associated with a decrease in fatty acid content and alteration in the composition of unsaturated and saturated fatty acids in various plant species (Xu *et al.*, 2011). Dehydration reduces the DBI of fatty acids in the plastidic membranes of leaves (Quartacci *et al.*, 2002). Following dehydrative stress, differences in the DBI values of four cultivars of grapevine (*Vitis vinifera* L.) that exhibit different levels of drought tolerance implied that specific adjustments in the level of unsaturation of lipids during stress could compromise stress tolerance (Toumi *et al.*, 2008). The ability to change DBI in order to maintain membrane integrity and fluidity during periods of stress is of major importance for plant survival, as discussed above, and may also be critical for seed rehydration or regrowth (Quartacci *et al.*, 1995; Bettaieb *et al.*, 2009; Toumi *et al.*, 2008). However, changes in DBI during imbibitional chilling and the effects of these changes on seed germination do not appear to have been reported.

Plant lipidomics analysis based on electrospray ionization tandem mass spectrometry (ESI-MS/MS) can rapidly measure the relative abundances of hundreds of different types of lipid molecules in vivo using small samples (Welti *et al.*, 2002), this enables accurate calculation of the DBI of all membrane glycerolipids measured. Several studies have employed lipidomics to profile changes in molecular species and DBI during plant growth and stress. For example, Zheng *et al.* (2011) used a lipidomics approach to profile the changes in molecular species of membrane glycerolipids and thus calculated the DBI in four plant species in response to frequent changes of temperature.

Seeds of most soybean varieties are sensitive to cold imbibition, albeit to various extents. Alleviation of this sensitivity poses one of the most important challenges to reliable and sustainable soybean production in Northeast China (Zheng, 1991). We found two soybean cultivars characterised by different levels of resistance to imbibitional chilling: 'LX' (chilling-tolerant) and 'R5' (chilling-sensitive). The purpose of this study was to use lipidomic analysis to determine: (i) how DBI changes in these two soybean cultivars during their imbibition at normal and cold temperatures; (ii) how DBI changes during PEG priming and how DBI changes in PEG-primed chilling-sensitive seeds subsequently imbibed at cold temperatures; and (iii) whether chilling tolerance is associated with a change in DBI during imbibition.

1 Methods and materials

1.1 Seeds

Soybean seeds of the two cultivars, LX and R5, were obtained from a commercial source. The seeds were equilibrated to 10% moisture over a saturated solution of LiCl (53% relative humidity) for one week and then stored at 15 ℃ until analysis.

1.2 Seed germination and sensitivity to imbibitional chilling injury

Seeds of the two cultivars were tested for sensitivity to imbibitional chilling. They underwent imbibition for 24 h in distilled water at 25 ℃ or 4 ℃, and were then transferred to 25 ℃ for four days during which seed germination was recorded using a camera. Percent germination was measured to determine the sensitivity to imbibitional chilling, four replications with 40 seeds each were germinated at 25 ℃ (unpulished data).

1.3 Seed priming

Dry seeds underwent imbibition in 33% PEG-6000 solution for three days at 15 ℃. And then the seeds were washed three times in distilled water and then desiccated for three days at room temperature to

adjust their water content to 10.3% on average (dry weight basis), which is similar to that of control untreated seeds. The control seeds and the primed seeds then underwent imbibition at 4 ℃ for 24 h for imbibitional chilling treatment. Untreated seeds were used as controls.

1.4 Lipid extraction and ESI/MS-MS analysis

The process of lipid extraction, ESI-MS/MS analysis, and quantification was performed as described previously with minor modification (Welti *et al.*, 2002; Li *et al.*, 2008; Zheng *et al.*, 2012). Each sample contained seed axes with a pooled dry weight of 10 to 20 mg; samples were harvested at the indicated sampling time. To inhibit lipolytic activity, seed axes were transferred immediately into 3 mL of isopropanol with 0.01% butylated hydroxytoluene in a 75 ℃ water bath. The tissue was extracted three times with chloroform/methanol (2∶1) with 0.01% butylated hydroxytoluene, with a week of agitation. The remaining plant tissue was dried overnight at 105 ℃ and weighed to give the dry weight of the tissue. Lipid samples were analysed on a triple quadrupole MS/MS equipped for ESI. Automated ESI-MS/MS analysis was performed in the Kansas Lipidomics Research Center Analytical Laboratory as described previously (Kansas Lipidomics Research Center, http: //www.k-state.edu/lipid/lipidomics).

