Expression of the \textit{ggpPS} gene for glucosylglycerol biosynthesis from \textit{Azotobacter vinelandii} improves the salt tolerance of \textit{Arabidopsis thaliana}

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Abstract

Many organisms accumulate compatible solutes in response to salt or desiccation stress. Moderate halotolerant cyanobacteria and some heterotrophic bacteria synthesize the compatible solute glucosylglycerol (GG) as their main protective compound. In order to analyse the potential of GG to improve salt tolerance of higher plants, the model plant \textit{Arabidopsis thaliana} was transformed with the \textit{ggpPS} gene from the \(\gamma\)-proteobacterium \textit{Azotobacter vinelandii} coding for a combined GG-phosphate synthase/phosphatase. The heterologous expression of the \textit{ggpPS} gene led to the accumulation of high amounts of GG. Three independent \textit{Arabidopsis} lines showing different GG contents were characterized in growth experiments. Plants containing a low (1–2 \(\mu\)mol g\(^{-1}\) FM) GG content in leaves showed no altered growth performance under control conditions but an increased salt tolerance, whereas plants accumulating a moderate (2–8 \(\mu\)mol g\(^{-1}\) FM) or a high GG content (around 17 \(\mu\)mol g\(^{-1}\) FM) showed growth retardation and no improvement of salt resistance. These results indicate that the synthesis of the compatible solute GG has a beneficial effect on plant stress tolerance as long as it is accumulated to an extent that does not negatively interfere with plant metabolism.

Key words: Compatible solute, sugar metabolism, stress, transgenic plant.

Introduction

Drought and increased soil salinity limit the distribution and productivity of crop plants worldwide. Therefore, improvement of desiccation and salt tolerance is an important challenge in modern plant breeding. In order to achieve this goal, classical breeding techniques as well as genetic engineering are used (Apse and Blumwald, 2002; Flowers, 2004). The avoidance of high concentrations of toxic ions in the cytoplasm by active extrusion or compartmentation of ions accompanied by the accumulation of so-called compatible solutes in metabolically active parts of the cell represent the general strategy for successful salt acclimation in plants. The compatible solutes act by enhancing the internal osmotic potential and thereby improving the water status of the cell. In addition, they are able to protect proteins and membranes directly against denaturation (Crowe et al., 1984; Luzardo et al., 2000; Borges et al., 2002; Hincha and Hagemann, 2004).

An increased accumulation of compatible solutes has been obtained by two strategies: (i) the enhancement of synthesis or the reduction of breakdown of naturally occurring compatible solutes, for example, proline (Hong et al., 2000) or trehalose (Goddijn et al., 1997); or (ii) the transfer of heterologous genes, which code pathways for the synthesis of compatible solutes originating from halotolerant micro-organisms, into crop or model plants (for reviews, see Nuccio et al., 1999; Apse and Blumwald, 2002; Chen and Murata, 2008). Genes coding for enzymes catalysing the synthesis of different classes of compatible
solutes were successfully transferred, for example, the carbohydrate trehalose (Holmström et al., 1996; Pilon-Smits et al., 1998), the polyols mannitol (Tarczyński et al., 1993; Thomas et al., 1995) and sorbitol (Sheveleva et al., 1998), or the amino acid derivatives glycine betaine (Lilius et al., 1996; Hayashi et al., 1997; Nuccio et al., 1998; Waditee et al., 2005) and ectoine (Nakayama et al., 2000). Although the transgenes were successfully expressed in different plant species, the amounts of the corresponding solutes detected were relatively low (0.01–4.6 μmol g⁻¹ FM). Despite this rather low accumulation, improved growth of the transgenic plants under drought, high salt, as well as cold or high light conditions, has been reported in many cases (Hayashi et al., 1997; Pilon-Smits et al., 1998; Waditee et al., 2005).

