Quantification and Fatty Acid Profiles of Sulfolipids in Two Halophytes and a Glycophyte Grown under Different Salt Concentrations

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This study was aimed at understanding the role of sulfolipids in salt tolerance mechanisms of the halophytes *Aster tripolium* L., Compositae, and *Sesuvium portulacastrum* L., Aizoaceae, and of the glycophyte *Arabidopsis thaliana* (L.) Heynh., Brassicaceae. In *Aster* and *Sesuvium* the sulfolipid contents increased significantly under salt stress conditions (517 mM or 864 mM). In *Arabidopsis*, changes in sulfolipid contents were not observed (NaCl up to 100 mM). The fatty acid profile of sulfoquinovosyldiacylglycerol (SQDG) in *Aster* was modified with increasing NaCl concentrations. LC-MS analyses of sulfolipids from *Aster* and *Sesuvium* revealed the presence of 18:3/18:3 and 16:0/18:3 molecules. Obviously, the function of sulfolipids during salt stress differs between halophytic species and between halophytes and glycophytes where sulfolipid accumulation was not observed.

Key words: Fatty Acids, Salt Tolerance, Sulfolipids

Introduction

Benson and co-workers (1963) reported a new sulfur-containing lipid in plants and named it sulfoquinovosyldiacylglycerol (SQDG). Since then, SQDG has been found in numerous photosynthetic plants, algae, cyanobacteria, purple sulfur and non-sulfur bacteria (*e.g.* Harwood, 1980; Barber and Gounaris, 1986), and also in substantial amounts in the non-photosynthetic bacterium *Rhizobium meliloti* (Cedergreen and Hollingsworth, 1994). Recently, the entire biosynthetic pathway was elucidated for higher plants (Yu *et al.*, 2002). In higher plants the glycolipid SQDG is localized in the thylakoid membranes of chloroplasts (7 mol% of all lipids in the spinach membrane) (Block *et al.*, 1983). The quantities of saturated SQDG (16:0/18:0) range from 26% (*Triticum aestivum* L.) to 62% (*Carica papaya* L.) (Kenrick and Bishop, 1986).

It has been shown previously that environmental and nutritional factors like water deficit (Muller and Santarius, 1978), temperature (Quartacci *et al.*, 1995; Sato *et al.*, 2003), sulfate deprivation (DeKok *et al.*, 1997), and phosphate starvation (Essigmann *et al.*, 1998; Yu *et al.*, 2002) influence the synthesis of SQDG. It has also been suggested that SQDG stabilizes protein complexes in chloroplast membranes such as photosystem II (PSII) (Minoda *et al.*, 2003) and CF_0–CF_1 ATPase (Pick *et al.*, 1985). Supporting evidence was given by the fact that ATPase activity was inhibited when SQDG tightly bound to the CF_0–CF_1 complex of chloroplast ATPase was removed (Barber and Gounaris, 1986).

The sea aster *Aster tripolium* L., Asteraceae, grows in diverse environments especially in the salt marshes along the coastlines in temperate regions but also in other inland places with saline soils (Wagenvoort *et al.*, 1989). *Sesuvium portulacastrum* L. belongs to the Aizoaceae family and is located naturally in the tropical and subtropical brackish areas around the world. These two halophytes along with the glycophyte *Arabidopsis thaliana* (L.) Heynh. (ecotype Wassilewska), Brassicaceae, were chosen for this study. Salt tolerance studies in *Aster* and *Sesuvium* are vital because both are potential vegetables for the future. The objective of this study was to know the effect of salt stress on sulfolipids and the possible contribution of unsaturated fatty acids in SQDG towards salt tolerance in these halophytes.

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Material and Methods

Biological material

Seeds of *Aster tripolium* (Dollart area, North Sea, Germany), *Sesuvium portulacastrum* (collected close to Dakhla, Morocco), and *Arabidopsis thaliana* (ecotype Wassilewska) were germinated on substrate TKS1 (Floragard, Oldenburg, Germany). *Aster* seeds needed a stratification period of 14 d at 4 °C on moistened TKS1. The seedlings were transplanted in TKS2 substrate (Floragard) into pots after about 30 d in the case of *Aster* and 20 d in the case of *Arabidopsis*. At the beginning of the salt treatments the *Aster* plants were about 40 d old having about 5 leaves and the *Arabidopsis* plants were 30 d old having 8 leaves. *Sesuvium* plants were propagated by cuttings. The cuttings developed roots in about 7 d. 15 d after making cuttings the salt treatment began. At this state the plants possessed about 8 leaves. The plants were watered daily with nutrient-supplemented tap water without or containing the respective concentrations of NaCl: for the treatment of *Aster* plants 258 mM and 517 mM, for *Sesuvium* plants 428 mM and 856 mM, and for *Arabidopsis* plants 50, 75, and 100 mM. Conditions in the greenhouse were a 16 h light/8 h dark rhythm at temperatures of 23 °C/21 °C.

