Plant Physiology and Biochemistry 142 (2019) 452–459

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Membrane sterols and genes of sterol biosynthesis are involved in the response of *Triticum aestivum* seedlings to cold stress

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ARTICLE INFO

Keywords:
Wheat
Cold stress
Sterols
Glycoceramides
Membrane stability
C24-sterol methyltransferases

ABSTRACT

Cold stress can significantly alter the composition and functioning of the major membrane lipids in plants. However, the roles of the sterol component of plant membranes in stress tolerance remain unclear. In the work presented here we investigated the role of sterols in the response of wheat to cold stress. Initial experiments demonstrated that the roots and leaves of wheat seedlings are differentially sensitive to low positive temperatures. In the roots, cold stress induced disturbance of membrane integrity and accumulation of ROS followed by the induction of autophagy. The absence of such changes in leaves suggests that in wheat, the roots are more sensitive to cold than the leaves. The roots display a time-dependent parabolic pattern of cold stress response, characterized by raised levels of sterols and markers of oxidative stress during short-term treatment, and a decline of these parameters after prolonged treatment. MβCD-induced sterol depletion aggravated the negative effects of cold on the roots. In the leaves the changes also displayed parabolic patterns, with significant changes occurring in 24-ethyl sterols and major PLs. Constitutively high levels of sterols, glycolipids and PLs, and up-regulation of *TaSMTs* in the leaves may provide membrane stability and cold tolerance. Taken together, results suggest that sterols play important roles in the response of wheat seedlings to cold stress.

1. Introduction

Temperature stress occurs commonly in plants growing in their natural environment or as crops. Defense reactions to cold involve a sharp rise in the amounts of endogenous cryoprotecting compounds such as sugars, proline, betaine, and activation of antioxidative systems (Bilska-Kos et al., 2017). Failure to develop a defense strategy to cold may cause disruptions to important physiological processes such as photosynthesis and respiration, and result in loss of turgor, chlorophyll destruction, accumulation of reactive oxygen species (ROS) and lipid peroxidation (Airaki et al., 2012).

Membranes are important sites of both cold perception and injury (Orvar et al., 2000). Initial symptoms of cold stress include changes in membrane fluidity and functional activity, including the activity of membrane-associated proteins (Los and Murata, 2004). Modification of membrane fluidity can influence gene expression. For example, membrane rigidification caused by DMSO treatment at +25 °C upregulates a cold-acclimation marker gene and increases freezing tolerance (Orvar et al., 2000). Membrane-associated histidine kinases (such as Hik33) are cold sensors (Suzuki et al., 2000). Hik33 contains putative membrane-spanning domains, and was therefore proposed to be a membrane-associated sensor that transduces a cold-elicited signal to a downstream transducer (Suzuki et al., 2000). Cold-induced Hik33-dependent gene expression is initiated by red light (Feduraev et al., 2018). Another cold sensor in mammals and some other organisms is the superfamily of transient receptor proteins (TRP), a group of calcium-permeable ion channels (García-Ávila and Islas, 2019), however, in plants such sensors have not been identified so far. It seems likely that not all receptors for cold stress are associated with membranes. For example, the phytochrome B photoreceptor, which is located in the nucleus, also participates in temperature perception in plants (Legris et al., 2016).

Membrane fluidity is determined by the relative proportions of their constituent lipids. It has been demonstrated that cold stress can significantly alter the membrane lipid composition, for example the degree

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https://doi.org/10.1016/j.plaphy.2019.07.026
Received 23 April 2019; Received in revised form 30 July 2019; Accepted 31 July 2019
Available online 31 July 2019

Abbreviations

DPG	diphosphatidylglycerol
FA	fatty acid
GlCer	glycoceramides
MSI	membrane stability index
MβCD	methyl-β-cyclodextrin
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
PL	phospholipids
ROS	reactive oxygen species
SMT	C24 sterol methyltransferases

of unsaturation of the fatty acid (FA) residues of phospho- and glycolipids, and also the relative proportions of sterols, sphingolipids and phospholipids (PL) (Vu et al., 2014). These changes help to enhance membrane cryo-tolerance and stability (Uemura and Steponkus, 1997). Unlike phospho- and glycolipids, information about the involvement of sterols in the response of plants to cold is rather limited. Davis and Finkner (1973) reported that in wheat shoots reducing the temperature from +10 °C to +1 °C results in a general decrease in sitosterol, stigmasterol, and campesterol. Authors suggested that temperature influences the movement of the sterol precursor mevalonic acid from the site of its synthesis to the site of campesterol, sitosterol, and stigmasterol synthesis. As structural membrane components, sterols can affect the physical status of a membrane during stresses not only *via* quantitative changes in total sterols but also *via* changes in the ratio of their molecular species (Senthil-Kumar et al., 2013). The characteristic feature of plant sterols is the availability of methyl or ethyl groups in the side chain at the 24th carbon atom, and they are referred to as methyl sterols or ethyl sterols, respectively. The balance between 24-methyl sterols and 24-ethyl sterols is specific for individual plant species. Moreover, the ratio of sterol molecular species, in particular *β*-sitosterol/stigmasterol, can influence the physical and chemical properties of ordered microdomains, the so-called “lipid rafts”, which are enriched in sphingolipids and sterols (Zauber et al., 2014). Therefore, changes in the ratio of sterol molecular species can modulate signaling and the defense responses of plants.