The heteroside glucosylglycerol (GG), which is composed of a sugar and a polyol part, is the main compatible solute of moderate halotolerant cyanobacteria. The molecular biology of cyanobacterial salt acclimation was characterized using the moderate halotolerant model strain Synechocystis sp. PCC 6803 (Hagemann et al., 1999). The biosynthesis of GG resembles the two-step mechanism of trehalose or sucrose synthesis. Thereby glycerol-3-phosphate and ADP-glucose are converted by the activity of the GG-phosphate synthase (ggPS) to GG-phosphate, which is subsequently dephosphorylated by the specific GG-phosphate phosphatase (ggPP or StpA) resulting in GG (Hagemann and Erdmann, 1994). The corresponding genes, ggPS and ggPP (stpA), have been characterized (Hagemann et al., 1997; Marin et al., 1998). Mutations of the GG synthesis genes resulted in salt-sensitive phenotypes (Hagemann et al., 1997; Marin et al., 1998), while feeding with GG or complementation of the GG-deficient mutants by the intact enzyme restored salt resistance in vivo (Mikkat et al., 1997; Hagemann et al., 2008). Moreover, addition of GG protected enzymes against denaturation and preserved membrane integrity in vitro (Borges et al., 2002; Hincha and Hagemann, 2004). Recently, GG-forming enzymes that occur in several heterotrophic bacteria such as Azotobacter vinelandii and Stenotrophomonas rhizophila have been characterized. These enzymes represent fusion proteins exhibiting both synthase and phosphatase activity and were named GgpPS. In contrast to the cyanobacterial GgpS, the GgpPS from heterotrophic bacteria accept UDP-glucose in addition to ADP-glucose as a precursor for GG synthesis (Hagemann et al., 2008). Besides bacteria, the accumulation of GG was only reported for the plants Myrothamnus flabellifolia and Lilium spp. (Kaneda et al., 1984; Bianchi et al., 1993). So far, nothing is known about the GG synthesis pathway in these plants.

Despite numerous reports on the transfer of pathways for the biosynthesis of compatible solutes into higher plants, no example has been reported for the class of heterosides such as GG. In the present study, the aim was to investigate whether the synthesis of GG can be achieved in higher plants by the transfer of microbial genes. It was found that the expression of ggP PS led to a significant accumulation of GG in all plant organs. Subsequently, three GG-accumulating lines showing different amounts of GG were selected and analysed regarding their tolerance to salt stress.

### Materials and methods

#### Plant material, bacteria and growth conditions

Plants of the Arabidopsis thaliana ecotype Columbia (Col-0) were obtained from the European Arabidopsis Stock Centre (NASC; Nottingham, UK). Seeds were incubated at 4 °C for at least 2 d to break dormancy prior to germination. Seedlings and adult plants were grown on soil (Type VM, Einheitserdewerk, Uetersen, Germany) and vermiculite (4:1 v/v) under a 12/12 h (22/18 °C) light/dark regime at 100–150 μmol photons m⁻² s⁻¹ in controlled environment chambers and watered with 0.2% Wuxal® (Aglukon Spezialdünger GmbH, Germany). For in vitro experiments, surface-sterilized seeds were plated on half-strength Murashige and Skoog (MS; Murashige and Skoog, 1962) basal medium (Duchefa Biochemie) solidified with 1% agar (Kobe I, Roth) and supplemented with different NaCl concentrations. Seedlings on MS agar plates were grown in Percival growth chambers under a 16/8 h (22/18 °C) light-dark cycle at 100 μmol photons m⁻² s⁻¹. The vectors pJIT117 (Guernneau et al., 1988), pLH9000 (Hausmann and Töpfer, 1999) and their derivatives were propagated in E. coli strain TG1 and Agrobacterium tumefaciens strain GV3101 grown on LB medium (Roth) at 37 °C and 30 °C, respectively.

#### Generation of transgenic Arabidopsis plants

The ggpPS gene was obtained via PCR from total chromosomal DNA isolated from lysozyme-treated cells of the heterotrophic bacterium Azotobacter vinelandii strain AvOP. DNA was purified using the detergent cetyltrimethylammonium bromide according to Hagemann et al. (2008). After treatment with the corresponding restriction enzymes (Table 1) the PCR product was inserted into the derive pJIT117-TP of the vector pJIT117 (Guernneau et al., 1988). The original pJIT117 vector contained a transit peptide coding sequence of rbcS from Pisum sativum mediating chloroplast import. To ensure cytoplasmic expression of the GgpPS protein, the transit peptide coding sequence was deleted from the original pJIT117 using HindIII and PaeI. Subsequently, the vector molecule was treated with Mung Bean Nuclease (New England Biolabs) and self-ligated generating the modified plasmid pJIT117-TP. The insertion of the ggpPS-PCR-fragment into pJIT117-TP resulted in its directed fusion with the double enhanced CaMV 35S promoter and the CaMV 35S terminator. Afterwards, the entire expression cassette composed of ggpPS and plant regulatory sequences was transferred as a KpnI fragment into the binary plant vector pH9000 (Hausmann and Töpfer, 1999), which contains the neomycin phosphotransferase gene (nptII) for plant selection. The generated vector was transferred into A. tumefaciens strain GV3101 by the freeze–thaw method. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998; Desfeux et al., 2000). Transgenic plants were screened on MS agar medium containing 1% sucrose and 50 μg ml⁻¹ kanamycin (Km). In order to select homozygous plants with a single T-DNA insertion, segregation tests were performed.
Table 1. Oligonucleotides used for amplification of the ggpPS gene from Azotobacter vinelandii and for detection of the transgenes in the genome of Arabidopsis plants

