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ORIGINAL ARTICLE

Accumulation of glycine betaine in transplastomic potato plants expressing choline oxidase confers improved drought tolerance

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Abstract

Main conclusion **Plastid genome engineering is an effective method to generate drought-resistant potato plants accumulating glycine betaine in plastids.**

Glycine betaine (GB) plays an important role under abiotic stress, and its accumulation in chloroplasts is more effective on stress tolerance than that in cytosol of transgenic plants. Here, we report that the *codA* gene from *Arthrobacter globiformis*, which encoded choline oxidase to catalyze the conversion of choline to GB, was successfully introduced into potato (*Solanum tuberosum*) plastid genome by plastid genetic engineering. Two independent plastid-transformed lines were isolated and confirmed as homoplasmic via Southern-blot analysis, in which the mRNA level of *codA* was much higher in leaves than in tubers. GB accumulated in similar levels in both leaves and tubers of *codA*-transplastomic potato plants (referred to as PC plants). The GB content was moderately increased in PC plants, and compartmentation of GB in plastids conferred considerably higher tolerance to drought stress compared to wild-type (WT) plants. Higher levels of relative water content and chlorophyll content under drought stress were detected in the leaves of PC plants compared to WT plants. Moreover, PC plants presented a significantly higher photosynthetic performance as well as antioxidant enzyme activities during drought stress. These results suggested that biosynthesis of GB by chloroplast engineering was an effective method to increase drought tolerance.

Keywords Compartmentation · Drought stress · Genetic improvement · Glycine betaine · Plastid transformation · Potato

Abbreviations

GB	Glycine betaine
COD	Choline oxidase
RWC	Relative water contents
MDA	Malondialdehyde
qP	Photochemical quenching
NPQ	Non-photochemical quenching
ETR II	Electron transport rate of PSII
ROS	Reactive oxygen species
SOD	Superoxide dismutase
POD	Peroxidase
CAT	Catalase
APX	Ascorbate peroxidase

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Introduction

Potato (*Solanum tuberosum* L.) as one of the staple food crops with annual production approaching up to 300 million tons (Camire et al. 2009) ranks the fourth in production after wheat, maize, and rice (Ünlü et al. 2006) and plays a vital role in ensuring food supply all over the world (Gerbens-Leenes et al. 2009). However, due to its sparse and shallow root system, most potato varieties are vulnerable

to a series of abiotic stresses, including severe temperature changes, drought and high salinity (Hijmans 2003; Ahmad et al. 2008), thus resulting in a reduction in tuber yield and quality (Jefferies 2010). Even short periods of drought stress can result in serious damage and cause a severe reduction in tuber production (Cho et al. 2016). Water deficit can inhibit and even completely stop many physiological processes such as photosynthesis, transpiration and enzymatic activities in potato (van Loon 1981). Therefore, there is an urgent need to develop drought-resistant potato variety to meet the challenge by global warming and desertification (Cho et al. 2016).

Glycine betaine (GB) is a fully *N*-methyl-substituted derivative of glycine, widely distributed in bacteria, marine invertebrates, higher plants and animals (Rhodes and Hanson 1993; Chen and Murata 2002). In both higher plants and *Escherichia coli*, GB is synthesized by two-step oxidation reaction from choline via betaine aldehyde, a toxic intermediate, while one-step synthesis pathway in the soil bacterium *Arthrobacter globiformis* is completed by a single enzyme–choline oxidase (COD), which can catalyze the direct conversion from choline to GB (Ikuta et al. 1977). As a low molecular weight metabolite, GB is highly soluble in water and non-toxic at high concentration (Wei et al. 2017) and plays a crucial role in protection of plant against various abiotic stresses.

GB has shown multiple biological functions (Rhodes and Hanson 1993; Chen and Murata 2008) including osmotic adjustment to maintain cellular water balance (Hasegawa et al. 2000; Mansour 2000; Al Hassan et al. 2016), stabilizing the secondary structure of enzymes and proteins (Demiral and Türkan 2004) and maintaining a highly ordered state of membranes (Papageorgiou and Murata 1995). In addition, it can efficiently protect the photosynthetic machinery, such as Rubisco and the oxygen-evolving photosystem II (PSII) complex under stress conditions (Murata et al. 2007).

Introduction of GB biosynthesis pathway genes into GB non-accumulating plants, such as potato, tomato, Arabidopsis and rice, can enhance their tolerance to various stresses (Hayashi et al. 1998; Sakamoto and Murata 2000; Chen and Murata 2002, 2008). For example, introduction of the *codA* gene from *A. globiformis* enhanced drought and salt tolerance in potato (Ahmad et al. 2008, 2010; Cheng et al. 2013), rice (Sakamoto and Murata 1998; Mohanty et al. 2002; Kathuria et al. 2009) and poplar (Ke et al. 2016), improved chilling (Park et al. 2004) and salt tolerance (Goel et al. 2011; Wei et al. 2017) in tomato, increased salt and cold tolerance (Hayashi et al. 1997), freezing (Sakamoto and Murata 2000) and photodamage tolerance (Alia et al. 1999) in Arabidopsis.