The key enzymes of the sterol biosynthesis that catalyze the formation of methyl and ethyl sterols are the C24 sterol methyltransferases (SMT). In plants, two types of SMTs carry out the primary (SMT1) and secondary (SMT2) methylation of the 24th carbon atom of the side chain of sterols (Nakamoto et al., 2015). The reaction product of primary methylation is campesterol (24-methyl sterol), and the end products of secondary methylation are *β*-sitosterol and stigmasterol (24-ethyl sterols). It has been shown that SMTs are essential for normal growth, development and the responses of plants to stress (Guan et al., 2017).

Earlier we demonstrated that sterol binding by nystatin and methyl-*β*-cyclodextrin (M*β*CD) causes not only sterol depletion but also has many effects on the physiology of wheat roots (Valitova et al., 2014, 2016). We also found that changes in sterol content and the activity of *TaSMT1* genes occur in roots subjected to oxidative and wounding stresses (Sulkarnayeva et al., 2014). The aim of present work was to understand the role of sterols in response of plants to low positive temperature (+4 °C) stress by analyzing lipid composition, membrane stability and redox changes in the roots and leaves of wheat seedlings. Our results demonstrate that cold treatment causes marked changes in the ratios between 24-methyl/ethyl sterols, PL and glycoceramides (GlCer) in the roots and leaves of wheat seedlings. Taken together, our results suggest that changes in sterols, together with other membrane lipids, are involved in physiological and biochemical responses of wheat roots and leaves to cold stress.

2. Materials and methods

2.1. Plant material

Wheat (*Triticum aestivum* L. cv. Kazanskaya Jubilejnaya; Niva Tatarstana, Kazan, Russia) seedlings were grown hydroponically in 0.25 mM $CaCl_2$ at +22 °C and a light intensity of 100 W/m^2 with a 12 h photoperiod for 4 d. To induce cold stress, 4 d old seedlings were transferred to +4 °C for 1 h and 12 h, and then roots and leaves were used for measurements.

2.2. Lipid extraction and analysis

Total lipids of roots and leaves of wheat seedlings were extracted with a mixture of isopropanol and chloroform (1:1), according to Nichols (1963) with modifications (Kotlova et al., 2009). Individual PLs and GlCer were analyzed with 2D TLC, according to Vaskovsky and Terekhova (1979) with modifications. A full description of the methodology of lipid analysis has been published previously (Valitova et al., 2011). Amounts of individual PLs and total GlCer were determined with a densitometer (DenSkan, Russia). Eluted sterols were dried and, after sample preparation, analyzed by GC-MS (Shimadzu, Japan). Sterol identification was performed using the mass spectral library of the GC-MS data system (McLafferty et al., 1999). Quantification was performed with the UNICHROM software (http://www.unichrom.com), with naphthalene as the internal standard. The instrumental error of measurements was less than 5%.

2.3. Membrane permeability for electrolytes

Membrane permeability was evaluated by the leakage of electrolytes from the tissues using the conductivity meter Cond 7310 (WTW, Germany), and the MSI was calculated as a percentage of the total yield of electrolytes.

2.4. H_2O_2 content and lipid peroxidation

The content of H_2O_2 was determined in the soluble fraction of homogenate derived from both leaves and roots using the xylenol orange assay (Gay and Gebicki, 2000). The H_2O_2 content was calculated by use of a calibration curve. Lipid peroxidation in the soluble fraction of the homogenate was assessed spectrophotometrically by measuring the content of TBA-reactive products (λ_{532}).

2.5. Fluorescence imaging of autophagosomes

Imaging of autophagosomes was performed using the fluorescence dye LysoTracker Red DND-99 (LT, Invitrogen, USA, λ_{ab} 577 nm/λ_{em} 590 nm) and a laser confocal microscope LSM-510 Meta (Carl Zeiss, Germany) (Valitova et al., 2014).

2.6. Analysis of gene expression by quantitative real-time PCR

Total RNA was isolated from wheat roots and leaves using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocols. RNA concentration and purity were assessed using the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA) and the integrity was verified by 1% agarose gel electrophoresis. The reverse transcription (RT) reaction was performed using C1000 Touch™ Thermal Cycler (Bio-Rad, USA) with reverse transcriptase RevertAid RT

(Thermo Scientific, USA) and Oligo $(dT)_{16}$ primers (Sintol, Russia) in 25 μl reaction volume according to the standard manufacturer's protocol.

