Title: Exogenous salicylic acid protects phospholipids against cadmium stress in flax (*Linum usitatissimum* L.)

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Abstract

Salicylic acid (SA) belongs to a number of chemicals that are normally efficient to promote plant defense response against toxic metal stresses. For this purpose, flax seeds were pre-soaked for 8 h to SA (0, 250 and 1000 μ M) and then subjected, at seedling stage, to cadmium (Cd) stress. Subsequently, the present study was aimed to investigate from the hypothesis that 8-h SA pretreatment, will make positive alterations in membrane lipids and the SA-induced tolerance is associated with the protection of their fatty acid composition from Cd toxicity. At 100 μ M CdCl₂, significant decreases in the percentages of phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and monogalactosyldiacylglycerol (MGDG) and changes in their relative fatty acid composition were occurred in Cd-treated roots in comparison with control. However, in roots of 8-h SA pretreated plantlets, results showed that the amounts of PC and PE were significantly higher as compared to non pretreated plantlets. Additionally, in both lipid classes the proportion of linolenic acid (18:3) increased upon the pretreatment with SA. As the exogenous application of SA was found to be positive for flax lipid metabolism, the possible mechanisms by which it could induce membrane-lipids protection against Cd stress in flax roots were discussed.

Key words: *Linum usitatissimum* L., membrane lipids, fatty acids, roots, salicylic acid, cadmium stress.

Introduction

Cadmium (Cd) is a rare element, uniformly distributed in the earth's crust, where its average concentration is 0.15 to 0.20 mg kg⁻¹ (Fleischer 1974). It is found as a contaminant in sediment, air, water (Waisberg et al. 2003), agriculture (Guo et al. 2014) and industrial wastes (He et al. 2005; Trinchella et al. 2006). Because of the food chain contamination risk, its uptake by roots has been much studied on various plant species. Most of them have been particularly focused on cereals, such as rice (Guo et al. 2007), maize (Ivanova et al. 2008) and wheat (Li et al. 2011), or vegetables such as lettuce (Costa and Morel 1994), radish (Vitoria et al. 2001) and onion (Jiang et al. 2001). Therefore, the exposure of roots to Cd pollution alters directly various physical properties and biological functions of cell membranes (Hernandez and Cooke 1997, Sanz et al. 2009) or by the indirect formation of reactive oxygen species (ROS) (Djebali et al. 2005, 2008). This can result in alteration of lipid-protein associations (Hernandez and Cooke 1997), protein oxidation (Belkadi et al. 2013), peroxidative degradation of lipids and decrease of membrane fluidity and permeability (Schützendübel and Polle 2002, Belkadi et al. 2010, Guo et al. 2013, 2014). An increased lipoxygenase (LOX) activity which is as well responsible of the peroxidative degradation of membrane lipids has also been reported in Cd-treated plants (Ben youssef et al. 2005, Djebali et al. 2005).

The Cd-related changes in biomembranes concern their permeability to water, nutrients and protons (H⁺) (Sanz et al. 2009), and also have a major impact on the activity of membrane-bound enzymes (Hernandez and Cooke 1997). To diffuse into the root system, Cd profits from the non-specificity of some channels/transporters of membranes (Clemens 2006) and the extremely negative membrane potential exceeding -200 mV in rhizodermis cells (Hirsch et al. 1998). Thus, maintaining this potential constant recognizes the excretion of H⁺ in the external environment via

H⁺-ATPase pump (Gaxiola et al. 2001). Indeed, under Cd stress, various plants for instance *Triticum aestivum* (Malik et al. 1992), *Capsicum annum* (Jemal et al. 2000), *Brassica juncea* (Ivanova et al. 2008) and *Karelian Birch* (Kuznetsova et al. 2008) have also to balance these changes by regulating the level of saturation of membrane lipid components, essentially the galactolipids (GL), phospholipids (PL) and neutral lipids (NL). Moreover, in rhizodermis cells, Cd is known to substitute calcium (Ca) at its essential sites on the apoplast, tonoplast, mitochondrial and endoplasmic reticulum membranes (Breckle and Kahle 1991). Furthermore, Ca entry into cytosol induces the phospholipases activities which leads to the membrane discharge of linoleic (C18:2) and linolenic (C18:3) acids serving as substrates for LOX or for the ROS that have been indirectly produced by Cd. Cd-induced decreases of these both polyunsaturated fatty acids in roots has also been reported in *Hordeum vulgare* (Vassilev et al. 2004) and *Oryza sativa* (Sanz et al. 2009).