The plastid is considered to originate from a formerly free-living cyanobacterium and has a prokaryotic-like genetic system. Plastid transformation holds couples of unique advantages compared to conventional nuclear transformation (Staub and Maliga 1995; Scott and Wilkinson 1999; Bock 2015), e.g., remarkable high expression levels due to high polyploidy plastid genome, absence of epigenetic transgene silencing, precision of the transgene into plastid genome and the increased biosafety by maternal inheritance (Bock 2015). It has been demonstrated that GB was synthesized in plastids and GB accumulation in plastids could be more effective than in the cytosol for protecting transgenic plants against abiotic stresses (Sakamoto and Murata 1998). There is a positive correlation between chloroplastic GB content and stress tolerance capacity (Park et al. 2007; Zhang et al. 2008). These findings prompted us to investigate whether directly engineering the GB synthesis pathway in plastids would efficiently increase the biosynthesis of GB in plastids and confer improved tolerance of plants against abiotic stresses. In this study, we obtained *codA*-transplastomic potato plants (referred to PC plants) by plastid transformation. Our results showed that GB stably accumulated in leaves as well as in tubers of PC plants. Compartmentation of GB in plastids of PC plants conferred considerably higher tolerance to drought stress compared to wild-type (WT) plants in terms of growth, photosynthetic performance and antioxidant enzyme activities. To the best of our knowledge, this is the first study showing enhancement of potato drought tolerance by plastid transformation.

Materials and methods

Plant material and growth conditions

Potato (*Solanum tuberosum* cv. Désirée) plants were grown under aseptic conditions on agar-solidified MS medium containing 3% (w/v) sucrose (Murashige and Skoog 1962). Regenerated homoplasmic shoots were rooted and propagated on the MS medium. Rooted plantlets were transferred and grown in greenhouse in 160 µmol m^{-2} s^{-1} constant light under a 16-h-light/8-h-dark photoperiod, at 22 °C/20 °C and 50% humidity.

Vector construction

The *A. globiformis codA* gene (Accession: AY589052) was codon optimized based on the codon usage preference of plastid gene expression and chemically synthesized (Online Resource S1) (GeneCreate, Wuhan, China). The *codA* gene was amplified with primer pairs codA-F (5′-CATGCCATGGATGGGGGAAGCGG TGATCG-3′) and codA-R (5′-CTAGTCTAGATTATTTGCCGACTACCTTGGTGATCT-3′), by introducing NcoI and XbaI restriction sites (underlined) at the 5′ end and 3′ end, respectively. The PCR product was digested with NcoI/XbaI (Takara), and cloned

into previously reported pYY12 plasmid (Wu et al. 2017) to replace the *gfp* reporter gene, generating plasmid pYY47 for potato transformation.

Plastid transformation in potato

Potato plastid transformation and regeneration of the transplastomic potato plants were carried out as previously described (Zhang et al. 2015). Plasmid DNA for plastid transformation was prepared using the Nucleobond Xtra Plasmid Midi Kit (Macherey-Nagel, Düren, Germany). Young leaves of potato plants grown under aseptic conditions were bombarded with DNA-coated 0.6-µm gold particles using a PDS-1000/He Biolistic Particle Delivery System (BioRad, Hercules, CA, USA). Homoplasmy of putative transplastomic events was confirmed by Southern blotting.

Isolation of nucleic acids and gel blot analyses

Total leaf cellular DNA was isolated from leaves of wild-type and transplastomic plants by a cetyltrimethylammonium bromide-based protocol (Murray and Thompson 1980). Total RNA was isolated using the Trizol Reagent (Invitrogen, Waltham, MA, USA) following the manufacturer protocol. For Southern-blot analysis, 5 µg total cellular DNA was digested with AgeI (NEB, Ipswich, MA, USA) for 3 h and MluI (NEB) for 12 h, separated by agarose gel electrophoresis on 1% agarose gels and transferred onto Hybond nylon membranes (GE Healthcare). A fragment covering a portion of *psbZ* gene amplified from potato plastid genome DNA using primer pairs St-S-psbZ-F (5′-GTGCGAATC CACCGGTCGATCTA-3′) and St-S-psbZ-R (5′-AAGTAG CAATTAATGCAAAAACA-3′) was used as a hybridization probe to verify plastid transformation and assess the homoplasmic status of transplastomic lines. For RNA gel blot analysis, RNA samples were denatured and separated in formaldehyde-containing 1% agarose gels and blotted onto Hybond nylon membranes (GE Healthcare). A 496 bp PCR product generated by amplification of a portion of *codA* gene using primers coda-nor-probe-F (5′-AGTTGAAGCTGGT CTGATGATCG-3′) and coda-nor-probe-R (5′-ATCTAG TTCCATCAGCTCGTCGA TTAAT-3′) served as probe to determine *codA* mRNA transcripts. Probes were labeled according to the manufacturer's protocol using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Basel, Switzerland). Hybridizations were performed at 42 °C for Southern- and Northern-blot analyses.

