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Genetic engineering of the biosynthesis of glycinebetaine leads to alleviate salt-induced potassium efflux and enhances salt tolerance in tomato plants

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ABSTRACT

Tomato (*Solanum lycopersicum* cv. 'Moneymaker') was transformed with the choline oxidase gene *codA* from *Arthrobacter globiformis*, which was modified to allow for targeting to both chloroplasts and the cytosol. Glycine betaine (GB) was accumulated in transformed plants, while no detectable GB was found in wild-type (WT) plants. Compared to WT plants, transgenic lines showed significantly higher photosynthetic rates (Pn) and antioxidant enzyme activities and lower reactive oxygen species (ROS) accumulation in the leaves when exposed to salt stress. Furthermore, compared with WT plants, K^+ efflux decreased and Na^+ efflux increased in roots of transgenic plants under salt stress; resulted in lower Na^+/K^+ ratios in transgenic lines. The exogenous application of GB also significantly reduced NaCl-induced K^+ efflux and increased Na^+ efflux in WT plants. A qRT-PCR assay indicated that GB enhanced NaCl-induced expression of genes encoding the K^+ transporter, Na^+/H^+ antiporter, and H^+-ATPase. These results suggest that the enhanced salt tolerance conferred by *codA* in transgenic tomato plants might be due to the regulation of ion channel and transporters by GB, which would allow high potassium levels and low sodium levels to be maintained in transgenic plants under salt stress condition.

1. Introduction

Among various abiotic stresses, salinity is one of the major environmental constraints to crop productivity, especially in the coastal zone [1]. Salt stress in plant cells is primarily caused by a combination of osmotic and ionic stress resulting from high salt concentrations in the soil [2,3]. Plants have developed mechanisms that allow them to withstand abiotic stresses. One of these mechanisms involves the accumulation of compatible solutes [4,5], which are low molecular-weight metabolites that are very soluble in water and are non-toxic at high concentrations. In respond to saline environment, some plants can significantly elevate the level of compatible solutes in the cytosol; allow them to ameliorate the detrimental effects of salinity [1,6].

Glycine betaine (GB), often referred to as compatible solute, has been found in marine invertebrates, bacteria, plants and mammals [7,8]. Exogenous application of GB or introduction of genes related to GB biosynthetic pathway to non-GB synthesizing plants, such as tomato [9], have potential in enhancing the tolerance against various environmental stresses [10,11,8]. Potassium, one of the most abundant cations in higher plants, plays prominent roles in balancing membrane potential and turgor, activating enzymes, regulating osmotic pressure, stoma movement and tropisms [12]. Potassium is not just involved as an activator or cofactor in many biochemical processes such as photosynthesis, but also as an important signaling agent mediating a wide range of plant adaptive responses to environment [13–15]. The K^+ homeostasis plays a crucial role in salt tolerance mechanisms in salinized plants [16]. Transformation of tomato with a bacterial *codA* gene, from *Arthrobacter globiformis*, for choline oxidase also enhances tolerance to salt and water stresses [17]. Sulpice et al. [18] reported that glycine betaine neither affect the distribution and levels of Na^+ and K^+ ions in transgenic *Arabidopsis* with *codA* gene in normal or salt shock condition. Application of GB *in vitro* could alleviate the efflux of K^+ induced by ROS in the root of *Arabidopsis* [19]. However, whether the accumulation of GB in vivo can maintain Na^+-K^+ homeostasis under salt stress has not been fully clarified. In the present study, we used

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GB-accumulating transgenic plants and the WT plants with exogenous application of GB to further investigate the mechanism of GB enhanced salt stress tolerance in tomato plants. We also explored whether GB-enhanced tolerance to salt stress might involve in Na^+ and K^+ homeostasis via the regulation of ion channels and transporters.

2. Materials and methods

2.1. Plant materials and salt stress treatments

Tomato (*Solanum lycopersicum* cv. 'Moneymaker') plants were transformed with a gene (*codA*) for cholineoxidase, which enabled the seedlings to accumulate glycine betaine (GB) in both chloroplasts and the cytosol [20]. Wild type (WT) tomato and transgenic lines (L1, L2 and L3) were grown from seeds in a greenhouse at 25/20 °C (day/night) with a photosynthetic photon flux density of 400 μmol m^{-2} s^{-1}, a relative humidity of 65–70% and a photoperiod of 16/8 h light/dark [21]. The plants were grown in plastic pod (10 × 10 × 15 cm, length × width × height) with sand, and were irrigated with Hoagland nutrient solution every day. After 6 weeks, plants were first treated with 50 mM NaCl in Hoagland nutrient solution for 1 day, and then the NaCl concentration increased by 50 mM each day up to 200 mM NaCl, plants were treated with 200 mM NaCl for 7 days. Functional leaves at the same position on all seedlings were used in the experiments.

2.2. Growth performance of WT and transgenic seedlings under salt stress

After salt stress for 7 days, plant height, fresh and dry weight of plants was quantified. This experiment was repeated three times, with 15 replicates for each line.