Salicylic acid (SA), which is an important signaling molecule in plants, could be a promising compound for the improvement of tolerance to various biotic and abiotic stresses (Horváth et al., 2007). Its exogenous supply influences a range of diverse processes in plants, including seed germination (Xie et al., 2007; Agami, 2013), ion uptake and transport (Dražić et al., 2006), enzyme activities (Guo et al., 2007; Ahmad et al., 2011), and photosynthesis (Shi et al., 2009). There are also evidence that SA can ameliorate the damaging effects of ozone (Pasqualini et al. 2002), salinity (Yusuf et al. 2008, Misra and Saxena 2009) and heavy metals (Choudhury and Panda 2004, Guo et al. 2007, 2013, Li et al. 2014). The protective function of SA mainly includes the regulation of ROS and antioxidants, induction of gene expression, and absorption and distribution of nutrient elements (Shi and Zhu, 2008). However, whether SA could increase membrane lipid production after exposure of plants to Cd has not been reported in roots. Zhang et al. (2011)

reported that SA was involved in alleviating Cd-induced oxidative stress in root apoplasts of *Phaseolus aureus* and *Vicia sativa* by reducing H₂O₂ accumulation. It has been also reported that SA reduced ROS and NO accumulation in roots (Shao et al. 2010). SA can be a key component of signal transduction pathways. It may also directly or indirectly influence the activities of membrane-bound enzymes under Cd stress. In SA-pretreated root cell plasma membranes, activities of H⁺-ATPase and Ca²⁺-ATPase were increased after exposure to Cd (Shao et al. 2010). Exogenous SA has also been shown to mitigate oxidative damages of Cd in *Oryza sativa* (Panda and Patra 2007), *Phaseolus aureus* and *Vicia sativa* (Zhang et al. 2011), *Glycine max* (Li et al. 2014), *Poa pratensis* (Guo et al. 2013) and *Gossypium hirsutum* (Liu et al. 2014). Furthermore, it has been shown previously that SA modulates the activation of lipid peroxidation and regulates the membrane fluidity and permeability in Cd-treated roots of *Oryza sativa* (Choudhury and Panda 2004, Guo et al. 2007) and *Linum usitatissimum* (Belkadi et al. 2013).

Based on these reports, the aims of this study were to respond to two main questions: (i) how does SA influences the composition of membrane lipid species in Cd-treated flax roots, and (ii) when SA and Cd-induced changes in lipid metabolism have synergistic or antagonistic effects. Finally, in addition to these original objectives, our comprehensive analysis allowed us to test the effects of SA on the protection of lipid membrane components against Cd toxicity, with regard to their relative fatty acid composition.

Materials and methods

2.1. Plant material and growth conditions

Flax seeds (cv. Viking), were soaked for 8 h in 250 and 1000 µM SA, which was acquired in free form as a powder, or in water (0 µM SA) as previously shown in Belkhadi et al. (2010). After that they were germinated for four days at 25 °C in the dark, uniform plantlets were

transferred to a continuously aerated nutrient solution (pH 5.5) containing 1mM MgSO₄, 2.5mM Ca (NO₃)₂, 1mM KH₂PO₄, 2 mM KNO₃, 2 mM NH₄Cl, 50 mM EDTA– Fe–K, 30 mM H₃BO₃, 10 mM MnSO₄, 1mM ZnSO₄, 1mM CuSO₄ and 30 mM (NH₄)₆Mo₇O₂₄. The nutrient solution was buffered with HCl/KOH and changed twice per week. After growing for 2 days, plantlets were subjected during 10 days to CdCl₂ appropriately from moderate to high concentrations (50 to 100 µM). Five replicates were in individual 6 l plastic beakers made for control and Cd treatments. Plantlets were grown in a growth chamber at a day/night cycle of 16 h/8 h, at 23 °C/18 °C, respectively, a relative humidity close to 75% and a light intensity of 200 µmol photons m⁻² s⁻¹. Roots were then detached and washed with deionized water. Three independent culture experiments were performed.

2.2. Cell-membrane stability

Membrane stability index (MSI) of flax roots, an indicator of membrane integrity and tolerance to Cd-induced oxidative stress, was determined by recording the electrical conductivity of root tissues in double distilled water at 40 and 100 °C as shown in Belkhadi et al. (2010). In brief, root samples (100 mg) were cut into discs of uniform size and taken in test tubes containing 10 ml of double distilled water in two sets. One set was kept at 40 °C for 30 min and another set at 100 °C in boiling water bath for 15 min and their respective electric conductivity's C1 and C2 were measured by conductivity meter. The MSI was determined using the following formula: $MSI = (1 - (EC1/EC2)) \times 100$.

2.3. Lipid extraction and analysis

Lipids in roots were extracted according to Garcés and Mancha (1993) using 30 mg plant tissue. One-dimensional separation was performed using silica-gel thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany), according to Nichols (1965). To visualize the bands

of different lipid classes, the plates were sprayed with I₂ vapor. Lipid classes were then identified against lipid standards using specific stains for PL, GL and NL. Identification of individual PL was recognized by molybdenum blue reagent, whereas phosphatidylethanolamine (PE) by ninhydrin. GL were detected by staining with α-naphthol reagent and NL by a methanolic solution of manganese (II) chloride.

The samples were quantified against a heptadecanoic acid (17:0) internal standard, and the amount of each lipid species in the sample could, therefore, be directly expressed as a percentage of the total lipid present in the sample. Fatty acids from lipid classes were transformed into their corresponding methyl esters by the addition of 2 ml of heptane, 660 mL of methanol, 40 mL of toluol, 40 mL of 2,2-dimethoxypropane and 20 mL of sulfuric acid. The heptanic phase containing fatty acid methyl esters (FAMES) was recovered and its volume was reduced in a stream of nitrogen, prior to analysis. Fatty acid percentages were separated and quantified using an integrator (Model 3390 A, Hewlett-Packard, USA) and a fused silica capillary column BPX 70 (SGE, Austin, TX, USA), length 50 m, 0.22 mm i.d., 0.25 mm film thickness. FAMES dissolved in hexane were injected using an autosampler CP 9050 (Chromapack, Middelburg, Netherlands). PL, GL and NL were injected manually. Helium was used as the carrier gas at a pressure of 150 kPa and nitrogen was used as make-up gas at a flow rate of 30 mL min⁻¹. Detector and injector temperatures were at 250 and 230 °C, respectively. The peak areas were identified by comparing their retention times and responses with those of a standard FAMES mixture, GLC-68 A (Nu-Chek Prep, Elysian, USA).