Quantification of glycine betaine

The mature leaves and tubers of WT and PC plants were sampled for GB analysis. Frozen dried plant materials were weighted and suspended in 1000 µL 50%/50% methanol/water solution. Four seconds on/off cycling program was used (eight cycles) for its in-solution ultrasonic extraction process (VX-130; Sonics, Newtown, CT, USA). Samples were centrifuged at 15,000*g* for 8 min, and the supernatants were then lyophilized and re-dissolved in 450 µL water. A 450 µL aqueous layer was transferred to a clean 2-mL centrifuge tube. Fifty microliters of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) standard solution (Anachro, Toronto, Canada) was added. Samples were mixed well before transferred to 5 mm NMR tube (Norell, Andover, MA, USA). Spectra were collected using a Bruker AV III 600 MHz spectrometer. The first increment of a 2D-^{1}H, ^{1}H-NOESY pulse sequence was utilized for the acquisition of ^{1}H-NMR data and for suppressing the solvent signal. Experiments used a 100 ms mixing time along with 990 ms pre-sa 128 scans over a period of 15 min.

Whole plant drought stress treatment

Plantlets were transplanted to nursery pots containing only vermiculite and grown in a greenhouse with 160 µmol m^{-2} s^{-1} constant light under a 16-h-light/8-h-dark photoperiod, at 22 °C/20 °C. The relative humidity was maintained at ~ 60%, and the plants were irrigated using 20-20-20 N-P-K Scotts Peters Professional water-soluble fertilizer through trays placed underneath the pots for 4 weeks. Subsequently, a total of 24 plants (12 for WT plants and 12 for PC plants) of similar height and health conditions were selected and divided into two groups. One group consisting of six WT plants and six PC plants was set as control, in which the plants were grown under normal growth conditions with regular watering. Another group was subjected to water deficit by withholding the water supply. Photographs were taken before stress, 9, 13 days after stress, and 2 days after re-watering, respectively. All experiments were repeated at least three times, and results from one representative experiment were shown.

Relative water contents

The degree of drought stress was assessed by the relative water contents (RWC) of leaves from WT and PC plants after 9 days of water withholding. The third to fifth fully developed leaves (counting from the top) of potato plants were collected at 9:00 am of each indicated day and used for RWC measurements (Kanamoto et al. 2006). The following formula was utilized to calculate the relative water content: RWC (%) = [(fresh weight − dry weight)/(turgid weight − dry weight)] × 100, in which fresh weight means immediate weight of freshly collected leaves, turgid weight of leaves was measured after incubation in water at 20 °C for 6 h and dry weight of leaves was measured after drying at 80 °C for 48 h.

Analysis of antioxidant enzyme activities as well as proline and malondialdehyde contents

Superoxide dismutase (SOD) activity was measured as the method described by Giannopolitis and Ries (1977), peroxidase (POD) activity as described by Maehly and Chance (1954). Catalase (CAT) and ascorbate peroxidase (APX) activities were analyzed according to the method previously described by Bartoli et al. (1999) and Pinhero et al. (1997). The malondialdehyde (MDA) content was determined as described by Heath and Packer (1968) and the proline content according to the method described by Bates et al. (1973).

Photosynthetic gas exchange measurements

The net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr) and substomatal CO_2 concentrations (Ci) were measured using a CIRAS-3 portable photosynthesis system (PP Systems, Amesbury, MA, USA) at 25 °C, 1000 µmol m^{-2} s^{-1} PPFD, 70% relative humidity and ambient CO_2 (360 ± 20 µL L^{-1}) between 9:00 a.m. and 11:00 a.m. The fourth and fifth fully developed leaves of each potato were selected, and eight replicates were determined each time.

Chlorophyll fluorescence parameters measurements

The assay was performed using a Dual-PAM100 chlorophyll fluorescence instrument (Walz, Effeltrich, Germany). The fourth and fifth fully developed leaves of the top leaves of each potato were selected, and the leaves were subjected to dark adaptation for 20 min before the assay. After dark adaptation, the initial fluorescence yield (Fo) was measured and then the maximum fluorescence yield (Fm) with application of a saturation pulse for 0.2-1.5 s. Subsequently, the actinic light was turned on (Fo') to set up the light intensity to 300 µmol m^{-2} s^{-1} till the fluorescence signal reached steady state (Fs, about 45 min), and the maximum fluorescence yield under light (Fm') adaptation was finally determined when exposed to the saturated pulsed light. Chlorophyll parameters were calculated according to the following formulae:

$$\text{qP (photochemical quenching)} = (Fm' - Fs)/(Fm' - Fo');$$

$$\text{NPQ (non-photochemical quenching)} = (Fm - Fm')/Fm' = Fm/Fm' - 1$$

$$\text{ETR (electron transport rate) (II)} = (Fm' - Fo)/Fm' \times PAR \times 0.5 \times 0.84,$$

where 0.5 is the parameter obtained by assuming that the absorbed light is equally divided by two optical systems and 0.84 is the absorption coefficient (Schreiber et al. 1995).