1.5 Data analysis

Data processing was performed as previously described (Devaiah *et al.*, 2006; Welti *et al.*, 2002; Zheng *et al.*, 2012). DBI were calculated using the formula: DBI = [∑(N×mol% lipid)]/100, where N is the total number of double bonds in the two fatty acid chains of each glycerolipid molecule (Zheng *et al.*, 2011; Osmond *et al.*, 1982; Rawyler *et al.*, 1999; Bakht *et al.*, 2006). Five replicates of each treatment were analysed. The Q-test was performed on the total amount of lipid in each class, and data from discordant samples were removed. The data were subjected to one-way analysis of variance (ANOVA) with SPSS 13.0. Statistical significance was tested by Fisher's least significant difference (LSD) method.

2 Results and discussion

2.1 LX and R5 cultivars differ markedly in their sensitivity to imbibitional chilling

After 24 h of imbibition at 25 ℃, both cultivars exhibited a seed germination rate of 100% (Fig. 1, Control), which indicated that an extremely high level of viability of these seeds. However, LX and R5 exhibited contrasting levels of resistance to imbibitional chilling (Fig. 1, Imbibition Chilling): (1) R5, chilling-sensitive: Imbibition at 4 ℃ caused large reductions in survival and seedling vigour, and no germination occurred (0 germination rate, unpulished data). The seeds were surrounded by a circle of a light-yellow, sticky substance, which may have been mildew or fungal rot. (2) LX, chilling-resistant: Imbibitional chilling had a small effect; a small reduction of radicle emergence was observed compared with the control (unpulished data), but normal radicle extension occurred. Therefore, we used LX as a chilling-resistant cultivar and R5 as a chilling-sensitive one for further comparative investigation of the effects of chilled imbibition on germination.

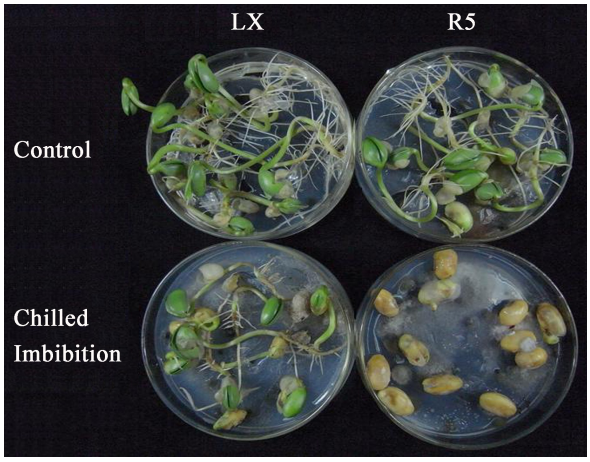

Fig. 1 The sensitivity of soybean cultivars to imbibitional chilling injury
Control: Seeds underwent imbibition for 24 h in distilled water at 25 ℃, followed by germination for four days at 25 ℃. Chilled imbibition: Seeds underwent imbibition for 24 h in distilled water at 4 ℃, followed by germination for four days at 25 ℃. LX, Liaoxin; R5, Riben 5

2.2 Effects of PEG priming on R5 seed germination

Many studies have indicated that osmopriming can accelerate seed germination. In order to deter-

mine whether PEG-osmoconditioned seeds were chilling-sensitive, they underwent imbibition at 4 ℃ for 24 h and were then transferred to 25 ℃. Figure 2 shows that control seeds lost their ability to germinate at 25 ℃ after imbibition at 4 ℃. However, the germination of PEG-osmoconditioned R5 seeds at 25 ℃ was almost unchanged after chilled imbibition (unpulished data). This experiment indicated that PEG osmoconditioning has clear advantages for improving the tolerance of R5 seeds to imbibitional chilling. Therefore, PEG was used to investigate the mechanisms of injury due to imbibitional chilling in sensitive seeds.