2.3. Extraction and quantification of GB

Six-week-old tomato plants treated with or without 200 mM NaCl for another 7 days were used in this experiment. GB was extracted and quantified as described by Rhodes et al. [22], with the following modifications. Leaf samples (T3 generation) were ground in a mixture of methanol: chloroform: water at 60 °C for 30 min. The aqueous phase was fractionated by ion-exchange chromatography. The GB fraction was eluted with 4 M NH_4OH. Then, the betaine in the preliminarily purified extract was analyzed by high-performance liquid chromatography (HPLC) on a liquid chromatograph (SCL-10AVP; Shimadzu, Kyoto, Japan) equipped with a Hypersil 10 SCX column. Millennium Chromatography Manager System Control software was used for the analysis.

2.4. Measurement of the net photosynthetic rate (Pn)

Net photosynthetic rate (Pn) was measured with a portable photosynthetic system (CIRAS-2, PP Systems, USA) under the following conditions: ambient CO_2 (360 μmol mol^{-1}), a PPFD of 800 μmol m^{-2} s^{-1}, a leaf temperature of 25 ± 1 °C and a relative air humidity of 60–70%. Functional leaves at the same position were used in the experiments. Five technical replicates were used for each measurement.

2.5. Measurement of chlorophyll fluorescence

Chlorophyll fluorescence was measured at room temperature with a portable fluorometer (FMS-2, Hansatech, UK) according to the protocol described by Kooten and Snel [23]. Leaves were then continuously illuminated with a white actinic light at an irradiance of 400 μmol m^{-2} s^{-1}. The steady-state value of fluorescence (Fs) was reached within approximately 6 min. After collecting measurements, a second saturating pulse at 8000 μmol m^{-2} s^{-1} was applied; the maximal fluorescence level in a light-adapted state (Fm') was then determined. The actual photochemical efficiency of PSII in a light-adapted state (Φ_{PSII}) was calculated as follows: Φ_{PSII} = (Fm'- Fs)/Fm'. Performance index (PIabs) was measured using a plant efficiency analyzer (PEA; Hansatech, UK) according to Strasser et al. [24]. Five technical replicates were used for each biological sample.

2.6. Measurement of K^+, Na^+ and Ca^{2+} in the leaves of WT and transgenic lines

Approximately 0.5 g of leaf tissue was sampled from plants either exposed to salt stress for 7 days or grown under normal conditions. Tissues were dried in an oven at 105 °C for 10 min, and then dried at 80 °C to a constant weight. Dry samples were finely ground, and the ground tissue was soaked for 12 h in digesting tubes with 5 mL H_2SO_4, 2 mL H_2O_2 mixture and distilled water to 250 mL. The amounts of K^+, Na^+ and Ca^{2+} were determined using an atomic absorption spectrophotometer (SP9-400, Pye, Unicam Ltd., Cambridge, England). The experiments were performed using three biological replicates.

2.7. Measurements of net K^+, Na^+ and H^+ flux

Net K^+, Na^+ and H^+ flux was measured by the non-invasive microelectrode ion flux measuring technique (NMT) (BIO-IM, Younger USA LLC, Amherst, MA, USA) [25,26]. WT and transgenic tomato seeds were germinated in Petri dishes on a sterile filter paper moistened with distilled water for a week, and then root tips were used for flux measurement. Net fluxes in the elongation zone of tomato roots were measured for 10 min. In our experiments, we used 5 mM lanthanum chloride ($LaCl_3$) and 5 mM EGTA to block the influx of extracellular Ca^{2+} via the PM Ca^{2+}-permeable channels [27,28]. Root tips were treated with several reagents for 1 h: orthovanadate (a specific inhibitor of PM H^+-ATPase) [29] and amiloride (an inhibitor of Na^+/H^+ exchange across the PM) [30,31]. All chemicals were purchased from Sigma. Each treatment had three replicates with 10 seeds per plate.

For K^+ ion flux measurements, roots were immobilized on liquid agar in a holding chamber placed in a bathing medium (0.1 mM KCl, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.5mMNaCl, 0.3mMMES, 0.2 mM Na_2SO_4, pH6.0). WT plants roots were incubated in solution with different concentration of GB for 2 h. Experiments commenced 15–30 min after immobilization; at this point, steady-state conditions were reached. For the controls, net ion fluxes were measured prior to treatment for 5 min; 80 mM NaCl was then added to the chamber. And then, net K^+ fluxes were measured for 30 min.

Short-term salt stress experiments involved the transfer of whole plants onto bathing medium containing 80 mM NaCl; this was followed by incubation and subsequent growth over 24 h. Control and NaCl-treated WT and transgenic tomato roots were incubated in inhibitors for 1 h before measurement: 100 μM amiloride, 500 μM sodium orthovanadate, 5 mM $LaCl_3$ or 5 mM EGTA. Afterwards, the ionic flux was measured at 400 μm at the root tips for approximately 10 min.

For Na^+ and H^+ ion flux measurement, WT and transgenic line L1 are used in this experiment. All lines were also pre-treated with 5 mM EGTA for 1 h. Roots treated with 80 mM NaCl or 80 mM NaCl with 5 mM GB for 24 h were incubated in the measuring solution (described above) to equilibrate for 10 min, the ion flux measurements were recorded for 10 min. Data and image acquisition and preliminary processing were performed using IMFLUX software, which also used for electrode positioner control and the stepper