| Name of oligonucleotide | Sequence (5′→3′) | Restriction site | Application |
|-------------------------|------------------|-----------------|-------------|
| AvGGPSJIT-5′            | GTTAACATGCTACTTGCGACCAGA | HpaI | Obtaining the ggpPS from A. vinelandii |
| AvGGPSJIT-3′            | GTTAACGCAGCGCATGTCGCGGA | HpaI | Screening of transgenic Arabidopsis plants |
| AvggpS-5′               | TCTGCGTCAGACTACA | – | Labelling of ggpPS probe |
| AvggpS-3′               | GTAGGCAGGTCTGCACTA | – | Screening of transgenic Arabidopsis plants |
| Kan-A                   | CGGCAAGCTCTTCAGCAGTC | – | Screening of transgenic Arabidopsis plants |
| Kan-S                   | AACAGAAATCGGCTGTCTGAGT | – | Screening of transgenic Arabidopsis plants |
| TDNA-AFfw               | ATGTAGATGTCCGCAGC | – | – |
| TDNA-AFrev              | GTCGGTAGAGGCGCATTT | – | – |

by growing the seedlings of the T2 generation under the selective conditions described above. The ratio of Km-resistant to Km-sensitive seedlings was evaluated. Lines with an expected ratio of 3:1 were further segregated to obtain homozygous plants. Homozygous plants of the third generation (T3) were used for growth experiments. The genotype of the transgenic plants was analysed by PCR using total DNA from transgenic plants and the primer combinations given in Table 1. The characterization of phenotypical differences in comparison to wild-type plants was performed according to Boyes et al. (2001).

Northern blot experiments

Total RNA from leaves of the transgenic Arabidopsis plants was extracted using the NucleoSpin® RNA Plant Kit (Machery-Nagel). For RNA gel blot analyses 1 μg of total RNA was separated in denaturing 1.3% agarose gels containing 17% formaldehyde. RNA was blotted by capillary transfer onto a nylon membrane. A gene-specific DNA probe was generated by PCR using the primers AvggpS-5′ and AvggpS-3′ (Table 1). Isolated DNA fragments were labelled with α-32P-dCTP using the Random Prime Labelling Kit (MBI Fermentas). Non-incorporated radioactivity was removed by gel-filtration using Sephadex G-25 microspin columns (Amersham Pharmacia Biotech). Hybridization was done using Church buffer (7% SDS, 50% formamide, 5× SSC, 2% blocking reagent (Roche), 50 mM K-phosphate buffer, 0.1% laurylsarcosine, and 25 μg ml−1 calf thymus DNA) at 52 °C. The filters were washed twice with 2× SSC/0.1% SDS for 15 min at room temperature and twice with 0.1× SSC/0.1% SDS for 2 h at 68 °C. Radioactive signals were detected by phosphoimaging.

Determination of glucosylglycerol and other soluble sugars

Plant sugar content was analysed by gas chromatography (GC) using a Focus GC (Thermo Scientific) equipped with a TR-5MS column (30 m×0.25 mm×0.25 μm, Thermo Scientific) and an AS 3000 autosampler. Extraction of soluble substances from plant material was accomplished twice with 80% ethanol at 68 °C. Sorbitol was added as an internal standard. The extracts were combined, dried in a vacuum centrifuge, and redissolved in 500 μl water. After centrifugation (15 000 g, 30 min) the supernatant was dried again. The extracted sugars were silylated by incubation with 65 μl pyridine/methoxyamine (20 mg ml−1, 90 min at 30 °C) and 35 μl N,O-bis(trimethylsilyl)-trifluoroacetamide (Sigma) for 60 min at 60 °C. Split-less liquid injection of a 1 μl sample was performed at 300 °C. The initial column temperature was set at 160 °C and increased with a rate of 5 °C min−1 to a final temperature of 280 °C that was held for 10 min at the end of the cycle. Nitrogen was used as carrier gas. Data were analysed with software ChromQuest 4.2.34 (Thermo Electron).

Salt-stress experiments

Salt-stress experiments were conducted according to Verslues et al. (2006). For growth experiments under salt-stress conditions, plants were precultivated for 6 weeks under standard conditions as described above. Salt stress was applied by irrigation with water containing various NaCl concentrations and 0.2% Wuxal® (Aglukon Spezialdünger GmbH). Wild-type and transgenic plants were grown in one pot to ensure the same salt concentration for both plants. The biomass of five individuals per transgenic line was estimated after at least 13 d of salt treatment. The rates of seed germination and seedling survival were analysed by plating surface-sterilized seeds on MS agar medium supplemented with various NaCl amounts and evaluating the proportion of germinated seeds every day. Germination was estimated as positive when the tip of the radicle fully penetrated the seed coat. Survival of seedlings was evaluated by the same procedure. The number of seedlings that successfully developed green cotyledons after 10 d was set as 100%. Seedlings were considered as dead when they were completely bleached. Root growth was analysed on vertical MS agar plates supplemented with 1% sucrose and various amounts of NaCl.