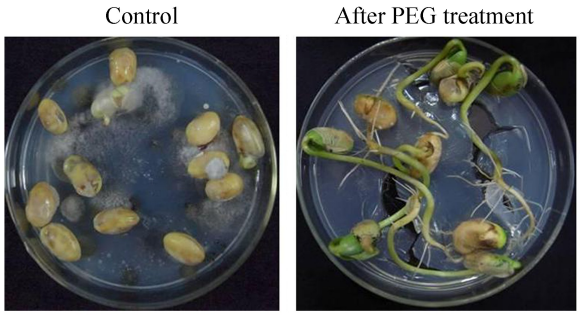

Fig. 2　Prior imibibition in solutions of PEG protects against chilling injury in germinating R5 soybean seeds

Controls: R5 seeds without PEG treatment underwent imbibition for 24 h in distilled water at 4 ℃, followed by germination for four days at 25 ℃. After PEG treatment: PEG-treated R5 seeds underwent imbibition for 24 h in distilled water at 4 ℃, followed by germination for four days at 25 ℃

2.3　Profiling DBI changes during germination

We harvested the embryo axes of LX and R5 seeds after their imbibition in water for 0 h (control), 3 h, and 24 h at 25 ℃ or 4 ℃, and then at one and three days of germination at 25 ℃ for lipidomics analysis. Base on the water uptake (unpulished data), these sampling times represent the three phases during germination (Bewley, 1997; Yin *et al.*, 2009; Han *et al.*, 2013). Imbibition for 3 h represented the period of rapid initial uptake (phase I), whereas imbibition for 24 h represented the plateau phase (phase II). Penetration of the seed radicles through the seed coat 1 day after the beginning of imbibition indicated that the seed had completed phases I+II and was just starting phase III (unpulished data). Using a lipidomics approach based on ESI-MS/MS (Welti *et al.*, 2002; Li *et al.*, 2008), we identified and quantified six head-group classes of phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidylglycerol (PG), as well as two head-group classes of galactolipids: monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) (Welti *et al.*, 2002), and thus we calculated their DBI during the three phases of germination (Table 1). From an overview of the DBI, it can be seen that there was diversity in the changes in DBI among the eight classes of lipids, differences between normal and chilling germination, and differences between PEG- and non-PEG-treated R5 seeds following chilled imbibition. Detailed analysis and discussion are presented in later sections.

2.4　Patterns of changes in levels of extraplastidic and plastidic DBI differ after imbibition at 25 ℃

To understand better the patterns of DBI change during germination, we profiled the DBI phase by phase from dry seeds to three days of germination in LX and R5 seeds imbibed at the normal temperature tested (25 ℃). Table 1 shows that LX and R5 had the same patterns of change after imbibition at 25 ℃; namely, the content of total DBI increased with time. After three days of germination, the contents of MGDG and DGDG increased markedly, whereas levels of PG significantly decreased; however, the contents of PI, PE, PC, PA, and PS changed very little.

The eight classes of lipids can be divided into extraplastidic and plastidic types. Plastidic lipids include MGDG and DGDG, which are the dominant components of chloroplast membranes (Jarvis *et al.*, 2000; Dubots *et al.*, 2010). Five of the other classes of lipids (PC, PE, PI, PS, and PA) belong to the extraplastidic type (Li *et al.*, 2008). PG lipids include both classes: 34∶4 PG (total carbon number: double-bond number), which harbours a 16∶1 acyl chain, is part of the plastidic membrane, whereas