The percentage of unsaturation was calculated using the following formula: % unsaturation = (18:1 + 18:2 + 18:3)/(16:0 + 16:1 + 18:0 + 18:1 + 18:2 + 18:3).

2.4. Data analysis

Statistical calculations were performed with SPSS-17 version 17.0 statistical software. Mean difference comparison among different treatments was done by analysis of variance (two-way ANOVA) followed by Tukey's HSD test at a 0.05 probability level.

Results

3.1. Effect of SA pretreatment on root membrane stability under Cd stress

The results showed that before Cd subjection, 1000 μ M SA-pretreated roots exhibited significant augmentation in the MSI that was 12.8% higher over the control plantlets (Fig. 1). The exposure of flax to Cd stress (100 μ M) significantly decreased the MSI values. This decrease was by about 40% over control. However, at the same Cd concentration, SA pretreatment led to the maintenance of the stability of cell-membranes (Fig. 1). Thus, at 100 μ M Cd-treated plantlets, SA (250 or 1000 μ M) enhanced the MSI in roots by about 75.7% and 74.4%, respectively, compared to non-pretreated plantlets.

3.2. Effect of SA pretreatment on lipids of root cell-membranes under Cd stress

Under normal conditions, PL were the major constituent lipid classes, accounting for more than 50% of the total lipids (TL). Moreover, in this lipid class the proportion of phosphatidylcholine (PC), about 24% of TL, was higher than that of phosphatidylglycerol (PG) and PE (Figure 2). However, phosphatidic acid (PA) and phosphatidylinositol (PI) were the minority (5% of TL). Furthermore, it was remarkable that the percentage of monogalactosyldiacylglycerol (MGDG) in TL of roots was lower than that of digalactosyldiacylglycerol (DGDG) representing 5.2 and 6.7% of TL, respectively.

As it was shown in Figs 2 and 3, the levels of PC, PG and PE were reduced in the presence of both Cd concentrations, whereas DGDG level in roots was significantly higher in the presence of 100 μ M Cd. It also seemed that for the class of GL, only MGDG was affected by the

highest Cd concentration (Fig. 3). Therefore, at 100 μ M Cd, the percentages of PC, PG, PE and MGDG were diminished by 10.7%; from 24.1% (control) to 21.5% (0 μ M SA + 100 μ M Cd), by 11.5%; from 14.2% (control) to 12.6% (0 μ M SA + 100 μ M Cd), by 10%; from 10.3% (control) to 9.3% (0 μ M SA + 100 μ M Cd) and by 25.5; from 5.1% (control) to 3.8% (0 μ M SA + 100 μ M Cd), respectively, compared to control. However, there were no significant changes in the levels of PA and PI compared to the control (Fig. 2). The levels of NL were significantly increased at 50 μ M Cd but there was no change at the concentration of 100 μ M Cd (Fig. 4).

In roots, exogenous SA was suspected of acting to protect cell membranes from Cd stress. In fact, the results showed that in 100 μ M Cd-treated roots; 250 μ M SA increased the levels of PC (+10.2%) and PE (21%) which varied from 21.5% (0 μ M SA + 100 μ M Cd) to 23.7% (250 μ M SA + 100 μ M Cd) and from 9.3% (0 μ M SA + 100 μ M Cd) to 11.2% (250 μ M SA + 100 μ M Cd), respectively, as compared to non-pretreated plantlets (Fig. 2). It was also important to mention that both Cd concentrations (50 and 100 μ M), enhanced the level of DGDG in comparison to the control, while no additional effect of SA was visible (Fig. 3). Additionally, SA did not change Cd-induced MGDG and NL levels (Figs 3 and 4).

3.3. Effect of SA pretreatment on fatty acid composition of root cell-membranes under Cd stress

Before the exposure of roots to Cd stress, tables 1, 2 and S2 showed that PL contained considerable levels of palmitic acid (C16:0) and C18:2. For GL and NL, they were rather rich in C16:0, C18:2 and C18:3 (Tables S1 and S3). The fatty acid composition of root membrane lipids was altered as well, by Cd stress and differed between different lipid species (Tables 1-2 and S1-S3). For PL, we noted that, in all PL constituent molecules, the action of Cd (50 and 100 μ M) at this level led to a reduction of the C18:2 (major fatty acid of roots) percentages (Tables 1, 2 and S2). This reduction was also accompanied by an increase in the proportion of C16:0 in comparison with the control. Furthermore, in both GL molecules of Cd-treated roots, there was a decrease in

C18:3 proportions accompanied by improvements in C18:0, C18:2 and C16:0, especially at 100 μ M Cd (Table S1). However, concerning NL, the increase in C16:0 amounts was linked to a diminution in both C18:2 and C18:3 levels at 100 μ M Cd-treated flax roots as compared to control (Table S3).