Results

Selection of transgenic Arabidopsis plants and analysis of transgene expression

In order to establish the two-step pathway for GG synthesis in higher plants, the ggpPS gene from the γ-proteobacterium Azotobacter vinelandii under control of the 35S promoter was
transferred into *A. thaliana*. Since this GG-synthesizing enzyme prefers UDP-glucose compared to ADP-glucose as substrate, it was expressed in the cytoplasm as the main site of UDP-glucose synthesis. The T-DNA-construct (Fig. 1A) was inserted into the genome of wild-type plants of *A. thaliana Col-0* via *Agrobacterium*-mediated gene transfer. About 30 independent Km-resistant transgenic Arabidopsis lines were obtained. In order to confirm the integration of the full T-DNA, selected Km-resistant plants were analysed by PCR using primer combinations for *nptII*, the border regions and *ggpPS*, respectively (Table 1). The PCR analysis confirmed the successful gene transfer by the detection of the expected DNA fragment pattern: (i) no fragments in wild-type plants, and (ii) fragments of *nptII*, *ggpPS*, and other T-DNA parts in independent AtAF-lines (Fig. 1B). Moreover, the expression of *ggpPS* was analysed on mRNA level in selected transgenic lines. RNA-blotting experiments revealed no signal for the *ggpPS* mRNA in wild-type plants, whereas in selected transgenic lines the *ggpPS* gene from *A. vinelandii* was clearly expressed (Fig. 1C).

**Verification of glucosylglycerol accumulation in the transgenic plants**

Transgenic plants of selected lines were subsequently analysed by GC regarding GG accumulation. The compatible solute GG was never detected in wild-type plants. By contrast, many transgenic lines showed a significant GG accumulation. The amounts of GG in leaves differed among the lines. Most of the transgenic lines contained rather low amounts of GG (between 1–2 μmol g⁻¹ FM), while only a few transgenic lines showed higher GG content. For further experiments three transgenic lines were selected, which accumulated low (line AtAF-19, 1–2 μmol g⁻¹ FM), moderate (AtAF-30, 2–8 μmol g⁻¹ FM) or high (AtAF-4, about 17 μmol g⁻¹ FM) amounts of GG (Fig. 2A). Thereby GG was detected in all the life phases of Arabidopsis (Fig. 2B). While in line AtAF-19 the amount of GG remained relatively constant, the level fluctuated in line AtAF-4 which displays a steadily increasing GG content throughout the whole life cycle. In addition to rosette leaves, GG was also detected in all other organs and tissues of these plants in a similar quantitative relation (Fig. 2C). The GG content correlated roughly to the observed expression level, since line AtAF-19 showed the lowest *ggpPS* mRNA level and the accumulation of GG was also rather low, while the lines AtAF-30 and AtAF-4 characterized by higher *ggpPS* mRNA contents revealed a higher accumulation of GG (Figs 1C, 2A). In order to analyse the effect of GG accumulation on the Arabidopsis phenotype, lines AtAF-19, AtAF-30, and AtAF-4 characterized by different contents of GG, were selected for further studies.

**Characterization of sugar level and plant phenotype**

None of the selected GG-accumulating, transgenic plants showed obvious morphological differences in comparison to wild-type plants (Fig. 3). However, the detailed analysis revealed that gradual growth retardation occurred with increasing GG accumulation. While plants of the line AtAF-19 characterized by a low GG content were not significantly different from wild-type plants, transgenic lines

