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Temperature-induced lipocalin (TIL) is translocated under salt stress and protects chloroplasts from ion toxicity

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ABSTRACT

Temperature-induced lipocalins (TIL) have been invoked in the defense from heat, cold and oxidative stress. Here we document a function of TIL for basal protection from salinity stress. Heterologous expression of TIL from the salt resistant poplar *Populus euphratica* did not rescue growth but prevented chlorophyll b destruction in salt-exposed *Arabidopsis thaliana*. The protein was localized to the plasma membrane but was re-translocated to the symplast under salt stress. The *A. thaliana* knock out and knock down lines At*til1-1* and At*til1-2* showed stronger stress symptoms and stronger chlorophyll b degradation than the wildtype (WT) under excess salinity. They accumulated more chloride and sodium in chloroplasts than the WT. Chloroplast chloride accumulation was found even in the absence of salt stress. Since lipocalins are known to bind regulatory fatty acids of channel proteins as well as iron, we suggest that the salt-induced trafficking of TIL may be required for protection of chloroplasts by affecting ion homeostasis.

Introduction

Plants exist under changing environmental conditions and, therefore, encounter external constraints such as pollution, chilling, high temperatures, drought, salinity, and many other stresses. To persist under fluctuating conditions plants contain constitutive and inducible defense systems. Genes that mediate increased resistance to a broad range of stresses are of particular interest because crop cultivation on arable land is strongly limited by drought and soil salinity, especially in arid countries, thus, requiring breeding of new, stress-resistant cultivars. The family of lipocalins is emerging as an interesting target for plant improvement because plant lipocalins are involved in the protection from a broad range of abiotic cues (Uemura et al., 2006; Frenette Charron, 2005; Charron et al., 2008; Chi et al., 2009; Kjellsen et al., 2010; Levesque-Tremblay et al., 2009).

Plant lipocalins compass, for example, the chloroplast lipocalins (CHL), which have been localized to the thylakoid lumen (Levesque-Tremblay et al., 2009) and the temperature-induced lipocalins (TIL, Frenette Charron, 2005; Grzyb et al., 2006). TILs have initially been detected as massively increased proteins in response to low temperatures (Kawamura and Uemura, 2003). In Siberian spruce they are among the most strongly increased proteins during cold acclimation (Kjellsen et al., 2010). TIL1 of Arabidopsis is localized in the plasma membrane (Frenette Charron, 2005) and its temperature response suggested a function in membrane stabilization during freezing stress (Uemura et al., 2006). In plants, the molecular targets of lipocalins are unknown, but evolutionary related proteins such as bacterial lipocalin Blc, insect Lazarillo protein, and mammalian apolipoprotein D bind for example retinoids, arachidonic acid, bilirubin, steroid hormones (progesterone and pregnenolone) and cholesterol, and play roles in immunity and signaling (Rassart et al., 2000).

Initial screening of stress responses of lipocalins in wheat and Arabidopsis showed strong increases in TIL expression in response to low and high temperatures (Frenette Charron, 2005). Reverse genetics using a knock-out line of TIL1 in Arabidopsis revealed the accumulation of H_2O_2 and increases in light and freezing sensitivity, probably as the result of increased oxidative stress (Charron et al., 2008). Chi et al. (2009) showed that AtTIL1 was important

Abbreviations: Chl, chlorophyll; EDX, Energy dispersive X-ray microanalysis; FAMEs, fatty acid methyl esters; OEX, overexpressor lines; OSE, of salt exposure; TIL, temperature-induced lipocalin; WT, wildtype.

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for both basal temperature and acquired temperature resistance. The chloroplast-localized lipocalin At*CHL* increased in response to drought, high light, paraquat and abscisic acid treatments, but not in response to heat shock or salinity (Levesque-Tremblay et al., 2009). Arabidopsis *chl* knock out lines showed strong increases in lipid peroxidation (Levesque-Tremblay et al., 2009). Notably, neither Arabidopsis nor wheat *TIL* levels increased in response to salinity (Frenette Charron, 2005; Brinker et al., 2010) although salinity is known to induce osmotic and oxidative stress (Chen and Polle, 2010).

In contrast to herbaceous plants, the *TIL1* ortholog Peu*TIL1* of the salt resistant tree species *Populus euphratica* increased rapidly after exposure to saline conditions (Brinker et al., 2010). When Arabidopsis mutant lines (At*til1-1* and At*til1-2*) with suppressed *TIL1* expression were exposed to high salinity, growth and biomass production were compromised (Brinker et al., 2010). This underpins the importance of TIL in mediating stress resistance and shows that TIL belongs to the innate constitute defense from abiotic stress in Arabidopsis (Brinker et al., 2010). However, the mechanisms that affect the salt resistance in TIL-deficient plants are still unclear.

To improve salt resistance of plants it is important to understand the adaptation processes for life in a saline environment. The goal of the present study was to characterize the phenotypic and biochemical response of At*til* knock out mutants and Peu*TIL* heterologous expression in *Arabidopsis thaliana*. In combination with anatomical and cytological studies we show that TIL specifically protects chlorophyll b from salt-induced degradation by preventing excess sodium and chloride accumulation in the chloroplasts, probably by a mechanism involving salt-responsive mobility of TIL between the plasma membrane and the cytosol.