both 34:1 PG and 34:2 PG are extraplastidic lipids (Welti *et al.*, 2002; Marechal *et al.*, 1997). In soybean seed axes, PG includes 34:1 PG, 34:2 PG, and 34:3 PG, but not 34:4 PG (data not shown). Accordingly, in this case, PG can be classified as extraplastidic lipids. As such, the data obtained in this study indicate that extraplastidic and plastidic lipids are affected differently by imbibition at 25 ℃. These differences may be associated with the special biological significance of extraplastidic and plastidic membranes. Dry seeds had very low levels of plastidic DBI (Table 1), which confirmed that plastids were scarce in dry seeds. The content of plastidic membrane DBI increased along with seed germination. This suggested that MGDG and DGDG synthesis and chloroplast development occurred during this period. Because MGDG and DGDG are the dominant components of thylakoid membranes, they are the main contributors to membrane unsaturation because they also harbour a relatively high level of trienoic fatty acids.

For all glycerolipid classes, the changes in DBI were small at phases I and II. However, during phase III, dramatic alterations took place in both the extraplastidic and plastidic lipids of the two cultivars. This was probably because, during imbibition (phases I and II), seeds mainly adjust their lipid composition using existing substances in order to respond to the level of hydration, whereas enzymes initiate metabolism and the synthesis of new polyunsaturated or saturated fatty acids in order to restore their integrity and fluidity state post-germination (phase III). Therefore, the DBI changed little during imbibition, but displayed significant changes at phase III; in addition, chloroplast development was the main occurrence during post-germination.

2.5 The effects of chilled imbibition on the DBI in chilling-tolerant seeds

To study how chilled imbibition affects DBI, we compared the DBI values of LX seeds imbibed at 25 ℃ and those imbibed at 4 ℃. The patterns of changes in the DBI of extraplastidic and plastidic lipids were similar between seeds imbibed at normal and cold temperatures (Table 1). Increases in the plastidic membrane DBI and decreases of extraplastidic PG DBI after imbibition at 4 ℃ imbibition were slower than those observed after imbibition at 25 ℃. For example, the DGDG DBI after imbibition at 4 ℃ (4.57) was lower than that at 25 ℃ (4.90) after one day of germination (Table 1). However, there was no difference after three days of germination. These results indicate that chilling delayed an increase in the DBI of plastidic lipids.

2.6 Differential changes in DBI between LX and R5 following chilled imbibition

Severe injury occurred in R5 seeds after imbibition at a low temperature. To reveal whether DBI change is associated with imbibitional chilling injury, we compared the extraplastidic and plastidic DBI of R5 with those of LX seeds subjected to chilled imbibition (Table 1). The DBI of the two cultivars exhibited different changes. In seeds of the tolerant cultivar LX, imbibition at 4 ℃ caused dramatic increases in the levels of total DBI and plastidic DBI (MGDG and DGDG), with MGDG increasing from 3.75 (control) to 5.76 (germination 3 d). However, MGDG and DGDG did not increase or even decreased in the sensitive cultivar R5 following imbibitional chilling: MGDG decreased from 5.21 (control) to 4.65 (germination 3 d). This indicated that the DBI of R5 seeds after imbibition at 4 ℃ differed markedly from that of LX seeds with normal germination. Low DBI levels of MGDG and DGDG in R5 seeds could be associated with their severe injury due to chilled imbibition. In other words, changes in the degree of unsaturation of membrane lipids of soybean seeds were suggested to be related to the tolerance of imbibitional chilling.

Given that dry seeds do not contain chloroplasts for photosynthesis, the development of chloroplasts to provide ATP during seed germination is essential for seeds to overcome imbibitional chilling and to grow. Photosynthesis requires high levels of membrane polyunsaturation (Mcconn and Browse, 1998). A high DBI helps to maintain the fluidity of mem-

branes in order to sustain the functional activity of membrane proteins and the membranes themselves. MGDG and DGDG, the dominant components of thylakoid membranes, are the main contributors to membrane unsaturation because they harbour a relatively high level of trienoic fatty acids.