Thin layer chromatography (TLC) of SQDG

For SQDG analysis the leaves were ground with a mortar and pestle to a fine powder in liquid nitrogen. 100 mg of the plant material was suspended in 300 μl of methanol/chloroform/water (65:25:4 v/v), mixed and centrifuged for 10 min at 16,000 × *g* at 4 °C. After centrifugation the lower phase (green) was transferred to a new tube and directly applied to a silica gel 60 TLC plate (20 × 20 cm; 0.25 mm layer thickness) (Merck, Darmstadt, Germany). TLC plates were activated at 120 °C for 1 h prior to sample application. After loading the samples as spots (5 mm) or streaks (2 cm long) in the case of fatty acids analysis, the plates were developed in solvent (chloroform/methanol/0.02% $CaCl_2$ 60:40:9, v/v) in a sealed glass TLC chamber (20 × 20 × 5 cm; Degussa AG, Hanau, Germany) at ambient temperature for about 75 min (modified after Murakami-Murofushi *et al.*, 1985; Archer *et al.*, 1997). For quantification a standard row using commercially available SQDG (Lipid Products, Redhill, UK) of 5 μg/μl diluted to 1:20 for quantification from plant lipid extracts was prepared and applied onto the same plate. Plates were dried for 30 min and then placed into a sealed glass chamber equilibrated before with iodine vapour formed by sublimation (Benning and Somerville, 1992). Spots appeared after about 5 min. Plates were immediately density-scanned because they loose their intensity due to the influence of light. The intensity of the spots was calculated using the TINA 2.0 software (Raytest, Straubenhardt, Germany).

Fatty acid analysis of SQDG by GC-MS

The SQDG containing fraction was scratched out from the TLC plate and the SQDG was desorbed with 30 ml of methanol under sonification. After concentration *in vacuo*, SQDG was saponified by adding 0.5 ml of 0.5 M methanolic sodium hydroxide and heating to 80 °C for 5 min in a tightly screwed derivatisation vial with a Teflon septum. For esterification, 1 ml of boron trifluoride methanol complex was added and the mixture was re-heated for 5 min (80 °C). After cooling and addition of 2 ml saturated sodium chloride solution, the fatty acid methyl esters (FAME) were extracted with 2 ml of *n*-hexane.

A Fisons 8060 gas chromatograph equipped with an on-column injector (100 °C) was directly coupled with a Fisons MD 800 mass spectrometer. Separation of FAME was achieved on a DB-WAX fused silica column (30 m × 0.32 mm i.d., 0.25 μm film thickness; J & W Scientific, Folsom, CA, USA) using helium as carrier gas (3.2 ml min^{-1}). The temperature program was as follows: 3 min isothermal at 100 °C, raised to 180 °C (1 min) at 5 °C min^{-1}, to 200 °C (1 min) at 1 °C min^{-1}, and to 230 °C (5 min) at 5 °C min^{-1}. The temperature of the ion source was 200 °C, the electron energy for the electron impact mass spectra was 70 eV. Assignment of the fatty acids was performed by comparison to the retention indices of authentic reference substances and on the basis of their respective mass spectra.

Mass spectrometry of SQDG

Sample application was performed by loop injection (200 μl min^{-1}, CH_3CN/H_2O 1:1 v/v) from a Waters Alliance (Milford, Massachusetts, USA) HPLC system. Mass spectra were recorded in the negative electrospray mode (cone voltage 30 V) by an LCT time of flight mass analyser (Micro-

mass, Manchester, UK), equipped with a LockSpray™ unit for exact mass measurement.

Miscellaneous

The salt treatment experiments were repeated three times independently. For the analysis four plants were combined at each time point. The determination of SQDG was done three times for each experiment. The mean and the standard error are given for each analysis.