Measurements of total chlorophyll content in potato leaves

Two leaf disks (diameter 0.5 cm) within the region of infiltration were excised, and total chlorophyll was extracted with 10 mL of 80% acetone in the dark for 72 h at room temperature. The extracts were analyzed using a UV–visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) according to the method described by Porra (2002).

Results

Vector construction for potato plastid transformation

The potato chloroplast transformation vector pYY47 was constructed to mediate the integration of the expression cassettes to the *trnf*M-*trn*G region of the plastid genome via homologous recombination. The pYY47 consists of the left (containing 3′ end of *psb*B, *trnf*M and *rps*14) and right (containing *psb*Z and *trn*G) homologous recombination regions and two expression cassettes that harbor spectinomycin resistance gene *aadA* and choline oxidase encoding gene *codA*, respectively. The *codA* gene was driven by tobacco plastid 16S ribosomal RNA operon promoter (P*rrn*) with fusion of 5′ untranslated region from *gene 10* of bacteriophage T7, a strong translation signal in nongreen plastid (Fig. 1a).

Generation of transplastomic potato plants

The construct pYY47 was introduced into potato plastid genome by biolistic transformation followed by selection of spectinomycin-resistant shoots as described previously (Zhang et al. 2015). Five transplastomic lines were obtained and Southern-blot analysis was performed to verify the homoplasmic status. Among them, two representative lines showed the presence of the 2.51 kb signal band while the absence of a hybridization signal for the WT genome (2.13 kb) (Fig. 1b), indicating homoplasmy of these two transplastomic lines. Due to site-directed transgene integration by homologous recombination and the absence of position effects and epigenetic gene silencing mechanisms from plastids, all the transplastomic lines generated with the same construct were identical and showed no variation in expression of transgene (Bock 2015; Zhang et al. 2015). To confirm this, we performed Northern-blot analysis, and the results revealed that two independent PC lines generated with the same construct showed identical transgene