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**Fig. 1.** Expression of the *ggpPS* gene from *Azotobacter vinelandii* in the transgenic lines AtAF-19, AtAF-30, and At-AF-4 obtained after transformation of Arabidopsis thaliana Col-0 wild type (WT) plants. (A) Schematic representation of the T-DNA region transferred into *A. thaliana*. (B) Electrophoretically separated DNA fragments obtained by PCR using specific primer combinations for *nptII* (lanes 1, Kan-A/-S), *ggpPS* (lanes 2, AvggpS-5’/-3’), and a T-DNA part at the left border (lanes 3, TDNA-AFfw/-rev) with DNA isolated from plants of the wild type (WT), transgenic lines AtAF-19, AtAF-30, and AtAF-4. (C) Expression of the *ggpPS* gene in plants of the wild type (WT) and transgenic plants of the lines AtAF-19, AtAF-30, and AtAF-4. The *ggpPS* mRNA was detected using a ³²P-labelled probe. The signal intensity of the 18S rRNA was used as loading control. Relative expression of the *ggpPS* gene in the transgenic lines was calculated by dividing the signal intensity for the *ggpPS* mRNA by the signal intensity of the 18S rRNA. Results from one representative experiment are shown. Abbreviations: BL, border left; BR, border right; P35S, cauliflower mosaic virus 35S promoter; dP35S, tandem cauliflower mosaic virus 35S promoter; T35S, cauliflower mosaic virus 35S terminator; *nptII*, neomycin phosphotransferase; *ggpPS*, combined glucosylglycerol-phosphate phosphatase/synthase from *A. vinelandii*; M, marker.
AtAF-30 and AtAF-4 with a moderate or high GG amount were slightly smaller than wild-type plants. Particularly, plants of the line AtAF-4 appeared retarded, indicated by a reduced total leaf number, rosette radius, and fresh weight (Fig. 3B, C). Interestingly, the ability to synthesize GG seems to interfere with the synthesis of other soluble sugars.

The amounts of glucose and fructose were negatively correlated with the accumulated amount of GG, whereas the sucrose content was reduced by about 50% in leaves of all three transgenic lines (Table 2). Thus the highest decrease in the hexose pool was observed in line AtAF-4. These negative correlations between GG and soluble sugars were found in rosette leaves during the vegetative growth phase as well as in several other organs of flowering plants (Table 3). With the exception of the sucrose content in leaves, a continuous reduction in the glucose, fructose, and sucrose contents was found with increased GG contents in all parts of the flowering transgenic plants in comparison to wild-type plants. The diminished amount of soluble sugars and the reduced size of the transgenic plants corresponded well and could be linked to each other.

Protective effects of GG accumulation

A central aim of the study was to analyse the protective effect of GG accumulation for the transgenic plants under salt-stress conditions, as it was reported for its microbial hosts. Therefore, three types of salt-stress experiments were performed. First, the protective effects of GG accumulation on seed germination and the survival of seedlings in the presence of different NaCl concentrations were tested on MS agar medium. These experiments revealed that the efficiency of seed germination was not positively influenced by the accumulation of GG (Fig. 4A). Seedlings of the lines AtAF-19 and AtAF-30, as well as the wild type, showed similar germination efficiency under salt stress, while seeds of line AtAF-4 exhibited clearly reduced germination rates. The analysis of soluble sugars verified the GG accumulation in these seeds in amounts similar to those of vegetative organs (Fig. 4B). Again the highest GG amounts were detected in seeds of line AtAF-4. As found before for other organs, the accumulation of GG led to a lower content of soluble sugars in seeds. Particularly in seeds of line AtAF-4 showing the lowest germination rate under salt stress, the amount of stored sucrose was significantly reduced (Fig. 4B).

A different picture appeared characterizing the salt resistance of juvenile plants after germination. In these experiments, seedlings of the transgenic line AtAF-4 containing the highest amount of GG showed a significant higher survival rate at a toxic salinity of 200 mM NaCl (Fig. 4C). Interestingly, seedlings of the other GG-accumulating lines were also able to resist longer under these conditions. Seedlings of line AtAF-30 having a moderate GG content survived longer than those of line AtAF-19 and significantly longer than wild-type seedlings.

In a second series of experiments, the survival of adult Arabidopsis plants in the vegetative growth phase was analysed after watering with different NaCl concentrations. The appearance of necrotic lesions on rosette leaves was taken as an indication of toxic NaCl effects. Generally, the application of NaCl stress conditions did not result in significantly changed GG contents of the transgenic plants (data not shown). In these experiments, plants of the

![Fig. 2. Accumulation of glucosylglycerol (GG) in plants of the wild type (WT) and the selected transgenic lines AtAF-19, AtAF-30, and AtAF-4 at different developmental stages and in different organs. Plants were grown under standard conditions on soil. Soluble sugars were extracted from plant material and analysed by gas chromatography. (A) Amount of GG in rosette leaves of the WT and the transgenic lines AtAF-19, AtAF-30, and AtAF-4. Plant material was collected before primary inflorescences appeared (growth stage 5.10). These data are means ± SD of five independent experiments each with three pots per line. (B) GG content in different plant organs and tissues of flowering plants (growth stage 6.50). Arabidopsis growth stages were defined according to Boyes et al. (2001). The data in (B) and (C) are means ± SD of triplicates from one representative experiment.](image-url)
The biosynthesis of GG was established in Arabidopsis, a model plant for studying plant biology. GG, which belongs to the class of heterosides, is synthesized from glucose and mannose. Many attempts have been undertaken to investigate the effect of heterologous compatible solutes on plant salt and drought resistance. The transfer of the capability to synthesize the compatible solute GG, which belongs to the class of heterosides, into higher plants by the expression of the corresponding bacterial genes, many attempts have been made to introduce mannitol, glycine betaine, and trehalose biosynthesis, respectively, into higher plants by the expression of the corresponding bacterial genes. Many attempts have been made to introduce mannitol, glycine betaine, and trehalose biosynthesis, respectively, into higher plants by the expression of the corresponding bacterial genes. Many attempts have been made to introduce mannitol, glycine betaine, and trehalose biosynthesis, respectively, into higher plants by the expression of the corresponding bacterial genes.