Results and Discussion

Effect of salt treatment on the phenotype of experimental plants

It was reported that *Aster* plants are able to grow at salt concentrations up to 500 mM (Kerstiens *et al.*, 2002). *Sesuvium* tolerates up to 1000 mM (Mssedi *et al.*, 2000), and *Arabidopsis* up to 200 mM (Shi *et al.*, 2003). In pre-tests NaCl concentrations were determined, which caused comparable effects on growth rates retardation and reduction in the fresh weight increase (data not shown). All experimental plants showed reduced growth after 10 d of salt treatment with the NaCl concentrations indicated. *Aster* was able to grow on NaCl levels between 0 and 517 mM, close to seawater salinity of 599 mM (Fig. 1). The succulent plant *Sesuvium* proved to be more tolerant to NaCl and could grow up to 856 mM NaCl concentration with a similar reduction in growth in comparison to the *Aster* plants. In the glycophyte *Arabidopsis* (ecotype Wassilewska) growth was severely affected already at 100 mM NaCl. At higher concentrations *Arabidopsis* plants showed necrotic cell death at the tips and margins of the older leaves (data not shown). The NaCl-stressed plants treated with the NaCl concentrations mentioned above could recover from stress after about 2 weeks. However, after treatment with higher NaCl concentrations (about 900 mM in the case of *Aster* and 1200 mM in the case of *Sesuvium*) for more than 10 d the effects of salt stress were not reversible any more.

Fig. 1. Effect of NaCl on the growth of *Aster tripolium* plants cultivated with different salt concentrations. *Aster* plants watered with 0 mM, 258 mM, and 517 mM NaCl in the watering solution (from the left to the right) for up to 10 d. Conditions in the greenhouse were a 16 h light/8 h dark rhythm at temperatures of 23 °C/21 °C and humidity of 55%. The plants showed decreased growth rates with increasing salt concentrations.

Effect of NaCl on the SQDG contents

In the halophytes *Aster* and *Sesuvium* the SQDG contents increased in comparison to the control plants watered without NaCl (Fig. 2A, B). In *Aster* the differences in SQDG contents between the control and 258 and 517 mM NaCl in the watering solution were already substantiated after 1 d and did not change during the duration of the experiment. After a 10-day-treatment with 856 mM NaCl in the watering solution the SQDG contents in *Sesuvium* were about twice as high as in the untreated control. In the glycophyte *Arabidopsis* no significant differences could be observed between control and salt-treated plants during the 10-day-exposure (Fig. 2C). The amount of SQDG in the plants investigated ranged from 140 to 580 μg per g fresh weight (FW).

Fatty acid patterns of SQDG under salt stress

The fatty acid profiles of SQDG from *Aster* and *Sesuvium* were analysed by means of GC-MS. *Sesuvium* and *Aster* were grown for 7 and 10 d, respectively, with and without salt as described in Material and Methods. The results of the fatty acid composition of SQDG are summarized in Table I. SQDG contained predominantly 16:0 (palmitic acid) and 18:3 (γ-linolenic acid), and trace amounts of 18:2 (linoleic acid). In *Aster* the degree of unsaturation slightly decreased after 1 d of salt treatment in comparison to control plants based on 18:3 and 18:2 species. During longer salt treatment (10 d) the degree of unsaturation slightly increased having less 16:0 and almost two-fold 18:2 species in comparison to the controls. In *Sesuvium* after 7 d of salt treatment the 18:2 species clearly increased in comparison to the control in a similar