Real-time qPCR was performed using an ICycler IQ 2 Multicolor Real-Time PCR Detection System (Bio-Rad, USA). The templates were amplified three times at 95 °C for 3 min followed by 40 cycles of amplification (94 °C for 10 s and 55/60 °C for 40 s). ADP-ribosylation factor (*TaARF*) and RNase L inhibitor-like protein (*TaRLI*) genes were used as reference genes (Paolacci et al., 2009). The gene-specific primers and TaqMan probes used for real-time qPCR are listed in Supporting Information Table S1. Quantification was performed according to Pfaffl (2001).

2.7. Statistics

All experiments were performed three times with at least three biological and four analytical replicates. Data presented are means and standard errors. Statistical analysis was performed using the two-tailed Student's *t*-test in Microsoft Excel 2013. Statistically significant differences from the control are marked with asterisks. One, two or three asterisks represent significant difference at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ respectively.

3. Results

3.1. The permeability of membranes and membrane stability index

In the control treatments the MSI was slightly higher in the roots than in the leaves (Table 1). In the roots, exposure of wheat seedlings to cold increased electrolyte leakage and reduced the MSI. Increasing the exposure time from 1 to 12 h enhanced these effects. By contrast, in the leaves these parameters did not change during cold treatment (12 h) (Table 1).

3.2. Redox status and autophagy

The cellular redox status of wheat seedlings was assessed by the changes in the level of lipid peroxidation and the content of H_2O_2. Cold treatment for 1 h induced pronounced redox changes in wheat roots. Lipid peroxidation increased by almost 50% and H_2O_2 content by more than 2-fold (Table 1). After 12 h of cold treatment the levels of lipid peroxidation and the H_2O_2 content returned to the control level (Table 1). By contrast, in the leaves cold treatment had no effect on the level of lipid peroxidation, while the H_2O_2 content slightly increased after 12 h (Table 1).

Cold treatment of wheat seedlings induced autophagy in the roots (Fig. 1a and b). Exposure of wheat seedlings to low positive temperature for 1 h or 3 h followed by transfer of the seedlings to RT for 2 h induced the accumulation of autophagosomes in the cytoplasm of cells. Autophagosomes appeared as bright-colored puncta (Fig. 1a). Interestingly, immediately after cold exposure autophagosomes were not visualized in the roots. Expression of the *TaATG8* gene was increased by up to 3.5 times by cold treatment in the roots (Fig. 1b), confirming the induction of autophagy.

3.3. Membrane parameters and the redox status in the presence of sterol binding agent MβCD

Treatment with the sterol-binding agent methyl-β-cyclodextrin (MβCD) had little effect on unstressed wheat seedlings. For example, 5 mM MβCD treatment caused only a non-significant decrease in MSI evidenced by a slight increase in electrolyte leakage from the roots and no change in H_2O_2 content. However, compared with roots treated with cold alone, 12 h pre-incubation of wheat seedlings with MβCD followed by 1 h cold treatment enhanced electrolyte leakage, decreased MSI and sharply (11-fold) increased the H_2O_2 content (Table 2).

3.4. Sterols

Short-term (1 h) cold treatment of wheat seedlings increased the total sterol content of the roots and leaves compared to the control (Fig. 2a). However, after 12 h of exposure to cold the total sterol content returned to control values in both the roots and leaves. The sterols β-sitosterol, stigmasterol (24-ethyl sterols), campesterol (24-methyl sterol) and small amount of cholesterol were identified (Table 3). In the control, the ratio of 24-methyl/ethyl sterols was lower in the leaves than in the roots (Table 4). In the roots of cold-treated seedlings a slight increase in the ratio of 24-methyl/ethyl sterols occurred as a result of an increase in campesterol. By contrast, in the leaves of cold-treated seedlings, the ratio of 24-methyl/ethyl sterols was reduced due to an increase in the amounts of β-sitosterol and stigmasterol (Tables 3 and 4).

3.5. Glycoceramides

In control seedlings the level of GlCer was higher in the roots than in leaves (Fig. 2b). Short-term cold response (1 h) of wheat seedlings significantly reduced total GlCer, while long-term cold treatment (12 h) increased the level of the total GlCer in both roots and leaves. These changes were more prominent in the roots than in the leaves (Fig. 2b).

3.6. Phospholipids

The total PL content in the leaves of the control plants (ca. 2350 μg/g FW) was double that in the roots (ca. 1240 μg/g FW) (Fig. 3). The relative contents of phosphatidylcholine (PC) in the roots (Fig. 3a) and leaves (Fig. 3b) was 2-fold higher than the relative content of phosphatidylethanolamine (PE). The other PLs such as phosphatidic acid (PA), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylinositol (PI), and phosphatidylserine (PS) were only present in small amounts (Fig. 3). Short-term cold treatment slightly reduced the content of major PLs in the roots (Fig. 3a). By contrast, in the leaves the PC content decreased, while the PE content increased (Fig. 3, Table 5). Long-term cold treatment resulted in more pronounced changes in the PL composition in the roots and leaves. In the roots the content of PE and PA significantly increased, while the level of PC declined (Fig. 3). In the leaves the content of PC and PA increased, while the amount of PE decreased. Therefore, the ratio of PC/PE changed in different ways in the roots and leaves, displaying upward parabolic-shaped response in

Table 1
The changes in membrane stability index (MSI), lipid peroxidation and H_2O_2 content in the roots and leaves of wheat seedlings under cold stress. Values represent the means ± SE. Two asterisks represent significant difference at $P \leq 0.01$ (two-tailed Student's *t*-test; $n = 6$).