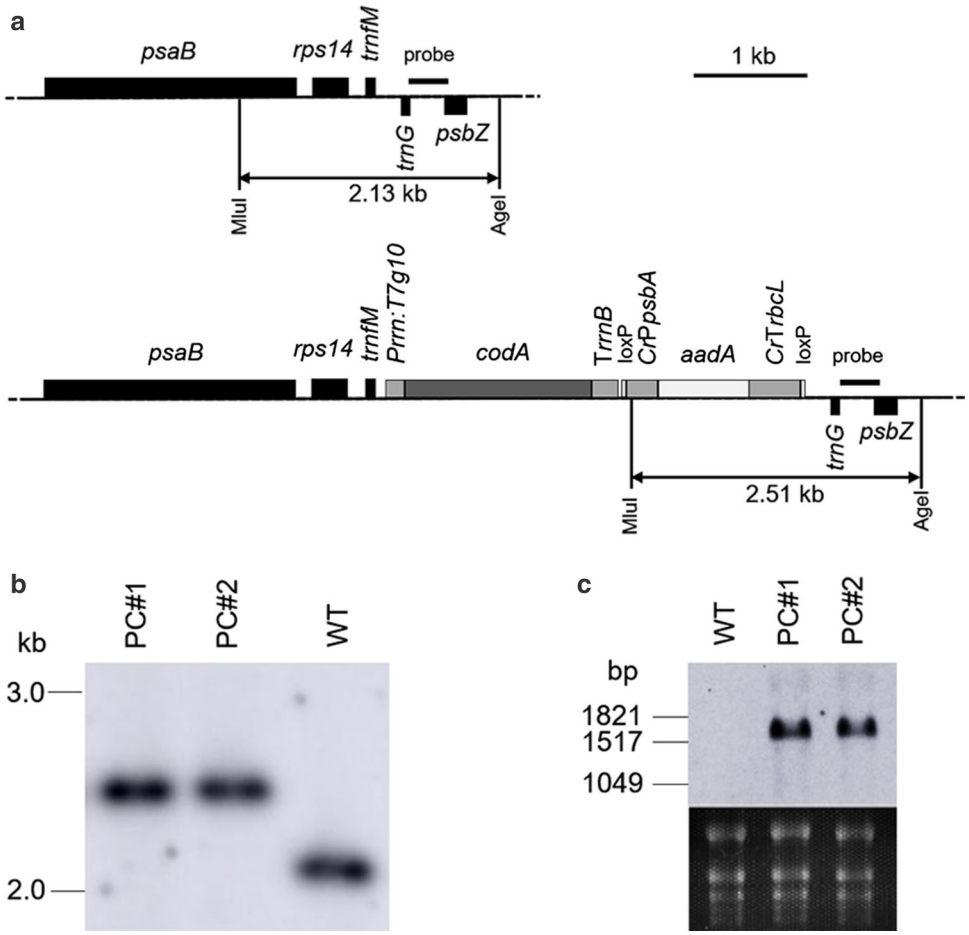

Fig. 1 Introduction of *codA* gene into potato plastid genome. **a** Physical map of the region in the plastid genome to which the *codA* is targeted and map of the plastid transformation vector containing *codA* expression cassette. **b** Southern-blot analysis of transplastomic potato lines. DNA samples were digested with MluI and AgeI and hydrized to a DIG-labeled probe as indicated. The absence of the hybridization signal diagnostic of the wild-type genome indicates the homoplasmy of the two transplastomic lines. Note that no difference of RFLP pattern in two independent transplastomic lines was observed. This is because of identical transgene integration into the plastid genome by homologous recombination and no position effects and/or transgene silencing of plastid transgenes. **c** Northern-blot analysis of *codA* mRNA accumulation in leaves of two independently generated PC lines. Two-microgram total cellular RNA of leaves was loaded. The ethidium bromide-stained gel prior to blotting is shown below the blot

expression levels (Fig. 1c). The phenotypes of two homoplasmic PC lines were phenotypically entirely normal and indistinguishable from WT plants under both mixotrophic growth conditions on synthetic sucrose-containing medium and autotrophic growth conditions in soil (Fig. S1). We therefore used one representative transplastomic line for the next physiology experiments.

Determination of *codA* expression level in leaf and tuber

To assess the *codA* mRNA transcripts accumulation in leaves and tubers, Northern-blot analysis was performed using a hybridization probe specific for the *codA* coding region. The *codA* mRNA accumulation in leaves was around ten times higher than that in tubers, as shown by strongly different intensities of signals on the blot among leaf and tuber samples (Fig. 2a). The full-length mRNA transcripts of the *codA* gene were detected in both leaves and tubers of PC plants. The less abundant and larger signals were also present on the blot (Fig. 2a), which could be the read-through transcripts by plastid-encoded RNA polymerase (Zhou et al. 2007).

Glycine betaine accumulation in *codA*-transplastomic potato plants

To determine whether the insertion of *codA* into potato plastid genome resulted in the increase in GB content in the PC plants, the GB was analyzed quantitatively by ^{1}H-NMR, a highly sensitive detection method. The *codA*-expressing plants accumulated GB in both leaves (~ 1.21 ± 0.36 µmol g^{-1} DW, dry weight) and tubers (~ 1.77 ± 0.21 µmol g^{-1} DW) at the similar level, which were much higher compared to WT plants (Fig. 2b). However, the GB contents in PC plants did not further increase when exposed to drought stress (Fig. S2).

GB accumulation enhances drought tolerance of transplastomic potato plants

To evaluate whether GB accumulation in plastid can increase the drought tolerance of PC plants, we subjected the plants to drought stress. WT and PC plants grown at similar stage withheld the water supply. At day 9 of water deficit, WT plants began to wilt, while PC plants stayed vigorous (Fig. 3a). After 13 days of drought stress, the WT

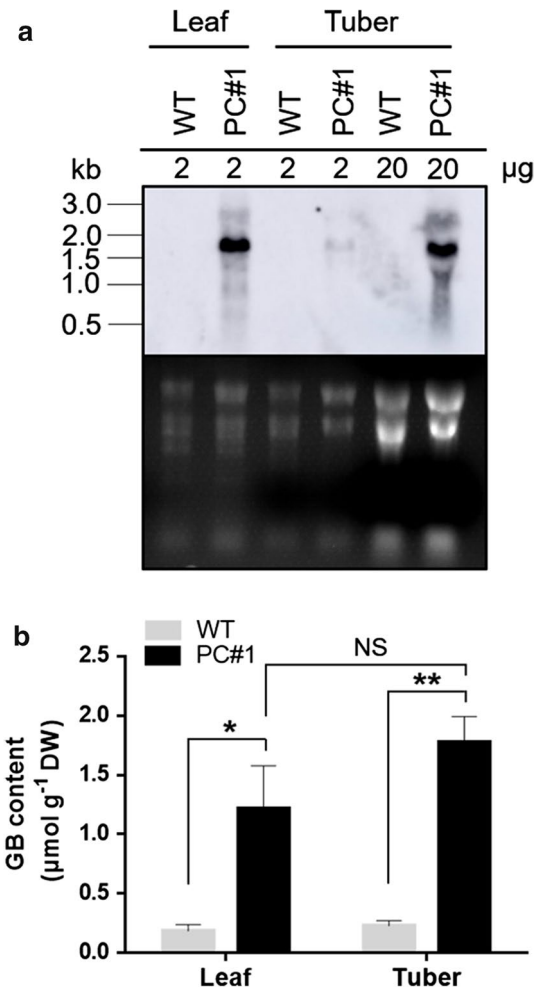

Fig. 2 Analysis of *codA* expression in *codA*-transplastomic (PC) potato plants. **a** Northern-blot analysis of *codA* mRNA accumulation in leaves and tubers of PC plants. The amount of total RNA loaded in each lane is indicated (μg), and the band sizes of the RNA marker are given on the left. The ethidium bromide-stained gel prior to blotting is shown below the blot. **b** GB content in leaves and tubers of wild-type (WT) and PC plants. Samples were collected under normal conditions. Data are expressed as the mean ± SD of three replicates. Significance of differences was analyzed by two-tailed Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$. *NS* no significance