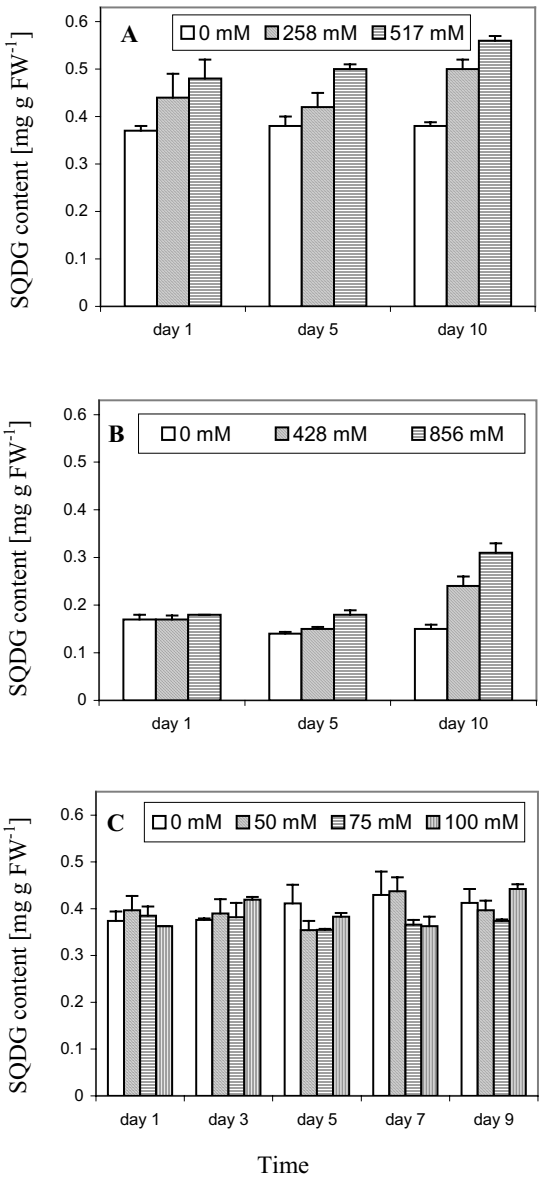

Fig. 2. Changes in the SQDG content in halophytes and glycophytes in response to salt treatment. (A) *Aster tripolium*, (B) *Sesuvium portulacastrum*, and (C) *Arabidopsis thaliana* (ecotype Wassilewska).

way as in *Aster*. To prove the reliability of the method for the determination of the fatty acid profiles the fatty acid composition of the SQDG from *Nicotiana tabacum* L. was determined and compared to published data (Bishop *et al.*, 1985) which are in agreement with our data (data not shown).

LC-MS of the SQDG pool

We were not only interested in knowing the percentage of fatty acids in all SQDG molecules in the halophytes, but also wanted to elucidate the fatty acid composition at *sn*-1 and *sn*-2 position. For the commercially available SQDG standard molecular ions were registered at 812.8796 Da and 834.8245 Da corresponding to $C_{43}H_{73}O_{12}S$ and to $C_{43}H_{71}O_{12}S$ (data not shown). In the SQDG isolated from *Aster*, ions were registered at 815.4979 Da and 837.4823 Da corresponding to elemental compositions of $C_{43}H_{75}O_{12}S$ (0.1 ppm) and of $C_{45}H_{73}O_{12}S$ (– 0.2 ppm), respectively (Fig. 3A, B). The molecular mass of 815.5008 Da corresponding to $C_{43}H_{75}O_{12}S$ is in good agreement with a 16:0/18:3 SQDG and the mass of 837.4813 Da ($C_{45}H_{73}O_{12}S$) matches the elemental composition of an 18:3/18:3 SQDG.

Physiological implications of the results obtained

The halophytes *Aster* and *Sesuvium* both contained increased SQDG contents when treated with salt in comparison to control plants. But the glycophyte *Arabidopsis* did not show any significant differences in its SQDG contents with respect to the salt treatment. Although both halophytes *Aster* and *Sesuvium* contained higher SQDG contents when treated with NaCl, their fatty acid composition differed based on their unsaturation capacity. Several reports describe the effect of salinity on the SQDG contents in glycophytes (Kuiper *et al.*, 1974; Pearcy, 1978; Stuiver *et al.*, 1981; Kettunen *et al.*, 1996). For example, when NaCl was added to the external medium of seedlings of the glycophyte *Hordeum vulgare* L., SQDG contents remained unchanged in comparison to the control (Muller and Santarius, 1978). Also in salt-sensitive lines of *Beta vulgaris* L. NaCl did not alter the sulfolipid contents, but in a salt-tolerant genotype of *Beta vulgaris* the SQDG content increased during NaCl treatment (Kuiper and Kylin, 1981; Stuiver *et al.*, 1981). Thus the formation of SQDG may be one important aspect of strategies involved in salt tolerance of halophytes.