	Treatment	MSI (%)	Lipid peroxidation (%)	H_2O_2 (mM g^{-1} FW)
Roots	Control	93.6	100 ± 1	6.3 ± 0.5
	Cold (+4 °C, 1 h)	91.6	157 ± 11**	13.2 ± 2.4**
	Cold (+4 °C, 12 h)	85.6	117.3 ± 7	5.4 ± 1.1
Leaves	Control	88.6	100 ± 3.1	5.9 ± 0.4
	Cold (+4 °C, 1 h)	87.3	89 ± 4.2	6.3 ± 0.7
	Cold (+4 °C, 12 h)	88.5	96.4 ± 8.1	8.1 ± 1.2

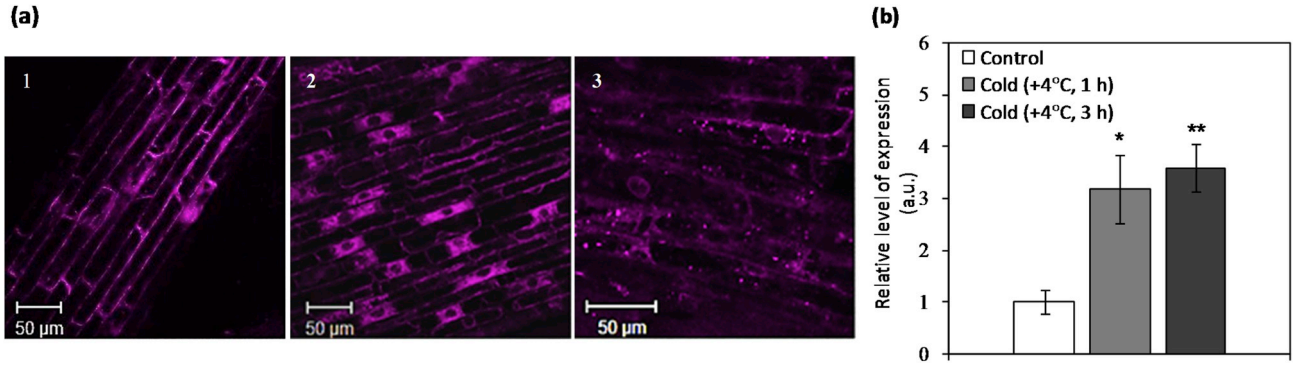

Fig. 1. (a) Induction of autophagy in cells of wheat roots treated with low temperature. Visualization of autophagosomes using LysoTracker Red in root cells was more prominent after 1 h cold treatment followed by keeping the seedlings at room temperature. Control (1), 1 h cold + 1 h RT (2), 1 h cold + 2 h RT (3). (b) The level of relative expression of autophagy marker *TaATG8* gene in cold treated wheat roots. Bars denote means ± SE of the mean. One or two asterisks represent significant difference at $P \leq 0.05$ and $P \leq 0.01$ respectively (two-tailed Student's *t*-test; $n = 6–9$). A. u., arbitrary units; RT, room temperature. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
The changes in membrane stability index (MSI) and H_2O_2 content in the roots of wheat seedlings treated with MβCD, cold stress and both MβCD and cold. Values represent the means ± SE. One, two or three asterisks represent significant difference at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ respectively (two-tailed Student's *t*-test; $n = 6$).

Treatment	MSI (%)	H_2O_2 (mM g^{-1} FW)
Control	93.6	6.9 ± 3
MβCD	90.6	5 ± 1.1
Cold (+4°C, 1 h)	91.6	13.5 ± 1.8*
MβCD + cold	89.3	55.4 ± 2.6***