8-h SA pretreatment led to significant differences in PL fatty acid composition of flax roots (Tables 1, 2 and S2). Consequently, at 100 μ M Cd, the rate of C18:3 in PC and PE of flax plantlets pretreated with 250 μ M SA increased by about 28%; from 5.8% (0 μ M SA + 100 μ M Cd) to 7.4% (250 μ M SA + 100 μ M Cd) and about 12%; from 10.1% (0 μ M SA + 100 μ M Cd) to 11.3% (250 μ M SA + 100 μ M Cd) compared to non-pretreated plantlets, respectively (Table 2). In addition, for GL (MGDG and DGDG) molecules, we observed in table S1 that in 100 μ M Cd-treated flax plantlets; the SA-effect was differentiated by an increase in the proportions of C16:0 and C18:0, essentially at the dose 250 μ M SA. On the other hand, although the NL percentages of Cd-treated roots did not differ quantitatively between SA-primed and non-primed plantlets, the fatty acids profiles were characterized by significant high amounts of stearic acid (C18:0) under both Cd concentrations (Table S3).

Discussion

Several studies have proved that, the higher unsaturation ratio would improve the resistance of plants to cadmium stress (Ben youssef et al. 2005, Djebali et al. 2005, Gondor et al. 2014). In our previous study, we demonstrated that SA-increased fatty acid unsaturation in flax roots may contribute to the membrane protection from lipid peroxidation and loss of integrity (Belkadhi et al. 2013). In this work, we investigate whatever these SA-induced modifications in membrane unsaturation may have direct impacts on the amount and fatty acid composition of some or all of the membrane lipid classes in Cd-treated roots.

SA could protect plants from Cd-induced oxidative stress by reducing ROS formation or by changing the activities of ROS-scavenging enzymes (Guo et al. 2013). Besides, our results showed that the exposure of flax to Cd stress (100 μ M) decreased by 40% the MSI values as compared to control plantlets (Fig. 1). This could be explained by the fact that the Cd-generated ROS were liberated and formed the lipid peroxides that destroyed biological membranes. As a consequence, the plant cell membranes are generally considered as primary sites of metal injury (Guo et al. 2014). In roots, we found that at the highest Cd concentration, MSI was higher in 250 or 1000 μ M SA-pretreated plantlets than non-pretreated ones (Fig. 1). Guo et al. (2013) reported that pretreatment with 500 μ M SA alleviated the toxicity generated by Cd stress in *Poa pratensis* by a reduction in hydrogen peroxide contents and lipid peroxidation. These authors also concluded that SA might regulate the antioxidant defense activities, reduce Cd uptake and stimulate nutrient elements absorption in Cd-treated seedlings to improve their resistance to Cd stress. Similarly, under Cd-stress conditions, Li et al. (2014) reported that SA might act as membrane stabilizer as well as ROS scavenger. Moreover, Rucinska and Gwóźdz (2005) reported that hydroperoxides formed from lipids may have been preferentially converted, at the highest Cd concentration, into more stable compounds including jasmonic acid and indicated rapid accumulation of jasmonic acid after heavy metal stress. Furthermore, under normal conditions, the MSI of SA-pretreated roots was higher than non-pretreated ones (Fig. 1). Those results depended on different factors, e.g. treatment concentrations, time of treatments and plant age (Li et al. 2012, Dresler et al. 2014). The results also demonstrated a common decrease in the relative percentages of C18:2 in all PL molecules of Cd-treated flax roots (Tables 1, 2 and S2). In Cd-contaminated roots, Hernandez and Cooke (1997) reported that alterations in PM-bound ATPase activity might partially explain the modification of PM permeability to solutes, but, the changes in its integrity results from alterations in its lipid composition. The shifts in polyunsaturated fatty acids are expected to be mainly due to the degradation of existing lipids as well as a decline in their

biosynthesis (Ben youssef et al. 2005, Djebali et al. 2005). Furthermore, there is evidence that this observed decrease might be the consequence of Cd-mediated lipid peroxidation (Guo et al. 2013, 2014). Likewise, Cd-induced reduction of membrane unsaturated fatty acids has also been reported in roots of *Pisum sativum* (Hernandez and Cooke 1997), pepper (Jemal et al. 2000) and *Zea mays* (Astolfi et al. 2005, Pal et al. 2006).

On the other hand, our results reported that Cd caused a significant increase in the levels of DGDG in SA-primed and non primed flax roots (Fig. 2). This is thought to be due to increased DGDG synthase activity (Andersson et al. 2003). Similarly to our observations, Popova and Hinch (2005) reported that under conditions of phosphate deficiency, it has been shown that plants reduce the amount of PL, which are a major phosphate sink in plant cells, in favor of DGDG. These authors also established that under the same conditions, DGDG was also found in extraplastidial membranes and could account for up to 70% of the total PM lipids. Furthermore, under some abiotic conditions and as part of a process aimed at maintaining phosphate homeostasis within the plant, Jouhet et al. (2007) indicated that an extensive lipid trafficking has been occurred from the chloroplast to the extraplastidial membranes by replacing their PL with chloroplast-made DGDG. In our study, we also noted a significant decrease in the percentages of polar lipids (PC, PG, PE and MGDG); mainly at 100 μ M Cd (Figs 2 and 3). These results are consistent with those of Jemal et al. (2000) and Ben youssef et al. (2005) who reported a similar decrease in PL contents of *Capsicum annum* and *Brassica napus* roots. Hence, these changes would basically concern the PM in which Cd could affect its fluidity, ion transport and permeability properties that are closely related to the metal effects on membrane-bound enzymes. In fact, Cd is also known to enhance the activities of different kinds of enzymes involved in the signaling responses of plants to heavy metal stress, for example, LOX, phospholipase and allene oxide synthase (Maksymiec 2007). In addition, these enzymes can as well, alter (reduce or

expand) the composition of membrane lipids and this in turn may lead to a decrease in the amounts of these polar lipids in Cd-treated plantlets.

