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

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ABSTRACT

Salicylic acid (SA) promotes plant defense responses against toxic metal stresses. The present study addressed the hypothesis that 8-h SA pretreatment, would alter membrane lipids in a way that would protect against Cd toxicity. Flax seeds were pre-soaked for 8 h in SA (0, 250 and 1000 μM) and then subjected, at seedling stage, to cadmium (Cd) stress. At 100 μM $CdCl_2$, significant decreases in the percentages of phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and monogalactosyldiacylglycerol (MGDG) and changes in their relative fatty acid composition were observed in Cd-treated roots in comparison with controls. 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. This resulted in a significant increase in the fatty acid unsaturation ratio of the root PC and PE classes. As the exogenous application of SA was found to be protective of flax lipid metabolism, the possible mechanisms of protection against Cd stress in flax roots were discussed.

1. Introduction

Flax is a prominent source for linoleic (C18:2) and linolenic (C18:3) acids (Kaplan et al., 2014) and can survive on cadmium (Cd)-contaminated soils with minimal effects on above and below-ground growth (Kaplan et al., 2014; Praczyk et al., 2015). Cd is a rare element, uniformly distributed in the earth's crust, where its average concentration is 0.15–0.20 mg kg^{-1} (Fleischer et al., 1974). It is found 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 in various plant species. Most of this research has focused on cereals, such as rice (Guo et al., 2007), maize (Ivanova et al., 2008) and wheat (Li et al., 2011), or vege such as lettuce (Costa and Morel, 1994), radish (Vitoria et al., 2001) and onion (Jiang et al., 2001). From these studies, it is known that the exposure of roots to Cd directly alters physical properties and biological functions of cell membranes (Hernandez and Cooke, 1997; Sanz et al., 2009). Cd also indirectly alters membranes by the 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 (Belkadhi et al., 2013), oxidative degradation of lipids and decrease of membrane fluidity and permeability (Schützendübel and Polle, 2002; Belkadhi et al., 2013; Guo et al., 2013, 2014). An increased lipoxygenase (LOX) activity, which is responsible for 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 affect 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 exploits the non-specificity of some channels/transporters of membranes (Clemens, 2006) and the negative membrane potential, which exceeds −200 mV in rhizodermis cells (Hirsch et al., 1998). Maintaining this potential requires excretion of H^+ into the external environment via H^+-ATPase (Gaxiola et al., 2001). Indeed, under Cd stress, species including *Triticum aestivum* (Malik et al., 1992), *Capsicum annum* (Jemal et al., 2000), *Brassica juncea* (Seth et al., 2007) 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 (GLs), phospholipids (PLs) and neutral lipids (NLs). Moreover, in rhizodermis cells, Cd is known to substitute calcium

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(Ca) at its essential sites on the apoplast, tonoplast, mitochondrial and endoplasmic reticulum membranes (Breckle and Kahle, 1991). Furthermore, Cd entry into cytosol induces the phospholipases activities which leads to the membrane discharge of C18:2 and C18:3 serving as substrates for LOX or for the ROS that have been indirectly produced by the metal (Chmielowska-Bak et al., 2014). Cd-induced decreases of these polyunsaturated fatty acids in roots have also been reported in many species such as *Hordeum vulgare* (Vassilev, 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 (Shi and Zhu, 2008), enzyme activities (Guo et al., 2007; Ahmad et al., 2011), and photosynthesis (Liu et al., 2014). There is 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 the root apoplast of *Phaseolus aureus* and *Vicia sativa* by reducing H_2O_2 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 Ca^{2+}-ATPase were increased after exposure to Cd (Shao et al., 2010). Exogenous SA has also been shown to mitigate oxidative damage caused by Cd in *O. sativa* (Panda and Patra, 2007), *P. aureus* and *V. 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 (Choudhury and Panda, 2004; Guo et al., 2007); however, ours is the first study showing the protective effects of exogenous SA in the unsaturated fatty acids of membrane lipids of roots, in order to reveal their regulatory mechanism under Cd stress.

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) whether 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.

2. 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 or in water (0 μM SA) as previously described by 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 1 mM $MgSO_4$, 2.5 Mm Ca $(NO_3)_2$, 1 mM KH_2PO_4, 2 mM KNO_3, 2 mM NH_4Cl, 50 mM EDTA– Fe–K, 30 mM H_3BO_3, 10 mM $MnSO_4$, 1 mM $ZnSO_4$, 1 mM $CuSO_4$ and 30 mM $(NH4)_6Mo_7O_{24}$. 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 $CdCl_2$ (50–100 μM). Five replicates were produced 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), 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 °C and 100 °C as described in Belkhadi et al. (2010). In brief, root samples (100 mg) were cut into discs of uniform size and placed 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 conductivities, C1 and C2**,** were measured by conductivity meter. The MSI was determined using the following formula: $MSI=(1-(EC1/EC2))\ 100$.

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 I_2 vapor. Lipid classes were then identified against lipid standards using specific stains for PLs, GLs and NLs. Identification of individual PLs was recognized by molybdenum blue reagent, whereas phosphatidylethanolamine (PE) by ninhydrin. GLs were detected by staining with α-naphthol reagent and NLs 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 different 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 heptanoic 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, finally, in hexane were injected using an autosampler CP 9050 (Chromapack, Middelburg, Netherlands). Each sample of PLs, GLs and NLs 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).