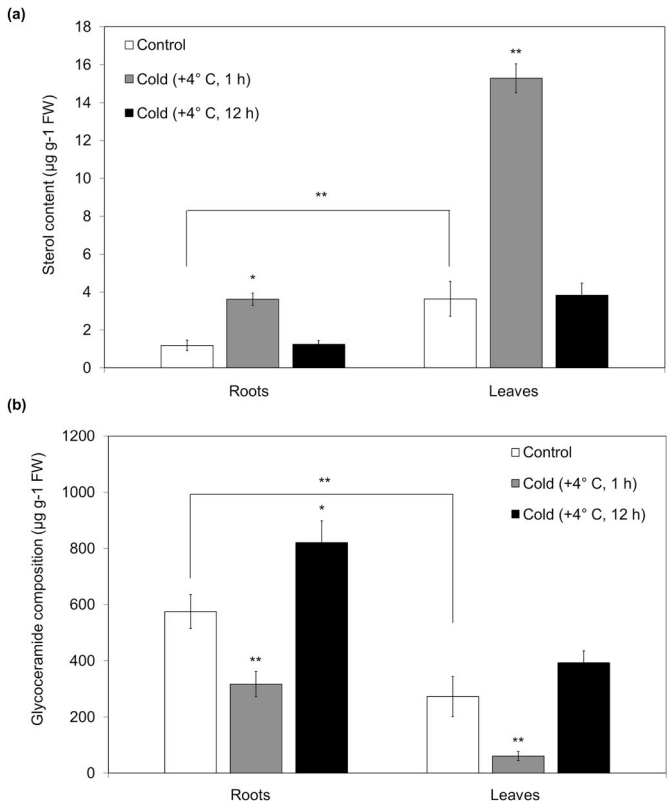

Fig. 2. The total content of (a) sterols and (b) GlCer in the roots and leaves of cold treated wheat seedlings. Bars denote means ± SE of the mean. One or two asterisks represent significant difference at $P \leq 0.05$ and $P \leq 0.01$ respectively (two-tailed Student's *t*-test; $n = 9$). FW, fresh weight; GlCer, glycoceramides.

the roots and downward parabolic-shaped response in the leaves (Table 5).

3.7. Differential expression of *TaSMT1* and *TaSMT2* genes

The expression of the *TaSMT1* and *TaSMT2* genes was estimated in the roots and leaves of control seedlings and cold stressed seedlings. The *TaSMT1* gene was more strongly expressed in the roots than in leaves (Fig. 4a), which suggests that the gene is differentially expressed in different plant organs. Cold stress up-regulated the activity of *TaSMT1* and *TaSMT2* genes both in the roots and leaves, with the greatest changes taking place in roots exposed to +4 °C for 12 h (a 4-fold increase for *TaSMT1* and 8-fold for *TaSMT2*).

4. Discussion

In nature, plants are regularly subjected to cold stress. Cold perception by membranes is suggested to occur *via* cold sensors such as the membrane-associated Hik33 (Suzuki et al., 2000). In addition, membrane lipids undergo complex changes following cold stress. Previous studies have emphasized the effects of cold on the saturation of FA in the PL and the total level of sterols (Uemura and Steponkus, 1997; Minami et al., 2010). Here we show that in wheat seedlings cold stress alters the ratio between various classes of membrane lipids, in particular the molecular species of sterols, and also the ratio between sterols and GlCer, resulting in changes in membrane permeability and cellular redox status. These cold-induced changes differ in the roots and leaves of wheat seedlings.

4.1. Roots are more sensitive to cold stress than the leaves in wheat

Measurements of membrane stability and redox status demonstrate that in wheat the roots are more sensitive to cold than the leaves. For example, cold treatment decreases the membrane stability of roots but has no effect on leaves (Table 1). A similar differential sensitivity to cold between the roots and leaves occurs in other plants (Davis and Finkner, 1973; Zhang et al., 2017). In addition, in wheat roots cold treatment for 1 h increases lipid peroxidation and the H_2O_2 content (Table 1), although after 12 h these parameters returned to the level of the control roots. By contrast, in the leaves cold reduces lipid peroxidation and has no effect on the H_2O_2 content. Therefore, the data on both membrane stability and redox status suggest that the roots are more sensitive to cold than the leaves.

A harmful consequence of oxidative stress is the accumulation of oxidized macromolecules in the cells. Autophagy is an effective mechanism for the removing oxidized macromolecules and damaged

Table 3
Change the composition and content (μg g^{-1} FW) of molecular species of sterols of cold treated wheat roots and leaves. Values represent the means ± SE. One, two or three asterisks represent significant difference at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ respectively (two-tailed Student's *t*-test; $n = 9$).

	Treatment	β-Sitosterol	Stigmasterol	Campesterol	Cholesterol
Roots	Control	0.72 ± 0.14	0.09 ± 0.05	0.33 ± 0.05	0.05 ± 0.04
	Cold (+4 °C, 1 h)	2.16 ± 0.12**	0.26 ± 0.01**	1.12 ± 0.19**	0.08 ± 0.01*
	Cold (+4 °C, 12 h)	0.81 ± 0.10	0.03 ± 0.01	0.35 ± 0.08	0.06 ± 0.01
Leaves	Control	2.23 ± 0.57	0.44 ± 0.10	0.86 ± 0.24	0.11 ± 0.01
	Cold (+4 °C, 1 h)	11.57 ± 0.34***	1.08 ± 0.19*	2.47 ± 0.22**	0.18 ± 0.02
	Cold (+4 °C, 12 h)	2.59 ± 0.42	0.31 ± 0.05	0.86 ± 0.15	0.07 ± 0.02

Table 4
The ratio of 24-methyl/ethyl sterols in the roots and leaves of cold treated wheat seedlings. Values represent the means ± SE. One asterisk represents significant difference at $P \leq 0.05$ (two-tailed Student's *t*-test; $n = 9$).