The desaturation of lipids in plants starts with the synthesis of 16∶0 and 18∶0 fatty acids (Wallis and Browse, 2002; Zheng *et al.*, 2011). Desaturation is mediated by a series of desaturases that are located

Table 1　Changes in the double-bond index (DBI) of each lipid class at 25 ℃ or 4 ℃ in LX and R5

Seeds underwent imbibition in water at 25 ℃ or 4 ℃ for 3 h and 24 h, and were then transferred to 25 ℃ for germination for one and three days. Values are means ± *SE* (n=4 or 5). Values in the same row with different letters are significantly different (P<0.05). DBI=(∑[N×mol% molecular species])/100, where N is the number of double bonds in each molecular species. LX, Liaoxin; R5, Riben 5

Lipid class	Treatment/℃		Double-bond index (DBI)				
			Control	Imbibition 3 h; phase I)	Imbibition 24 h; phase II	Germination 1 d; phase III	Germination 3 d; phase III
PG	LX	25	2.37 ± 0.03^{b}	2.48 ± 0.02^{a}	2.36 ± 0.01^{b}	1.69 ± 0.05^{c}	1.64 ± 0.02^{c}
		4	2.37 ± 0.03^{b}	2.40 ± 0.03^{b}	2.48 ± 0.03^{a}	1.88 ± 0.08^{c}	1.59 ± 0.04^{d}
	R5	25	2.43 ± 0.01^{a}	2.43 ± 0.03^{a}	2.41 ± 0.02^{a}	1.98 ± 0.06^{b}	1.59 ± 0.06^{c}
		4	2.43 ± 0.01^{a}	2.33 ± 0.01^{b}	2.44 ± 0.03^{a}	2.09 ± 0.03^{c}	1.93 ± 0.05^{d}
PI	LX	25	2.51 ± 0.01^{ab}	2.52 ± 0.01^{a}	2.50 ± 0.01^{b}	2.42 ± 0.01^{d}	2.68 ± 0.02^{c}
		4	2.51 ± 0.01^{b}	2.54 ± 0.02^{a}	2.53 ± 0.01^{a}	2.38 ± 0.01^{c}	2.52 ± 0.02^{ab}
	R5	25	2.53 ± 0.02^{b}	2.54 ± 0.01^{b}	2.49 ± 0.01^{c}	2.36 ± 0.01^{d}	2.70 ± 0.01^{a}
		4	2.53 ± 0.02^{a}	2.54 ± 0.02^{a}	2.56 ± 0.01^{a}	2.39 ± 0.02^{b}	2.36 ± 0.01^{b}
PE	LX	25	3.29 ± 0.03^{a}	3.24 ± 0.06^{b}	3.24 ± 0.03^{b}	3.13 ± 0.02^{c}	3.28 ± 0.01^{ab}
		4	3.29 ± 0.03^{a}	3.28 ± 0.03^{a}	3.24 ± 0.07^{ab}	3.19 ± 0.01^{b}	3.21 ± 0.04^{b}
	R5	25	3.34 ± 0.01^{b}	3.37 ± 0.03^{a}	3.28 ± 0.01^{c}	3.16 ± 0.01^{d}	3.28 ± 0.02^{c}
		4	3.34 ± 0.01^{a}	3.30 ± 0.05^{a}	3.34 ± 0.04^{a}	3.22 ± 0.02^{b}	3.24 ± 0.01^{b}
PC	LX	25	3.45 ± 0.04^{bc}	3.41 ± 0.03^{c}	3.41 ± 0.04^{c}	3.47 ± 0.07^{b}	3.67 ± 0.01^{a}
		4	3.45 ± 0.04^{bc}	3.47 ± 0.03^{b}	3.39 ± 0.01^{d}	3.43 ± 0.03^{c}	3.56 ± 0.01^{a}
	R5	25	3.48 ± 0.01^{b}	3.46 ± 0.02^{b}	3.49 ± 0.05^{b}	3.42 ± 0.03^{c}	3.65 ± 0.02^{a}
		4	3.48 ± 0.01^{ab}	3.49 ± 0.02^{a}	3.45 ± 0.03^{b}	3.46 ± 0.02^{ab}	3.35 ± 0.03^{c}