plants were severely wilted, but the PC plants just started to wilt (Fig. 3a). Subsequently, plants were re-watered after 13 days of water withholding. After 2-day re-watering, the PC plants were fully recovered from damage by drought and continued to grow, while the WT plants were hardly capable to recover and finally died (Fig. 3a). RWC in leaves of PC plants was remarkably higher than that of WT plants after 9-day drought stress (Fig. 3b). When WT plants and PC plants were subjected to drought stress, the RWC in WT leaves decreased from 96 ± 3 to 52 ± 2%. In contrast, the RWC was still maintained at 90% ± 2% in leaves of PC plants (Fig. 3b). There was no significant difference in the chlorophyll content between WT and PC plants under normal growth conditions (Fig. 3c). But under drought stress, the chlorophyll content of PC plants was higher than that of WT plants while the chlorophyll content of WT plants decreased significantly. Another transplastomic line (PC#2) also showed the similar phenotype to line PC#1 in tolerance to drought stress (Fig. S3).

Effects of drought stress on antioxidant enzyme activities

Reactive oxygen species (ROS) normally accumulate when plants are exposed to drought stress. In order to determine whether the PC plants have better antioxidant responses to drought stress, we measured the activities of SOD, POD, APX and CAT, enzymes linking to ROS metabolism, in WT and PC plants under normal and drought stress conditions, respectively. As shown in Fig. 4, the SOD, POD and APX activities between WT and PC plants were almost at the same level under normal condition, but significantly higher in PC plants compared to the WT plants (Fig. 4a–c) under drought stress condition. Comparably, CAT activity had no significant difference between WT and PC plants irrespective of stress or non-stress treatment (Fig. 4d).

Effects of drought stress on MDA and proline contents

Our results indicated that there was no significant difference in MDA and proline contents between WT and PC plants under normal condition (Fig. 4e, f). After 9-day water withhold, MDA content increased remarkably in the WT plants, nearly three times higher as that in the PC plants (Fig. 4e), indicating a less cell membrane damage in the PC plants. In addition, increased levels of proline in response to drought stress were observed in both WT and PC plants. The proline content in PC plants was comparably higher than that in WT plants (Fig. 4f).

Effects of drought stress on gas exchange

Under normal conditions, there was no significant difference in photosynthetic gas exchange parameters, including net photosynthetic rate (Pn), substomatal CO_2 concentrations (Ci), stomatal conductance (Gs) and transpiration rate (Tr) between WT and PC plants. After 9 days of drought stress treatment, the Pn, Ci, Gs and Tr of WT and PC plants decreased significantly; however, the Pn, Gs and Tr values of PC plants were higher than those of WT plants. The Ci value of PC plants was significantly lower than that of WT