In Cd-contaminated roots, we found that pretreatment with 250 μ M SA increased the levels of PC and PE (Fig 2). Moreover, we also noticed that 8-h SA pretreatment (250 μ M) induced increases in the levels of PC-C18:3 and PE- C18:3, especially at the highest Cd concentration (Table 2). These observations could be justified by the role of SA in mitigating Cd-induced oxidative damages to lipids and proteins (Guo et al. 2007, Zhang and Chen 2011, Guo et al. 2013). **Furthermore, our previous study also showed that SA inhibited lipid peroxidation and protein carbonylation, by increasing the activities of H₂O₂-scavenging enzymes in flax roots (Belkadhi et al. 2013).** Besides, as PC is a major constituent in apoplast, endoplasmic reticulum and mitochondria membranes and PE is only a minor constituent of plastid membranes, the SA-increased PC and PE amounts in Cd-treated roots may also be due to its possible role in increasing the quantity or the shape of almost all cell organelles, particularly the vacuoles (Djebali et al. 2005). The SA-increased percentage of unsaturated fatty acid (C18:3; Table 2) could play as well, an important role in promoting Cd chelation and sequestration mechanisms in different cell compartments of roots where Cd is accumulated preferentially (Maksymiec 2007, Guo et al. 2014). Furthermore, exogenous application of SA has been shown to increase tolerance of plants to various stressors (Li et al. 2014) and can elicit responses similar to those induced by biotic or abiotic stresses (Saltveit et al. 2005). However, these induced physiological responses have been elicited over a wide range of SA concentrations (Kang and Salveit 2002). Additionally, at cell-membrane level, Saltveit et al. (2005) reported that exogenous SA application inhibited allene oxide synthase; enzyme involved in phospholipid-signaling pathway. Exogenous SA was also found to alleviate the negative effects generated by other heavy metals like lead and mercury by decreasing LOX activity (Mishra and Choudhuri 1999). Canakci and Karaboga (2013) also

established that the pretreatment with SA exerted a protective effect against Cd toxicity on the membrane stability judging by the increased levels of fatty acid methyl esters and by the changes in their fatty acid composition. To conclude, the SA-induced protection of membrane lipids, in 16-d-old flax plantlets treated during 10 days with toxic Cd concentrations, gave some enlightenment about to its potential function as Cd-stress elicitor and membrane stabilizer in roots.

Conclusion

In conclusion, the purpose of this study was to provide basic knowledge about the mechanisms of cadmium tolerance improvement at the level of bio-membranes. Moreover, a potential applicative aspect of this research was the use of SA for the purpose to protect root cells from Cd toxicity. Thus, we have investigated the effects of exogenous SA on the lipid membrane composition of flax roots after their exposure to 50 and 100 μ M Cd. Noticeable modifications in membrane composition were observed after 10 days of exposure to Cd. Some specific SA-induced changes have been noticed and were clearly pronounced at the highest Cd concentration (100 μ M). It is also debatable whether these changes represent a protection against the toxic effects of Cd-induced ROS, or from SA-initiated action to enhance membrane stabilization. We suggest that the lesser loss of membrane stability in SA-pretreated flax roots under Cd stress may be related to a minor rate of the lipid oxidation and degradation. Therefore, use of SA pretreatment may be an efficient agricultural practice to improve plant growth and tolerance to contaminated soils.

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Figure captions

Fig. 1 Membrane stability index (MSI) of roots of salicylic acid (SA)-pretreated flax plantlets grown in hydroponic cultures and subjected for 10 days to cadmium (Cd) stress. Results are the means of 5 replicate experiments $\pm$ SE. Error bars with common letters are not significantly different at $P \leq 0.05$, according to Tukey's test.

Fig. 2. The effects of salicylic acid (SA) on phospholipid classes (PL) in roots of flax plantlets grown in hydroponic cultures and subjected for 10 days to cadmium (Cd) stress. Results are the means of 5 replicate experiments $\pm$ SE. Results with common letters are not significantly different at $P \leq 0.05$, according to Tukey's test. PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol.

Fig. 3. The effects of salicylic acid (SA) on galactolipid classes (GL) in roots of flax plantlets grown in hydroponic cultures and subjected for 10 days to cadmium (Cd) stress. Results are the means of 5 replicate experiments $\pm$ SE. Results with common letters are not significantly different at $P \leq 0.05$, according to Tukey's test. MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol.