Treatment	Ratio of 24-methyl/ethyl sterols (a.u.)	
	Roots	Leaves
Control	0.41 ± 0.03	0.32 ± 0.05
Cold (+4°C, 1 h)	0.46 ± 0.01*	0.20 ± 0.01*
Cold (+4°C, 12 h)	0.41 ± 0.02	0.30 ± 0.03

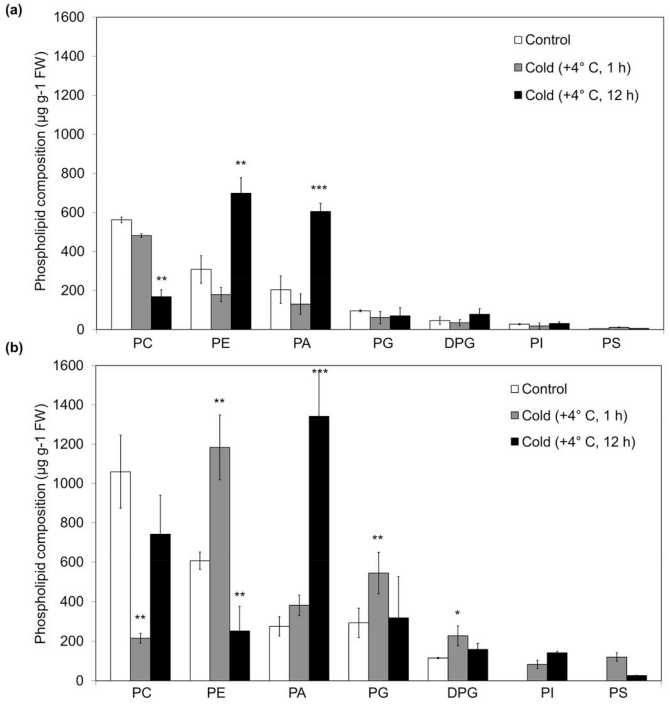

Fig. 3. The PL content in the (a) roots and (b) leaves of cold treated wheat seedlings. Bars denote means ± SE of the mean. One, two or three asterisks represent significant difference at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ respectively (two-tailed Student's *t*-test; $n = 9$). PL, phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PG, phosphatidylglycerol, DPG, diphosphatidylglycerol, PI, phosphatidylinositol; PS, phosphatidylserine; FW, fresh weight.

Table 5
The ratio of PC/PE in the roots and leaves of cold treated wheat seedlings. Values represent the means ± SE. One or two asterisks represent significant difference at $P \leq 0.05$ and $P \leq 0.01$ respectively (two-tailed Student's *t*-test; $n = 9$).

Treatment	Ratio of PC/PE (a.u.)	
	Roots	Leaves
Control	1.83 ± 0.2	1.75 ± 0.3
Cold (+4°C, 1 h)	2.68 ± 0.2*	0.18 ± 0.4**
Cold (+4°C, 12 h)	0.24 ± 0.4**	2.95 ± 1.0

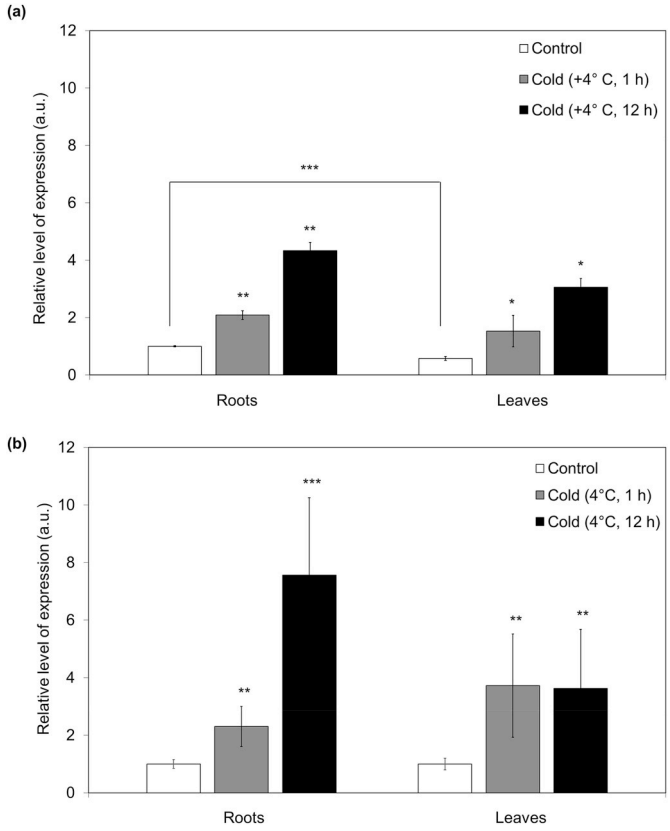

Fig. 4. Relative expression level of (a) *TaSMT1* and (b) *TaSMT2* genes assessed by real-time qPCR in cold treated wheat roots and leaves. Bars denote means ± SE of the mean. One, two or three asterisks represent significant difference at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ respectively (two-tailed Student's *t*-test; $n = 6$–9). A. u., arbitrary units.