PA	LX	25	3.34 ± 0.03^{b}	3.41 ± 0.03^{a}	3.41 ± 0.06^{a}	3.20 ± 0.04^{c}	3.35 ± 0.01^{b}
		4	3.34 ± 0.03^{b}	3.43 ± 0.02^{a}	3.43 ± 0.02^{a}	3.26 ± 0.05^{c}	3.29 ± 0.05^{c}
	R5	25	3.40 ± 0.01^{b}	3.58 ± 0.04^{a}	3.45 ± 0.02^{b}	3.30 ± 0.06^{c}	3.29 ± 0.03^{c}
		4	3.40 ± 0.01^{c}	3.47 ± 0.02^{b}	3.55 ± 0.02^{a}	3.38 ± 0.04^{c}	3.31 ± 0.04^{d}
PS	LX	25	2.24 ± 0.02^{c}	2.30 ± 0.03^{b}	2.29 ± 0.03^{b}	2.22 ± 0.01^{c}	2.37 ± 0.02^{a}
		4	2.24 ± 0.02^{b}	2.32 ± 0.09^{a}	2.26 ± 0.01^{ab}	2.24 ± 0.01^{b}	2.30 ± 0.01^{ab}
	R5	25	2.27 ± 0.03^{c}	2.35 ± 0.06^{ab}	2.32 ± 0.02^{b}	2.22 ± 0.02^{d}	2.38 ± 0.02^{a}
		4	2.27 ± 0.03^{b}	2.50 ± 0.16^{a}	2.30 ± 0.01^{b}	2.25 ± 0.01^{b}	2.25 ± 0.01^{b}
MGDG	LX	25	3.95 ± 0.16^{d}	4.33 ± 0.11^{c}	4.36 ± 0.06^{c}	5.54 ± 0.04^{b}	5.80 ± 0.01^{a}
		4	3.95 ± 0.16^{d}	4.02 ± 0.48^{cd}	4.37 ± 0.04^{c}	5.29 ± 0.14^{b}	5.76 ± 0.02^{a}
	R5	25	5.21 ± 0.05^{b}	4.40 ± 0.09^{d}	4.44 ± 0.09^{d}	4.99 ± 0.10^{c}	5.79 ± 0.01^{a}
		4	5.21 ± 0.05^{a}	4.09 ± 0.16^{d}	4.36 ± 0.03^{c}	4.68 ± 0.01^{b}	4.65 ± 0.13^{b}
DGDG	LX	25	4.56 ± 0.07^{c}	4.48 ± 0.10^{c}	4.27 ± 0.02^{d}	4.90 ± 0.06^{b}	5.13 ± 0.01^{a}
		4	4.56 ± 0.07^{b}	4.60 ± 0.08^{b}	4.34 ± 0.05^{c}	4.57 ± 0.06^{b}	5.17 ± 0.03^{a}
	R5	25	4.66 ± 0.10^{b}	4.60 ± 0.02^{b}	4.46 ± 0.02^{c}	4.49 ± 0.08^{c}	5.11 ± 0.01^{a}
		4	4.66 ± 0.10^{b}	4.82 ± 0.13^{a}	4.59 ± 0.06^{b}	4.27 ± 0.01^{c}	4.24 ± 0.07^{c}
Total	LX	25	3.17 ± 0.02^{c}	3.19 ± 0.08^{c}	3.20 ± 0.03^{c}	3.31 ± 0.01^{b}	3.74 ± 0.02^{a}
		4	3.17 ± 0.02^{c}	3.24 ± 0.05^{b}	3.22 ± 0.03^{b}	3.24 ± 0.03^{b}	3.58 ± 0.03^{a}
	R5	25	3.20 ± 0.02^{d}	3.28 ± 0.02^{c}	3.24 ± 0.04^{cd}	3.39 ± 0.03^{b}	3.76 ± 0.04^{a}
		4	3.20 ± 0.02^{ab}	3.28 ± 0.03^{a}	3.26 ± 0.01^{a}	3.24 ± 0.03^{a}	3.16 ± 0.04^{b}

in the endoplasmic reticulum and chloroplasts; these molecules have similar catalytic sequences within their active sites. Although little is known about the enzymatic mechanism by which desaturated fatty acids are saturated, low DBI levels in R5 seeds could result from inhibition of lipid synthesis processes at a low temperature. Accordingly, higher levels of unsaturated lipids in plastidic membrane might account for the tolerance of LX seeds to chilled imbibition.