Fig. 4. The effects of salicylic acid (SA) on neutral lipid (NL) class in roots of flax plantlets grown in hydroponic cultures and subjected for 10 days to cadmium (Cd) stress. Results are the means of 5 replicate experiments $\pm$ SE. Error bars with common letters are not significantly different at $P \leq 0.05$, according to Tukey's test.

Tables

Table 1

Effects of SA on fatty acid composition (% of TL) in root phosphatidylglycerol (PG) of 16-d-old flax plantlets treated with different Cd treatments.

SA (μM)	Cd (μM)	Fatty acids						
		<i>C16:0</i>	<i>C16:1 cis</i>	<i>C18:0</i>	<i>C18:1</i>	<i>C18:2</i>	<i>C18:3</i>	<i>Unsaturation</i>
0	0	27.48±0.60 a	Tr	9.69±0.28 b	10.81±0.28 ab	46.12±0.04 c	5.92±0.01 bc	62.85 c
0	50	32.09±0.09 bc	Tr	13.09±0.05 c	13.13±0.03 bc	38.04±0.01 ab	3.66±0.07 a	54.83 b
0	100	28.14±0.01 a	Tr	14.66±0.01 cd	14.98±0.02 bc	38.47±0.06 ab	3.76±0.10 a	57.21 bc
250	0	28.58±0.21 a	Tr	10.39±0.08 bc	11.18±0.15 ab	45.17±0.28 bc	4.70±0.01 bc	61.05 c
250	50	34.31±0.03 d	Tr	14.18±0.13 cd	14.11±0.22 b	34.12±0.06 b	3.29±0.01 a	51.52 ab
250	100	32.65±0.69 bc	Tr	9.07±0.12 b	10.10±0.11 ab	45.19±0.47 bc	3.00±0.01 a	58.29 bc
1000	0	30.64±0.01 b	Tr	11.76±0.07 bc	13.17±0.04 b	40.39±0.01 b	4.06±0.01 b	57.62 bc
1000	50	35.16±0.05 d	Tr	16.34±0.91 d	15.38±0.15 c	29.98±0.04 a	3.16±0.07 a	48.52 a
1000	100	38.14±0.01 e	Tr	6.84±0.36 a	8.74±0.26 a	42.48±0.08 b	3.82±0.01 ab	55.04 b

^a Data are means of three independent experiments (±SE).

^b Means with different letters indicate statistically different results at $P \leq 0.05$, according to Tukey's (HSD) test.

581

582 **Table 2**

583 Effects of SA on fatty acid composition (% of TL) in root phosphatidylcholine (PC) and
 584 phosphatidylethanolamine (PE) of 16-d-old flax plantlets treated with different Cd treatments.

SA	Cd	Fatty acids						
(μM)	(μM)	<i>C16:0</i>	<i>C16:1 cis</i>	<i>C18:0</i>	<i>C18:1</i>	<i>C18:2</i>	<i>C18:3</i>	<i>Unsaturation</i>
PC								
0	0	21.76±0.24 a	Tr	6.90±0.16 a	13.43±0.19 d	49.57±0.53 d	8.35±0.05 c	71.53 bc
0	50	28.50±0.78 b	Tr	10.39±0.03 b	11.60±0.07 c	34.08±1.07 b	5.45±0.19 a	51.13 ab
0	100	28.63±0.03 b	Tr	10.82±0.09 b	9.92±0.03 b	34.81±0.06 b	5.84±0.21 a	50.57 ab
250	0	24.84±0.92 a	Tr	7.88±0.09 a	12.77±0.11 cd	47.27±0.97 c	7.26±0.07 b	67.30 b
250	50	31.96±0.97 c	Tr	12.69±0.21 c	10.94±0.01 c	38.87±0.16 a	5.56±0.60 a	55.37 a
250	100	27.82±0.39 b	Tr	11.76±0.60 c	4.75±0.88 a	48.22±0.68 c	7.46±0.78 b	60.43 ab
1000	0	27.70±0.16 b	Tr	10.20±0.09 b	12.17±0.19 cd	44.55±0.91 b	5.40±0.79 a	62.12 ab
1000	50	33.51±0.03 c	Tr	12.74±0.11 c	10.31±0.11 b	38.87±0.90 a	4.59±0.87 a	53.77 a
1000	100	26.01±0.05 b	Tr	13.61±0.87 d	4.16±0.26 a	48.21±0.42 c	8.03±0.76 c	60.40 ab
PE								
0	0	30.55±0.87 a	Tr	9.14±0.29 a	9.69±0.19 ab	40.45±0.95 d	10.19±0.40 c	60.33
0	50	35.97±0.01 b	Tr	12.58±0.81 b	11.80±0.29 b	19.83±0.30 b	9.84±0.21 b	41.47
0	100	33.81±0.03 ab	Tr	12.39±1.07 b	11.52±0.78 b	22.15±1.67 bc	10.14±0.22 c	43.81
250	0	31.32±0.42 a	Tr	9.63±0.28 a	10.33±0.04 ab	37.80±0.01 b	10.94±0.19 d	59.07
250	50	37.14±0.01 b	Tr	13.16±0.02 c	12.04±0.01 c	27.85±0.01 a	9.83±0.01 b	49.72
250	100	38.39±0.97 bc	Tr	12.44 ±0.02 b	7.52±0.75 a	30.32±0.02 b	11.34±0.17 d	49.18
1000	0	34.44±0.11 ab	Tr	11.47±0.96 ab	10.28±0.90 ab	33.87±0.24 bc	9.95±0.08 b	54.10
1000	50	39.81±0.01 bc	Tr	13.67±0.01 c	12.25±0.01 c	24.68±0.15 a	9.61±0.12 b	46.54
1000	100	42.18±0.06 c	Tr	10.01±0.24 a	6.01±0.24 a	32.68±0.67 bc	9.13±0.13 a	47.82

585 ^a Data are means of three independent experiments ($\pm$ SE).