organelles. It is well established that many abiotic and biotic stresses, such as pathogens, drought, starvation, salinity and prooxidants induce autophagy in plant cells (Minibayeva et al., 2012). Unfortunately, only limited information is available on the activation of autophagy in plants in response to the cold stress, although recently Zhai et al. (2016) reported that cold stress up-regulates autophagic genes and induces autophagosome formation in the leaves of the pepper plants. Results obtained from the present study showed that in wheat roots cold induces the accumulation of autophagosomes and up-regulates *TaATG8* gene expression (Fig. 1), suggesting that the induction of autophagy is a part of the response of plants to cold stress.

4.2. Sterols are important components of tolerance to cold stress in wheat

To tolerate low positive temperature stress, plants must maintain membrane fluidity and prevent a lipid phase transition. Previous studies have shown that cold stress significantly alters the lipid composition of the membranes. These changes include the degree of saturation of phospho- and glycolipids and the relative proportion of sterols, PLs and sphingolipids (Bohn et al., 2007). By reinforcing the cell membrane, plant sterols, especially β-sitosterol, campesterol and their esters, may have a role in tolerance to abiotic stresses (Kumar et al., 2015). To understand the physiological roles of sterols in the response of plants to stress, we carried out experiments using the sterol-binding agent MβCD, which considerably decreases the total level of sterols (Valitova et al., 2014). Interestingly, treatment of control plants with MβCD has no significant physiological consequences such as changes in membrane stability or the generation of H_2O_2 (Table 2). However, pre-treatment of roots with MβCD for 12 h followed by cold treatment for 1 h sharply (11-fold) increases the H_2O_2 content of roots, and also increases electrolyte leakage and reduces the MSI (Table 2). It is known that sterols play an important role in the response of the cell to oxidative stress. According to the literature data β-sitosterol has a high antioxidant activity (Vivancos and Moreno, 2005). In particular, it can neutralize free radicals of diphenylpicrylhydrazyl (a superoxide anion donor). Mutants with higher contents of β-sitosterol showed higher resistance to oxidative stress compared to the wild type (Pose et al., 2009). It the present study, it seems most likely that MβCD reduced the level of the antioxidant β-sitosterol, enhancing the effect of cold stress on cell membranes. Results are consistent with the view that the sterol component of the membrane plays an important role in reducing oxidative stress during cold treatment.

The time-course of cold-induced changes in the total sterols (Fig. 2a) and individual molecular species (Table 3) in the roots and the leaves displayed a parabolic pattern, with a significant increase after short-term (1 h) and a decrease after a long-term (12 h) cold exposure. Bohn et al. (2007) have shown that the cold acclimation of wheat seedlings for 3 weeks at +2 °C increased the level of free sterols by 13–16%. In rye, cold treatment also increased the concentration of membrane sterols, and this effect was more pronounced in resistant varieties of rye (Uemura and Steponkus, 1994). Thus, initial increases in sterol levels are similar to those reported in other species. However, the latter decline in sterols by 12 h may be due to modifications of free sterols, such as acylation and glycosylation, which have been shown to occur during cold acclimation in some cereals (Takahashi et al., 2016).

Stress can affect the ratio between 24-methyl- and ethyl sterols; this ratio is specific for an individual plant species, and even within species can vary between different organs and tissues (Haubrich et al., 2015). The ability of plants to synthesize 24-alkyl sterols and more specifically 24-ethyl sterols is unique to the plant kingdom (Neelakandan et al., 2009). The alkylation process proceeds in two steps: primary methylation of cycloartenol catalyzed by SMT1 (EC 2.1.1.142) and resulting in the formation of 24-methyl sterols (campesterol), and secondary methylation of 24-methylenelophenol catalyzed by SMT2 (EC 2.1.1.143) and resulting in the formation of 24-ethyl sterols (β-sitosterol, stigmasterol) (Schaeffer et al., 2000). The reaction catalyzed by the SMT2 diverts carbon flux towards the 24-ethyl sterols and represents the final stage of plant sterol biosynthesis. Interestingly, in both the roots and the leaves of cold-treated seedlings, the ratios of 24-methyl/ethyl sterols changed with time in a parabolic manner, but in opposite directions. In the roots, the ratio increased then decreased, while in the leaves the ratio initially decreased and then increased due to an increase in the relative proportion of β-sitosterol and stigmasterol (Table 4). Changes in the level of free sterols (mainly β-sitosterol) in the plasma membrane and lipid rafts have been also demonstrated in oat during cold acclimation (Takahashi et al., 2016). It has been suggested that the ability of plants to synthesize the 24-ethyl sterols β-sitosterol and stigmasterol may be a part of an evolutionary adaptation to stresses and maintenance of important membrane-associated metabolic processes (Dufourc, 2008). It is known that the presence of an additional ethyl group branched on the alkyl chain of β-sitosterol and stigmasterol can enhance van der Waals interactions with the alkyl chains of sphingolipids and PLs, leading to greater membrane cohesion and, hence, a lower temperature sensitivity (Beck et al., 2007). Therefore, the increase in the levels of 24-ethyl sterols observed in the roots may represent an adaptation mechanism to cold stress.