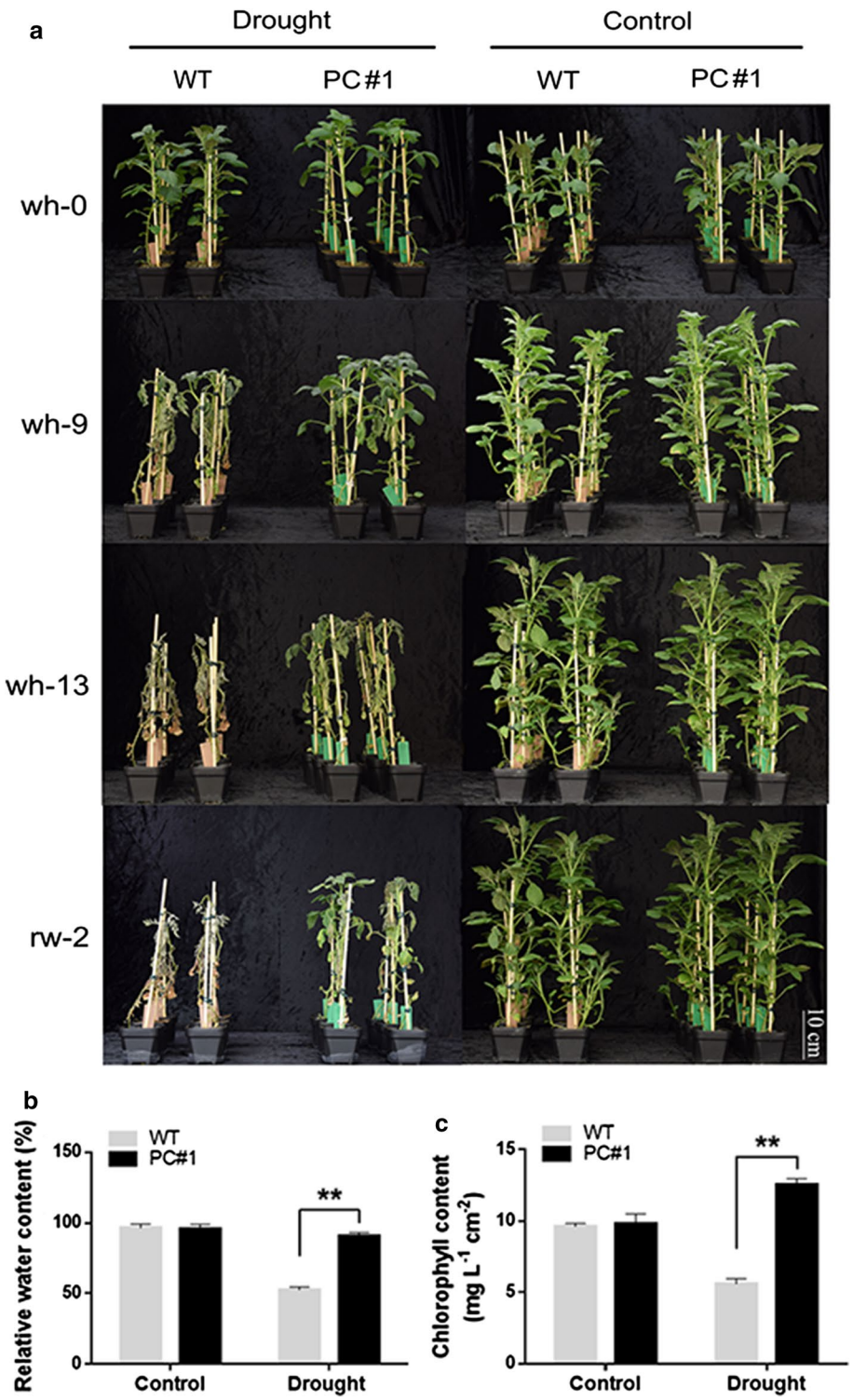

Fig. 3 Effect of drought stress on potato wild-type (WT) and PC plants grown in a greenhouse. **a** Plantlets grown in MS medium were transplanted to pots filled with only vermiculite for 4 weeks; WT and PC plants ($n=6$) were grown under normal conditions or withheld from water, respectively. Photographs were taken 0 (wh-0), 9 (wh-9) and 13 (wh-13) days after withholding from water, and 2 (rw-2) days after re-watering. Bar = 10 cm. Relative water content (**b**) and chlorophyll content (**c**) were measured under normal conditions and after drought stress treatment for 9 days. Data are shown as the mean ± SD of three replicates. Significance of differences was analyzed by two-tailed Student's *t* test. $^{**}P<0.01$

plants (Fig. 5). These results indicate that under drought stress, the transplastomic potato plants maintain a higher photosynthetic rate and show a reduced damage of the photosynthetic apparatus.

Effects of drought stress on PSII photochemistry

NPQ indicates non-photochemical quenching in fluorescence quenching, which is an effective way for plants to dissipate excess excitation energy and an effective indicator