2.7 Effects of PEG priming on DBI of membrane lipids

The above experiment indicates that PEG osmotic pretreatment (priming or osmoconditioning) reduced the damage due to imbibitional chilling in R5 seeds. We profiled the DBI in R5 seeds osmoconditioned for one and three days as well as PEG-treated dry seeds to test whether PEG treatment changed the seed DBI (Table 2). The results indicate that PEG osmopriming slightly but significantly changed the DBI of membrane lipids in chilling-sensitive seeds (R5), with the small changes being very similar to the pattern of LX imbibition at 25 ℃ during phase I. This finding suggested that PEG priming did not directly cause the main changes in DBI, but rather induced changes in DBI in preparation for germination.

2.8 Dramatic changes in DBI profiles occur in PEG osmoconditioned R5 seeds under imbibitional chilling stress

To examine how the effects of PEG osmopriming occur under imbibitional chilling, we analysed the DBI of membrane lipids in PEG-treated R5 seeds during imbibition at 4 ℃ for 3 and 24 h, followed by germination at 25 ℃ for one and three days (Table 3). Levels of the plastidic lipids MGDG and DGDG increased continually over the period studied: MGDG increased from DBI of 5.13 (control) to 5.72 (germination 3 d), whereas DGDG increased from

Table 2 Changes in double-bond index (DBI) of each lipid class in R5 soybean seeds during the PEG priming. Values are means ± *SE* (n=4 or 5). Values in the same row with different letters are significantly different (P<0.05)

Lipid class	Double-bond index (DBI)			
	Control	PEG 1 d	PEG 3 d	After PEG priming
PG	2.44 ± 0.07^{c}	2.62 ± 0.06^{b}	2.77 ± 0.04^{a}	2.78 ± 0.05^{a}
PI	2.52 ± 0.02^{b}	2.52 ± 0.01^{b}	2.53 ± 0.02^{b}	2.58 ± 0.02^{a}
PE	3.50 ± 0.03^{a}	3.37 ± 0.02^{b}	3.39 ± 0.01^{b}	3.39 ± 0.02^{b}
PC	3.55 ± 0.03^{a}	3.38 ± 0.03^{b}	3.35 ± 0.02^{b}	3.41 ± 0.03^{b}
PA	3.35 ± 0.02^{a}	3.34 ± 0.01^{a}	3.31 ± 0.01^{b}	3.28 ± 0.02^{b}
PS	2.47 ± 0.06^{b}	2.51 ± 0.03^{b}	2.67 ± 0.07^{a}	2.55 ± 0.09^{ab}
MGDG	4.93 ± 0.12^{b}	4.92 ± 0.11^{b}	5.21 ± 0.08^{a}	5.13 ± 0.17^{ab}
DGDG	4.59 ± 0.13^{a}	4.48 ± 0.08^{ab}	4.51 ± 0.11^{ab}	4.34 ± 0.08^{b}
Total	3.19 ± 0.03^{a}	3.13 ± 0.01^{b}	3.11 ± 0.02^{b}	3.12 ± 0.02^{b}

Table 3 Changes in double-bond index (DBI) of each lipid class during imbibitional chilling and post-germination for R5 soybean seeds after PEG priming. Values are means ± *SE* (n=4 or 5). Values in the same row with different letters are significantly different (P<0.05)