4.3. Glycoceramides and phospholipids in cold-treated wheat seedlings

In membranes, sterols interact with GlCer and PL. Glycoceramides are the main plant sphingolipids, comprising a sphingoid base, long-chain saturated fatty acids and a carbohydrate moiety (Cacas et al., 2013). An important feature of GlCer is their high affinity for sterols, caused by the interaction of the side chains of sterols with saturated alkyl chains of sphingolipids. This allows them to display dense packing and facilitates the formation of lipid microdomains (“rafts”). In the literature, these two classes of lipids are often called raft-forming lipids (Furt et al., 2011). These microdomains may play an important role in signal transduction into the cell and serve as platforms for enzyme signaling complexes.

In the present study we found that cold stress changes the content of GlCer. After 1 h cold-treatment the GlCer content significantly decreases (Fig. 2b), accompanying an increase in sterols in the roots and leaves (Fig. 2a). After exposure of the seedlings to cold for 12 h the GlCer content increases, along with a dramatic decline in the level of sterols. We have previously observed a similar inverse relationship between sterols and GlCer in wheat roots following treatment with sterol-binding agents (Valitova et al., 2014). These data suggest a possible functional relationship between sterols and GlCer, including the raft formation, although the precise ways that these molecules interact requires further study.

In contrast to sterols and GlCer, the major PLs undergo diverse changes in cold treated roots and leaves. In the control seedlings, the level of most PLs is higher in the leaves than in the roots (Fig. 3). This appears to be typical for plants; for example, in barley the amount of PL in the leaves is more than double that in the roots (Rochester et al., 1987). Interestingly, in response to cold the concentrations of PC and PE, the two major phospholipid classes, change in different ways with time of exposure to cold in the roots and leaves (Fig. 3). During cold stress response, in the leaves the PC/PE ratio declines after 1 h, returning to its initial level after 12 h (Table 5). Conversely, in the roots the PC/PE ratio increases after 1 h and then decreases after 12 h, and this is accompanied by decreased membrane stability (Table 1). The PC/PE ratio is a key regulator of membrane integrity (Li et al., 2006). Our data confirm that decreased PC/PE ratio is correlated with a loss of membrane integrity in the roots, while the higher PC/PE ratio in leaves might contribute to maintaining cell membrane integrity based on the biophysical properties of these PLs (Jia and Li, 2015).

4.4. Sterol biosynthesis genes expression of roots and leaves of cold-treated wheat seedlings

Cold stress has been shown to cause the up-regulation of several genes of sterol biosynthesis (Byun et al., 2014), in particular those that encode enzymes that catalyze the formation of methyl and ethyl sterols, the SMTs. Transcriptional regulation of plant *SMT1* and *SMT2* genes of the sterol pathway has been studied in various plant species (Luo et al., 2008). In the roots of wheat seedlings *TaSMT1* and *TaSMT2* genes are continuously up-regulated during whole duration of cold treatment (Fig. 4). Conversely, in the leaves the expression of *TaSMT1* gene is continuously up-regulated during cold treatment, while the expression of *TaSMT2* gene increased after 1 h and remained unchanged with longer cold exposure (Fig. 4b). Earlier Neelakandan et al. (2009) demonstrated that in soya several biotic and abiotic factors, including

dehydration, cold and abscisic acid (ABA), stimulate the expression *GmSMT2* genes. Stress sensitive *cis*-elements and transcription factor binding sites in the promoter region were shown to regulate *GmSMT2* activity (Neelakandan et al., 2009). Therefore, SMTs are necessary not only for the normal growth and development but also for the response of plants to stress.

5. Conclusions

Results of the present study demonstrate that sterols are important components of the response of wheat seedlings to cold. This is supported by the observation that depletion of sterols by MβCD exacerbates the negative effects of cold stress. The effects of stress are organ-specific; roots appear to be more sensitive to cold than the leaves. Cold-induced changes in the ratios of lipid raft forming sterols and GlCer and also in the PC/PE ratios might facilitate membrane integrity and flexibility contributing to the stress signaling and cold acclimation.

Contributions

Conception and design (JV, FVM); Analysis and interpretation of the data (AR, JV, SD, FM); Drafting of the article (JV, AR, FVM); Critical revision of the article for important intellectual content (RPB); Final approval of the article (RPB, FVM); Statistical expertise (AR).

Conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgements

This study was partly supported by the Russian Foundation for Basic Research (№ 17-04-01562), the government assignment of FRC KSC RAS and partly performed according to the Russian Government Program of Competitive Growth of Kazan Federal University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.07.026.

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