586 ^b Means with different letters indicate statistically different results at $P \leq 0.05$, according to Tukey's (HSD) test.

Figures

Figure 1

Figure 3

Figure 4

Tables Supplementary data

Table S1

Effects of SA on fatty acid composition (% of TL) in root monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) of 16-d-old flax plantlets treated with different Cd treatments.

SA	Cd	Fatty acids						
(μM)	(μM)	<i>C16:0</i>	<i>C16:1 cis</i>	<i>C18:0</i>	<i>C18:1</i>	<i>C18:2</i>	<i>C18:3</i>	<i>Unsaturation</i>
MGDG								
0	0	27.41±0.19 a	Tr	8.54±0.09 a	15.19±0.21 e	33.80±0.01 b	15.08±0.06 c	64.07
0	50	32.88±0.50 c	Tr	11.19±0.28 b	11.09±0.18 c	32.09±0.09 a	12.77±0.52 b	55.95
0	100	32.08±0.89 c	Tr	10.37±0.06 b	10.23±0.39 c	37.22±0.34 d	10.11±0.10 a	57.56
250	0	28.22±0.21 a	Tr	9.09±0.15 a	14.61±0.58 e	33.84±0.13 b	14.26±0.10 c	62.71
250	50	35.71±0.03 d	Tr	11.48±0.19 b	10.41±0.11c	31.52±0.24 a	10.93±0.09 a	52.86
250	100	36.19±0.96 d	Tr	11.05±0.59 b	5.61±0.19 a	30.83±0.88 a	16.33±0.16 d	52.77
1000	0	29.87±0.11 b	Tr	9.18±0.28 a	12.70±0.02 d	35.33±0.99 c	12.94±0.83 b	60.97
1000	50	38.37±0.09 e	Tr	12.11±0.19 c	9.06±0.09 b	30.90±0.18 a	9.56±0.19 a	49.52
1000	100	31.16±0.20 c	Tr	13.14±0.11 d	9.37±0.11 b	33.53±0.01 b	12.82±0.01 b	55.72
DGDG								
0	0	22.22±0.09 a	Tr	6.39±0.06 a	14.79±0.01 c	29.22±0.06 c	27.39±0.04 c	71.40
0	50	30.61±0.04 d	Tr	9.64±0.11 c	11.31±0.09 ab	26.21±0.28 b	22.25±0.30 b	59.77
0	100	28.10±0.17 c	Tr	7.68±0.07 a	11.11±0.02 ab	31.03±0.21 d	22.10±0.01 b	64.24
250	0	23.58±0.19 a	Tr	7.30±0.09 a	13.86±0.11 c	28.39±0.09 c	26.89±0.10 c	69.14
250	50	31.85±0.09 d	Tr	8.63±1.12 b	10.62±0.72 a	25.66±0.70 a	23.26±0.38 b	59.54
250	100	29.20±0.29 c	Tr	9.43±0.25 bc	10.92±0.02 a	29.94±0.01 d	20.52±0.01 a	61.38
1000	0	26.18±0.11 b	Tr	9.11±0.03 b	11.88±0.11 b	26.24±0.28 b	26.61±0.04 c	64.73
1000	50	34.44±1.05 e	Tr	9.40±0.13 bc	10.24±0.25 a	24.28±0.49 a	21.65±0.69 a	56.17
1000	100	31.80±1.40 d	Tr	9.96±0.10 c	10.39±0.30 a	25.28±0.22 ab	22.58±0.98 b	58.25

^aData are means of three independent experiments ($\pm$ SE).

^bMeans with different letters indicate statistically different results at $P \leq 0.05$, according to Tukey's (HSD) test.

Table S2

Effects of SA on fatty acid composition (% of TL) in root phosphatidylinositol (PI) and phosphatidic acid (PA) of 16-d-old flax plantlets treated with different Cd treatments.