Lipid class	Double-bond index (DBI)				
	After PEG treatment	Imbibition 3 h	Imbibition 24 h	Germination 1 d	Germination 3 d
PG	2.78 ± 0.05^{a}	2.73 ± 0.11^{a}	2.78 ± 0.05^{a}	1.96 ± 0.01^{b}	1.53 ± 0.01^{c}
PI	2.58 ± 0.03^{b}	2.58 ± 0.03^{b}	2.64 ± 0.01^{a}	2.37 ± 0.01^{c}	2.61 ± 0.01^{b}
PE	3.39 ± 0.02^{a}	3.38 ± 0.01^{a}	3.37 ± 0.02^{a}	3.18 ± 0.02^{c}	3.29 ± 0.01^{b}
PC	3.41 ± 0.03^{c}	3.38 ± 0.04^{c}	3.52 ± 0.02^{b}	3.42 ± 0.01^{c}	3.67 ± 0.04^{a}
PA	3.28 ± 0.02^{b}	3.21 ± 0.03^{c}	3.47 ± 0.06^{a}	3.20 ± 0.03^{c}	3.31 ± 0.06^{b}
PS	2.55 ± 0.09^{a}	2.50 ± 0.07^{a}	2.27 ± 0.02^{b}	2.21 ± 0.02^{c}	2.30 ± 0.02^{b}
MGDG	5.13 ± 0.17^{b}	4.70 ± 0.19^{c}	4.38 ± 0.05^{d}	5.32 ± 0.13^{b}	5.72 ± 0.01^{a}
DGDG	4.34 ± 0.08^{c}	4.48 ± 0.13^{c}	4.25 ± 0.13^{c}	4.77 ± 0.08^{b}	5.15 ± 0.03^{a}
Total	3.12 ± 0.02^{d}	3.17 ± 0.01^{c}	3.35 ± 0.01^{b}	3.39 ± 0.04^{b}	3.54 ± 0.02^{a}

4.34 (control) to 5.15 (germination 3 d). For extraplastidic lipids, the levels of PG decreased continually, from 2.78 (control) to 1.53 (germination 3 d). These remarkable changes occurred mainly during phase III. These patterns were the same as those for the imbibition of LX and R5 seeds at 25 ℃. These results indicate that PEG osmopriming had a large effect on DBI in chilling-sensitive seeds during phases II and III. The effect of PEG priming was to increase the degree of unsaturation of plastidic membrane in order to enhance the tolerance of imbibitional chilling. PEG-osmoprimed R5 seeds could synthesise desaturases under chilled imbibition in order to change their membrane fluidity and enhance photosynthesis. This result further demonstrates that the DBI of seed membrane lipids is positively correlated with their resistance to imbibitional chilling.

3 Conclusion

The present study provides evidence that the level of unsaturation of membrane lipids is involved in the tolerance of soybean seeds to imbibitional chilling. Extensive increases in the extent of total lipid unsaturation occurred during imbibition and after germination. LX exhibited similar changes to R5 after imbibition at 25 ℃. The content of total DBI increased during germination, which was mainly due to the marked increase in the content of plastidic DBI. There were increases of plastidic DBI in LX after imbibition at 4 ℃, but these increases were significantly slower than those after imbibition at 25 ℃. However, the patterns of change in R5 significantly differed from the patterns that occurred upon normal imbibition, when even plastidic DBI decreased. PEG priming slightly changed the DBI in sensitive seeds (R5). It improved their resistance to injury due to imbibitional chilling, mainly by increasing their plastidic DBI during phases II and III. Given that increases of plastidic DBI favor the resistance of membranes to low temperature injury, those seeds with higher DBI values for plastidic lipids might be more likely to germinate after imbibitional chilling stress. The delay or prevention of an increase in the degree of unsaturation in plastidic membrane lipids was thus the cause of injury due to imbibitional chilling. The increase in the DBI of plastidic lipids is associated with the restoration of functional chloroplastic membranes needed to support photosynthesis and membrane fluidity.

The results of this study suggested that an increase in the degree of unsaturation in plastidic membrane lipids is positively associated with the tolerance to imbibitional chilling in soybeans. These findings have not been reported previously for soybean seeds, and should provide further insight into the mechanisms of adaptation to, and survival of, imbibitional chilling. Such insights might guide the development of chilling-resistant seeds. Further analyses should be undertaken to study the mechanisms that underlie differences between these two cultivars in the way that chilled imbibition affects DBI.

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