Materials and methods

Plants and salt treatments

Arabidopsis thaliana wildtype Col 0 (WT) was used. The T-DNA insertion mutants in the gene locus At5g58070, denominated as temperature-induced lipocalin (TIL) (Salk_136775 = At*til1-1* and Salk_150259 = At*til1-2*) were obtained from Nottingham Arabidopsis Stock Centre (NASC, http://nasc.nott.ac.uk) and homozygous lines isolated by Brinker et al. (2010) were used. The heterologous overexpressor (OEX) lines for TIL from *Populus euphratica* (PeuTIL) T5, T6 and T21 were constructed as described below. Seeds were sterilized in 5% $Ca(Cl_2O)$ (Merck, Darmstadt, Germany) containing 0.02% Triton X-100 (Serva, Heidelberg, Germany) for 5 min, washed 3 times with distilled water, sown in Petri dishes on MS medium (Murashige and Skoog, 1962) containing 2.5% sucrose and 0.3% gelrite (Duchefa, St. Louis, MO) and stratified for 3 d at 4 °C in darkness. For germination, the plates were kept for 10 d in a culture room (150 μmol m^{-2} s^{-1} photosynthetic active radiation (PAR) 16 h, 8 h darkness, 20 °C, 60% air humidity).

To test salt stress in plate assays, the MS medium in the Petri dishes contained either no salt (control) or 50 mM NaCl. Each plate contained 5 WT and 5 transgenic seeds. In each experiment 6 plates per line and treatment were tested. Each experiment was repeated three-times.

To test long-term salt resistance, the 10 d-old seedlings were planted into pots containing 115 g soil (Fruhstorfer Erde, Type T 25, Archut, Germany) and grown with 8 h light (75 μmol PAR m^{-2} s^{-1}) at 20 °C and a relative air humidity of 60%. Initially, the plants were watered regularly with tap water containing 0.22 mM Na^+, 0.75 mM Ca^{2+}, 0.002 mM K^+, and 0.22 mM Cl^-. After 10 days the plants were divided in a control group and a salt-treated group. All plants were irrigated regularly with 1/8 strength MS medium, but the medium for the salt-treated plants contained additionally increasing concentrations of NaCl according to the following scheme: 5 d exposure to 50 mM, subsequently 5 d to 100 mM (10 days of salt exposure [OSE]) and finally 10 d to 200 mM NaCl (20 d OSE). At 10 d OSE and 20 d OSE 6 plants per treatment and line were harvested. The experiment was repeated two times. Data are means ± SE.

Plant harvest

Before each harvest the plants were photographed (Nikon CoolPix 4, Nikon, Tokyo, Japan) and the images were used to measure projected leaf area with the software Blattfläche (version 1.0, DatInf GmbH 2005, Tübingen, Germany). At harvest fresh mass of the whole rosettes was determined. One fresh leaf was immediately used to excise discs for electrolyte leakage measurements. Three leaves were removed and dried for 3 days at 65 °C. The remaining parts of the rosettes were kept at −80 °C.

Electrolyte leakage

Electrolyte leakage was determined according to Tripathy et al. (2000). Five leaf disks (6 mm diameter) were cut, weighed and subsequently placed at room temperature into Falcon tubes (Sarstedt, Numbrecht, Germany) containing 25 mL double distilled water (ddH_2O). Conductivity of the water was measured at time zero (EC_{t0}) and after 24 h (EC_{t24}) using a conductivity meter (LF 315/Set, WTW Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). To determine maximum electrolyte leakage (EC_{max}) the samples were autoclaved (121 °C, 20 min, HST 6 × 6 × 6 Zirbus technology GmbH, Bad Grund, Germany) and cooled to RT before measurement. The relative electrolyte leakage (REL) was calculated as

$$\mathrm{REL}\,(\%) = (\mathrm{EC_{t24}} - \mathrm{EC_{t0}}) \times \frac{100}{\mathrm{EC_{max}} - \mathrm{EC_{t0}}}$$

Chlorophyll determination

Pigments were analyzed after Lichtenthaler and Wellburn (1983). Leaf material was powdered in liquid nitrogen. Frozen leaf powder (FW = 30 mg) was extracted for 20 min in darkness in a volume (V) of 5 mL 80% acetone. The extract was centrifuged (4 °C, 15 min, 470 g) and the absorbance of the supernatant was measured in a spectrophotometer (UV-DU® 640, Beckman Instrument Inc., Fullerton, USA) at wavelengths of 663 nm and 646 nm, respectively. The concentrations of chlorophyll a (Chl a) and Chl b were calculated as:

$$\mathrm{Chl\ a}\,[\mu g\ mg^{-1}] = (12.21A_{663} - 2.81A_{646}) \times \frac{V}{FW}$$

$$\mathrm{Chl\ b}\,[\mu g\ mg^{-1}] = (20.13A_{646} - 5.03A_{663}) \times \frac{V}{FW}$$

Total fatty acid determination

Total fatty acids (FA) were extracted according to Przybyla et al. (2008) with some modifications. 50 mg of ground leaf material were extracted by adding 4 mL of extraction medium (n-hexane: 2-propanol, 3:2 (v/v) with 0.0025% (w/v) butylated hydroxytoluene) and 20 μg triheptadecanoate as internal standard for quantification. The extract was shaken for 10 min and centrifuged at 3200 × *g* at 4 °C for 10 min. The clear upper phase was collected, and 2.5 mL of 6.7% (w/v) solution of potassium sulfate was added. After vigorous shaking and centrifugation at 3200 × *g* at 4 °C for 10 min, the upper hexane-rich layer was subsequently dried under streaming nitrogen. Subsequently, the total fatty acids were derivatized into