Until now, the fatty acid composition of SQDG was predominantly analysed in glycophytes, namely spinach, tobacco, wheat, and cucumber (Bishop *et al.*, 1985). Also the characterization of lipids from the chloroplast envelope in glycophytes has been well investigated (Heinz, 1973; Siebertz *et al.*, 1979). The positional distribution of

Table I. Fatty acid patterns of SQDG in the leaves of *Aster tripolium* (top) and *Sesuvium portulacastrum* (bottom) watered with different concentrations of NaCl (*Aster*, 0 mM, 258 mM, and 517 mM; *Sesuvium*, 0 mM, 428 mM, and 856 mM NaCl). The composition is given in mol%. The values are the means of three independent experiments.

(NaCl)	16:0	18:0	18:1	18:2	18:3
[mM]			day 1		
0	32.4 ± 6.6	10.1 ± 1.9	11.0 ± 4.0	17.1 ± 6.1	29.8 ± 5.1
258	30.0 ± 4.6	13.1 ± 2.2	13.3 ± 3.8	17.4 ± 1.7	25.5 ± 4.7
517	32.6 ± 7.8	8.3 ± 1.7	9.8 ± 2.2	25.5 ± 7.3	23.4 ± 3.9
			day 10		
0	34.5 ± 4.0	6.0 ± 1.2	11.4 ± 2.4	14.4 ± 3.2	38.4 ± 5.4
258	34.9 ± 4.7	5.2 ± 2.5	9.8 ± 0.7	25.2 ± 2.4	24.9 ± 4.1
517	31.1 ± 8.5	5.7 ± 1.5	9.8 ± 2.8	29.9 ± 6.6	23.1 ± 3.0

(NaCl)	16:0	18:0	18:1	18:2	18:3
[mM]			day 1		
0	33.1 ± 1.1	6.1 ± 0.5	20.4 ± 0.05	25.7 ± 1.8	15.1 ± 2.9
428	38.3 ± 6.3	7.6 ± 1.3	18.4 ± 3.0	23.3 ± 8.1	14.3 ± 3.3
856	39.8 ± 5.7	6.4 ± 0.5	15.9 ± 6.6	25.3 ± 6.6	12.3 ± 2.3
			day 7		
0	35.9 ± 2.8	8.7 ± 1.8	19.3 ± 0.8	23.3 ± 2.2	12.8 ± 0.4
428	37.8 ± 4.1	7.5 ± 2.5	18.6 ± 1.7	21.7 ± 4.4	12.4 ± 1.7
856	33.1 ± 4.8	4.5 ± 0.9	17.2 ± 0.5	30.9 ± 5.6	13.9 ± 2.8

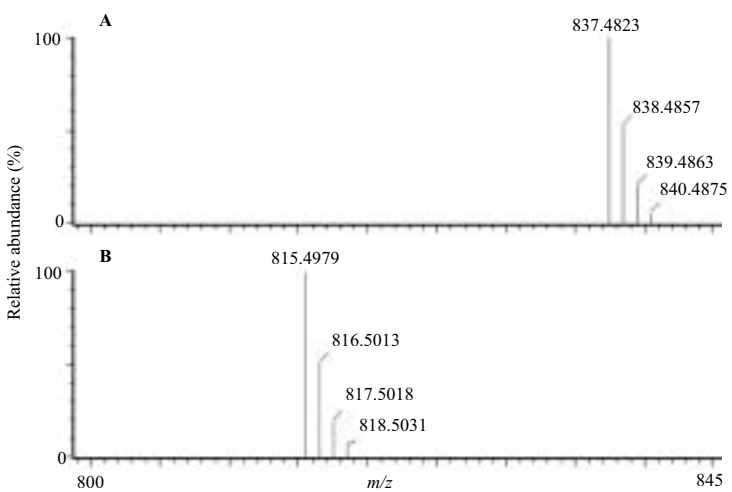

Fig. 3. LC-MS spectra of SQDG extracted from *Aster tripolium* showing two different molecular ion peaks at (A) 837 Da and (B) 815 Da, in the complete mass spectrum with 815 and 837 Da peaks. Single mass analysis with 0.1 and – 2.0 ppm tolerance for 815 and 837 Da, respectively. Mass spectra were recorded in the negative electrospray mode (cone voltage 30 V) by an LCT time of flight mass analyser (Micromass, Manchester, UK), equipped with a LockSpray™ unit for exact mass measurement.