SA	Cd	Fatty acids						
(μM)	(μM)	<i>C16:0</i>	<i>C16:1 cis</i>	<i>C18:0</i>	<i>C18:1</i>	<i>C18:2</i>	<i>C18:3</i>	<i>Unsaturation</i>
PI								
0	0	29.31±0.01 a	Tr	15.29±0.01 ab	8.25±0.09 b	36.78±0.09 d	10.39±0.02 b	55.42
0	50	34.05±0.01 b	Tr	17.85±0.21 b	7.59±0.09 ab	30.12±0.04 b	9.42±0.09 b	47.13
0	100	35.11±0.18 b	Tr	17.75±0.22 b	6.50±0.12 a	31.10±0.09 b	9.55±0.01 b	47.15
250	0	29.21±0.84 a	Tr	20.25±0.07 d	8.56±0.24 b	33.49±1.66 bc	8.51±0.50 a	50.56
250	50	36.77±0.01 c	Tr	19.08±0.11 c	6.85±0.10 a	28.38±0.19 ab	8.94±0.03 ab	44.17
250	100	40.18±0.01 d	Tr	10.27±0.01 a	6.76±1.06 a	34.11±0.11 c	8.70±1.19 a	49.57
1000	0	31.53±0.27 a	Tr	16.62±0.23 ab	8.05±0.10 b	33.92±0.04 c	9.89±0.19 b	51.86
1000	50	40.11±0.01 d	Tr	19.61±0.28 c	6.84±0.28 a	24.59±0.01 a	8.87±0.01 ab	40.30
1000	100	42.18±0.01 d	Tr	9.70±0.95 a	6.80±0.98 a	33.23±0.15 bc	8.11±0.19 a	48.14
PA								
0	0	28.15±0.01 a	Tr	9.42±0.01 b	11.67±0.21 b	38.16±0.02 d	12.62±0.21 c	62.45
0	50	32.79±0.01 c	Tr	11.66±0.01 bc	12.98±0.01 c	31.53±0.06 ab	11.06±0.03 b	55.57
0	100	33.09±0.11 cd	Tr	12.22±0.07 c	13.37±0.13 c	30.43±0.04 ab	10.91±0.13 b	54.71
250	0	28.43±0.05 a	Tr	10.49±0.01 b	11.97±0.02 b	36.57±0.05 cd	12.56±0.01 c	61.10
250	50	35.81±0.03 d	Tr	13.91±0.08 d	13.59±0.09 c	26.41±0.18 a	10.28±0.04 a	50.28
250	100	34.27±0.29 cd	Tr	10.10±0.10 b	7.62±0.38 a	35.26±0.10 c	12.76±0.10 c	55.64
1000	0	30.67±0.04 b	Tr	11.23±0.04 b	12.29±0.48 c	33.51±0.02 b	12.31±0.50 c	58.11
1000	50	38.08±0.03 de	Tr	13.89±0.20 d	13.93±0.13 c	23.87±0.04 a	10.24±0.07 a	48.04
1000	100	37.98±0.09 e	Tr	7.01±0.12 a	7.80±0.41 a	35.11±0.19 c	12.11±0.20 c	55.02

^aData are means of three independent experiments ($\pm$ SE).

^bMeans with different letters indicate statistically different results at $P \leq 0.05$, according to Tukey's (HSD) test.

Table S3

Effects of SA on fatty acid composition (% of TL) in root neutral lipid (NL) of 16-d-old flax plantlets treated with different Cd treatments.

SA (μ M)	Cd (μ M)	Fatty acids						
		<i>C16:0</i>	<i>C16:1</i> <i>cis</i>	<i>C18:0</i>	<i>C18:1</i>	<i>C18:2</i>	<i>C18:3</i>	<i>Unsaturation</i>
0	0	25.18 $\pm$ 0.04 a	Tr	8.05 $\pm$ 0.11 a	13.67 $\pm$ 0.01 d	35.25 $\pm$ 0.09 d	17.87 $\pm$ 0.01 c	56.79
0	50	31.52 $\pm$ 0.01 ab	Tr	9.73 $\pm$ 0.05 b	14.82 $\pm$ 0.16 d	29.21 $\pm$ 0.09 c	14.74 $\pm$ 0.21 b	58.77
0	100	50.07 $\pm$ 0.08 d	Tr	8.91 $\pm$ 0.06 a	9.43 $\pm$ 0.06 a	20.24 $\pm$ 0.02 a	11.35 $\pm$ 0.10 a	41.02
250	0	28.73 $\pm$ 0.01 ab	Tr	8.57 $\pm$ 0.01 a	13.06 $\pm$ 0.01 c	34.55 $\pm$ 0.03 d	15.11 $\pm$ 0.06 bc	62.72
250	50	37.02 $\pm$ 0.01 b	Tr	11.04 $\pm$ 0.02 c	13.28 $\pm$ 0.11 c	25.48 $\pm$ 0.05 bc	13.19 $\pm$ 0.02 b	51.95
250	100	40.12 $\pm$ 0.02 c	Tr	12.50 $\pm$ 0.78 d	13.34 $\pm$ 0.07 c	22.69 $\pm$ 0.92 b	11.36 $\pm$ 0.09 a	47.39
1000	0	29.87 $\pm$ 0.01 ab	Tr	9.37 $\pm$ 0.01 b	13.26 $\pm$ 0.01 c	32.34 $\pm$ 0.02 cd	15.18 $\pm$ 0.01 bc	60.78
1000	50	37.06 $\pm$ 0.11 b	Tr	12.48 $\pm$ 0.21 d	12.43 $\pm$ 0.01 b	25.33 $\pm$ 0.14 bc	12.72 $\pm$ 0.19 ab	50.48
1000	100	38.49 $\pm$ 0.52 b	Tr	12.80 $\pm$ 0.59 d	13.34 $\pm$ 0.08 c	24.18 $\pm$ 0.05 bc	11.20 $\pm$ 0.20 a	48.72

^aData are means of three independent experiments ($\pm$ SE).

^b Means with different letters indicate statistically different results at $P \leq 0.05$, according to Tukey's (HSD) test.