Table 2. Changes in the content of soluble sugars in leaves of the transgenic lines AtAF-19, AtAF-30, and AtAF-4 in comparison with wild-type plants (growth stage 5.10; Boyes et al., 2001)

The rosette leaves of transgenic plants used for the evaluation of GG accumulation (see Fig. 2A) were also analysed regarding the levels of sucrose, glucose, and fructose. Data are relative values calculated as fold-changes of the corresponding wild-type concentrations (means ±SE of six independent experiments).

| Sugar   | AtAF-19  | AtAF-30  | AtAF-4   |
|---------|----------|----------|----------|
| Sucrose | 0.524±0.109 | 0.798±0.088 | 0.753±0.050 |
| Glucose | 0.514±0.154 | 0.703±0.069 | 0.677±0.042 |
| Fructose| 0.515±0.136 | 0.653±0.108 | 0.525±0.064 |

Table 3. Sugar levels in different organs from flowering plants (growth stage 6.50; Boyes et al., 2001) of GG-accumulating, transgenic lines in comparison to wild-type plants

Data are relative values calculated as fold-changes of the corresponding wild-type concentrations (means ±SD of three independent experiments).

| Sugar   | AtAF-19  | AtAF-30  | AtAF-4   |
|---------|----------|----------|----------|
| Sucrose | Flower: 1.144±0.263 | 1.097±0.331 | 0.723±0.068 |
|         | Siliq: 2.546±1.084 | 0.744±0.182 | 0.269±0.084 |
|         | Stem: 5.352±2.680 | 0.590±0.148 | 0.462±0.189 |
|         | Rosette leaf: 0.276±0.182 | 0.341±0.176 | 0.661±0.311 |
|         | Root: 6.174±1.881 | 1.187±0.668 | 0.418±0.144 |
| Glucose | Flower: 1.149±0.083 | 0.835±0.167 | 0.900±0.026 |
|         | Siliq: 0.889±0.270 | 0.591±0.115 | 0.523±0.037 |
|         | Stem: 0.573±0.141 | 0.421±0.081 | 0.268±0.035 |
|         | Rosette leaf: 0.497±0.343 | 0.281±0.057 | 0.296±0.008 |
|         | Root: 1.121±0.103 | 1.544±0.288 | 1.190±0.338 |
| Fructose| Flower: 1.191±0.182 | 0.713±0.077 | 0.942±0.135 |
|         | Siliq: 1.378±0.274 | 0.866±0.122 | 0.650±0.059 |
|         | Stem: 1.497±0.321 | 0.917±0.097 | 0.437±0.036 |
|         | Rosette leaf: 1.813±1.375 | 0.400±0.100 | 0.326±0.080 |
|         | Root: 0.995±0.260 | 1.344±0.242 | 0.945±0.234 |

Similar results were obtained in the third experimental series to test for alterations in salt tolerance. Roots are known to react most sensitively to increased amounts of toxic salt such as NaCl (Wang et al., 2008). Therefore, root growth was quantified on MS agar medium supplemented with different amounts of NaCl. A significantly improved root growth could be observed for seedlings of line AtAF-19 compared with the root growth of wild-type plants on media supplemented with 50 and 100 mM NaCl, respectively (Fig. 5C, D). By contrast, the root length of seedlings of line AtAF-30 did not differ from wild-type roots, while the seedlings of line AtAF-4 always exhibited shorter roots independently of the applied salt-stress conditions.