fatty acids on *sn*-1 and *sn*-2 was investigated by Bishop *et al.* (1985). All samples of SQDG isolated from different glycophytic species contained higher contents of 16:0 than of the corresponding glycolipids occurring in plant membranes monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). The positional distribution of 16:0 demonstrated that in the 16:3-plants significant amounts of these acids are present at both the *sn*-1 and *sn*-2 positions, demonstrating

that both prokaryotic and eukaryotic synthesis mechanisms for DAG production contribute to the biosynthesis of SQDG. In the 18:3-plants 16:0 acids are predominantly located in the *sn*-1 position of SQDG, again demonstrating the origin of the DAG as being the eukaryotic pathway. It is not known to which group of plants *Aster* and *Sesuvium* belong to. To our knowledge this is the first report of a clear increase of SQDG in halophytes due to salt stress and 16:0/18:3 and 18:3/18:3 SQDGs in the halophytes *Aster* and *Sesuvium*.

The effect of low temperatures on the lipid content and composition of the thylakoid membrane was investigated in the glycophyte *Pisum sativum* L. (Chapman *et al.*, 1983). Both, relative amounts of lipid classes and degree of saturation, were not changed significantly during growth at cold and warm conditions. However, it was demonstrated that in cold-grown plants there were slightly higher 18:3 and lower 18:2 contents for all three glycolipids, MGDG, DGDG, and SQDG. In our experiments temperature effects can be excluded, as temperature in the green house was controlled. Therefore direct effects of the salt treatment on the SQDG contents can be assumed.

It was hypothesized that SQDG is involved in stabilization of ATPase complexes and PSII (Pick *et al.*, 1985). This hypothesis seems to match our experimental results on F-ATPase activities. *Aster* F-ATPase activity increased with increasing NaCl concentration in the medium of growth (Ramani, 2004). Recently, Minoda *et al.* (2002, 2003) confirmed the role of SQDG in the functionality of PSII in more detail. The authors demonstrated that a *Chlamydomonas reinhardtii* mutant deficient in SQDG biosynthesis showed almost 40% reduction in PSII activity. The activity could be restored when SQDG was supplemented; furthermore chemical modifications of SQDG impaired the activity of PSII. Taken together SQDG clearly contributes to maintain the conformation and efficiency of PSII complexes in *Chlamydomonas*. Similar effects were reported for a *Chlamydomonas SQD1* mutant. This genetically well characterized mutant showed increased sensitivity to a diuron herbicide, a herbicide binding to the Q_B receptor site of PSII. PSII activity was blocked in the *SQD1* mutant suggesting a role for SQDG in photosynthesis (Riekhof *et al.*, 2003).

The nutritional status of the plants also influences the SQDG content, mainly sulfur and phosphorus: Sulfur deprivation has a decisive impact on lipid metabolism in *Brassica oleracea* (DeKok *et al.*, 1997). There was a substantial decrease in the levels of SQDG in both roots and shoots when expressed in a total lipid basis. A major role of SQDG in glycophytes might be the exchange of phospholipids to SQDG during phosphate starvation. Under normal growth conditions about 30% of P_i are bound in phospholipids which might be released under phosphate deficiency when increasing amounts of SQDG take over the role of phospholipids, at least in *Arabidopsis* (Essigmann *et al.*, 1998). This mechanism still remains to be proved in halophytes.

The increasing amounts of SQDG during salt treatment might also play a role in signalling processes. It was shown that spinach annexin binds in the presence of 100 μM Ca^{2+} not only to envelope membranes of purified chloroplasts, but also to vesicles consisting of phosphatidylcholine (PC) and SQDG (Seigneurin-Berny *et al.*, 2000). However, only SQDG and not PC was accessible in the asymmetric outer envelope membrane and might communicate any signals via annexin to the cytoplasm or *vice versa*.

Based on the experimental results of SQDG quantification and fatty acids analysis in *Aster* and *Sesuvium*, it might be suggested that in some halophytes SQDG plays a protecting role during salt stress. To follow this hypothesis we need to construct and analyse halophytic mutants. It would be very useful and interesting to start a lipomics project. This project aims at investigating the abundance of all lipids and their fatty acids in different membranes of the cell during application of various abiotic stresses. That kind of analysis might help to reveal the role of SQDG in the plastids during salt stress.

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