**Discussion**

Since the pioneering work of Tarczynski et al. (1993), Lilius et al. (1996), and Holmström et al. (1996) which introduced the mannitol, glycine betaine, and trehalose biosynthesis, respectively, into higher plants by the expression of the corresponding bacterial genes, many attempts have been undertaken to investigate the effect of heterologous compatible solutes on plant salt and drought resistance. The transfer of the capability to synthesize the compatible solute GG, which belongs to the class of heterosides, into the model plant A. thaliana is reported here for the first time. The biosynthesis of GG was established in Arabidopsis after
having received the ggpPS gene from the γ-proteobacterium \textit{A. vinelandii}, which codes for a fusion protein exhibiting the GG-phosphate synthase and the GG-phosphate phosphatase activity on one protein (Hagemann et al., 2008). Using this gene for transformation many transgenic GG-accumulating lines were obtained. The plants contained GG at rather high amounts without severely affecting the plant phenotype. This verified that the heteroside GG is, in principle, compatible with plant metabolism. Moreover, similar attempts were undertaken with genes for the GG synthesis originating from several other bacteria (data not shown). Thereby, a significant GG accumulation was also detected in independent \textit{Arabidopsis} lines transformed with the ggpPS gene of \textit{Stenotrophomonas rhizophila}, which is very similar to the ggpPS gene of \textit{A. vinelandii} (Hagemann et al., 2008). Furthermore, the two genes for GG synthesis from the cyanobacterium \textit{Synechocystis} sp. strain PCC 6803 (ggpS and ggpP), for which the synthesis of the compatible solute GG was originally characterized at the molecular level (Hagemann et al., 1999), were also transferred into \textit{Arabidopsis}. However, despite the relatively high expression of the corresponding genes, no GG accumulation could be detected in these plants. One possible explanation is the strict dependency of the cyanobacterial GgpS activity on ADP-glucose, a substrate that occurs only to a limited amount in plants. By contrast, the UDP-glucose pool is much higher in leaves (Baroja-Fernandez et al., 2004). The combined GgpPS of \textit{A. vinelandii} can accept UDP-glucose as well as ADP-glucose, which could be the reason for the successful GG synthesis derived by the expression of this particular gene.

Beside the observation that GG seemed to be, in principle, compatible with plant metabolism, slight growth retardations were observed correlating with increasing levels of GG accumulation. This was particularly true for plants of the transgenic line AtAF-4, in which very high amounts of up to 30 μmol GG g\textsuperscript{-1} FM (GG at about 7% (w/w) of the leaf dry mass; see Fig. 2C) were reached in the final developmental stage. Comparable results were reported for transgenic, sorbitol-accumulating tobacco plants, which showed severe changes in the plant phenotype with increasing sorbitol accumulation (Sheveleva et al., 1998). Plants that accumulated low sorbitol amounts in leaves (less than 2–3 μmol g\textsuperscript{-1} FM) developed like wild-type plants, but
necrotic lesions, a reduced root growth, and infertility were found more frequently for plants that accumulated sorbitol in higher amounts (>5 μmol g⁻¹ FM). Transgenic plants with sorbitol levels exceeding 17.5 μmol g⁻¹ FM were completely infertile, whereas amounts higher than 30 μmol g⁻¹ FM resulted in plant death. However, necrotic lesions and infertility were never monitored in GG-accumulating Arabidopsis plants (Fig. 3A), although the GG concentrations in line AtAF-4 reached levels similar to those reported for the infertile sorbitol-accumulating plants. Severe effects on the plant phenotype were never observed in the early attempts to establish the synthesis of the compatible solute trehalose in transgenic tobacco. Later on, it became clear that wild-type plants also perform an active trehalose metabolism, which is of high importance for the regulation of sugar metabolism and growth (reviewed in Goddijn and van Dun, 1999). In particular, the intermediate trehalose-6-phosphate plays an important regulatory role (Schluempmann et al., 2003).

The observed growth retardation in transgenic plants accumulating high GG amounts might have several reasons. It is known that GG and trehalose biosynthesis depend on the precursor UDP-glucose. Therefore, it could be possible that the GG accumulation in transgenic lines negatively interferes with the trehalose biosynthesis. Furthermore, the decreased availability of precursors may also affect other carbohydrate levels such as those observed for sucrose and hexoses in GG-accumulating plants, which could have
versatile effects on plant growth. As shown by Jang et al. (1997), altered levels of glucose affect hexose kinases and act as signals for glucose-mediated gene regulation. The importances of a sufficient precursor amount and the multiple interactions by the introduction of a biosynthetic pathway for a heterologous compatible solute have been intensively investigated for glycine betaine-accumulating transgenic plants by Nuccio et al. (1998).

After establishing the synthesis of the compatible solute GG in the model plant Arabidopsis it was interesting to evaluate if the GG accumulation improved the salt-stress tolerance as it does for microbial cells accumulating GG (Hagemann et al., 1999, 2008). For this purpose three transgenic lines were selected showing different levels of GG accumulation. Initially, it was assumed that a positive correlation between the amount of GG and salt tolerance might exist. However, the observed growth retardation in plants accumulating high GG amounts under control conditions made this assumption questionable. In order to evaluate changes in the salt resistance of GG-accumulating plants, different test systems were applied as suggested by Verslues et al. (2006). Thereby, the germination of seeds was analysed in the presence of various salt concentrations. In contrast to the positive effects shown for mannitol-accumulating (Thomas et al., 1995) as well as for glycine betaine-accumulating (Hayashi et al., 1998; Waditee et al., 2005) transgenic Arabidopsis plants, the accumulation of GG had no protective effect on the germination of seeds under salt-stress conditions. Seeds of the line AtAF-4 (high GG accumulation) even showed reduced germination under salt conditions, which could result from the decreased internal sucrose level in seeds of these plants. Sucrose is one of the most abundant sugars in mature Arabidopsis seeds and is thought to represent a significant and rapidly available energy source during germination (Baud et al., 2002). Probably, non-sugar compatible solutes such as glycine betaine do not interfere to such a high extent with the accumulation of sucrose in seeds as GG. Generally, it has been critically discussed whether the evaluation of seed germination can be used as an indication for salt-stress tolerance, since in many cases a high rate of germination under salt stress was found to be not well correlated with salinity tolerance at later developmental stages (Verslues et al., 2006). In contrast to the results of seed germination, it was found that the survival of seedlings under toxic salt conditions was positively affected in correlation with an increasing GG-amount. Such a beneficial effect under high salt conditions was expected, since it was confirmed for living cells in vivo (Hagemann et al., 1999) as well as for enzymes (Borges et al., 2002) and membranes (Hincha and Hagemann, 2004) in vitro. Consequently, increasing protection was assumed with higher GG concentrations. However, despite the prolonged survival of GG-accumulating seedlings under these conditions they did not show a substantial increased salt tolerance, since no seedling of the lines tested developed into a mature plant.

Test systems examining whole plants or roots are discussed as the most valuable systems to assess changes in the salt tolerance of different plants (Verslues et al., 2006). Such experiments indicated clearly that low or moderate GG amounts improved the growth of whole plants on salt-watered soil or roots on NaCl-supplemented MS-medium. In both test systems an improved salt tolerance was observed for plants of the transgenic line AtAF-19, which accumulated GG-amounts of about 1–2 µmol g⁻¹ FM. By contrast, higher GG contents had no or even a negative effect on root growth. This is in agreement with the observed growth retardation for these plants under control conditions, which indicates that GG synthesis interferes negatively with regular plant development while this effect is more pronounced under stress conditions. The age-dependent differences of salt-dependent growth between the three transgenic lines might reflect the different degrees of interference of GG-synthesis with sugar metabolism at these developmental stages and/or the age-dependent appearance of different salt-sensitive targets directly protected by the heterologous compatible solute GG.

Summarizing, results presented here show that the synthesis and accumulation of the heteroside GG in higher plants is possible. Moreover, a certain accumulation level of the compatible solutes GG had beneficial effects on plant salt stress tolerance as shown by the enhanced survival rates of seedlings, and the improved growth of roots and whole plants on soil. However, in these test systems the positive effects were only observed for plants accumulating rather low GG amounts of about 2 µmol g⁻¹. FM. It should be noted that this GG content is similar to the level reported for other heterologous compatible solutes in transgenic plants, which were obtained after the transfer of microbial genes and showed an improved stress tolerance (Hayashi et al., 1997; Pilon-Smits et al., 1998; Waditee et al., 2005; Chen and Murata, 2008). Obviously, a newly expressed compatible solute only exhibits a protective effect up to a certain extent of accumulation, which does not dramatically limit or alter the normal plant metabolism. The protective effect of GG at a rather low accumulation level makes an osmotic effect for the increased salt tolerance unlikely. Preliminary experiments indeed showed that the content of water and inorganic ions was not significantly altered in the salt-treated, GG-accumulating lines in comparison to wild-type plants (data not shown). Therefore, it is assumed that the low GG accumulation provides a protective effect in vivo on sensitive enzymes or membrane complexes similar to the results observed in vitro (Borges et al., 2002; Hincha and Hagemann, 2004).

Moreover, salt acclimation in plants is a multifactorial process. Beside the accumulation of compatible solutes, ion export and/or compartmentation play a crucial role in plant salt tolerance. It has been reported that the expression of ion transporter genes also improved the salt tolerance of Arabidopsis (Apse et al., 1999). Therefore, the joint improvement of compatible solute accumulation and an increased ability of ion compartmentation in combination with a salt stress-regulated expression of the corresponding genes may offer opportunities to increase plant salt tolerance further by genetic engineering.
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