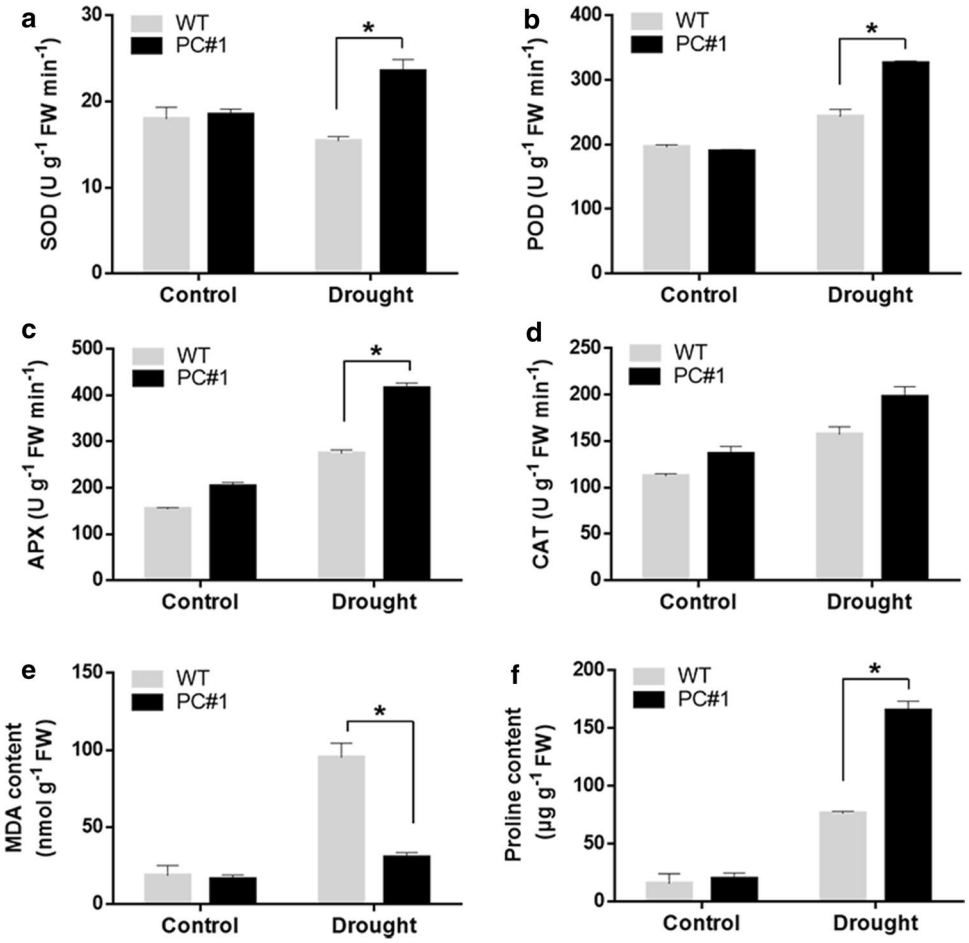

Fig. 4 Changes in activities of SOD (**a**), POD (**b**), APX (**c**) and CAT (**d**), and contents of MDA (**e**) and proline (**f**) in leaves of potato WT and PC plants under normal conditions and drought stress for 9 days. Data are the mean ± SD of three replicates. Significance of differences was analyzed by two-tailed Student's *t* test. $^{*}P < 0.05$

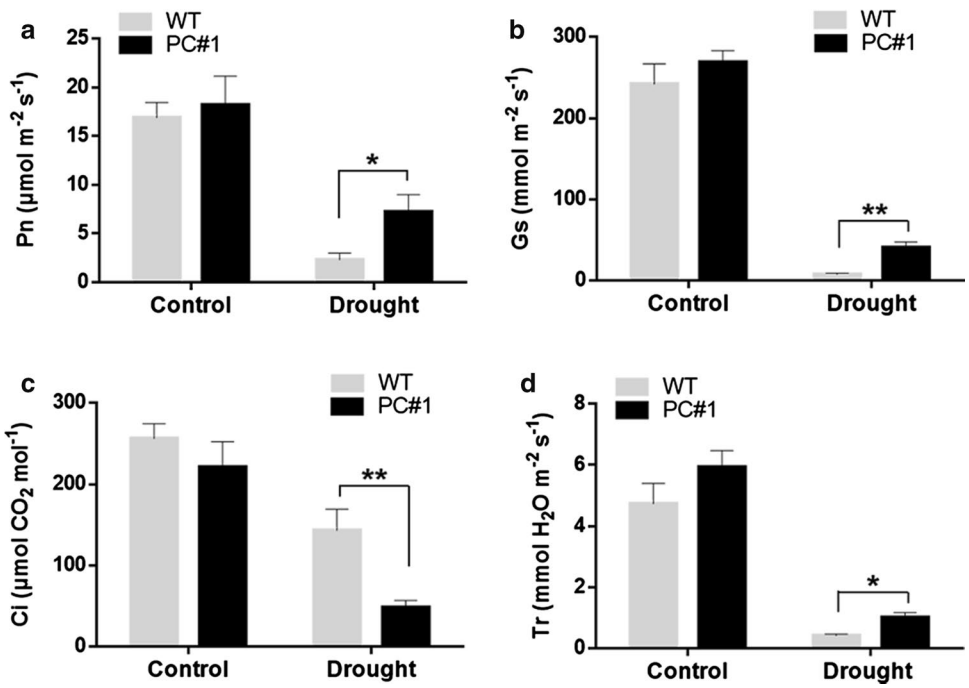

Fig. 5 Changes in photosynthetic rate (Pn, **a**), stomatal conductance (Gs, **b**), intercellular CO_2 concentration (Ci, **c**) and transpiration rate (Tr, **d**) in leaves of potato WT and PC plants under normal conditions and drought stress for 9 days. Significance of differences was analyzed by two-tailed Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$. Error bars indicate SD of eight replicates

of the ability of non-radiative dissipation (Demmig-Adams and Adams 1996; Ort 2001). As shown in Fig. 6a, under normal growth conditions, the NPQ value of PC plants was higher than that of WT plants, indicating that the ability of PC plants regulating excess excitation energy was stronger than that of the WT plants under normal conditions. Under drought stress, the NPQ values of WT and PC plants increased significantly, and the increase of PC plants was more significant (Fig. 6a), which indicated that PC plants could protect PSII by consuming more excess excitation energy than WT plants. Different from NPQ, qP indicates photochemical quenching in fluorescence quenching. Under normal growth conditions, there was no significant difference in qP values between WT and PC plants. Nevertheless, under drought stress, the qP values of potato leaves decreased significantly, and the decline of PC plants was less than that of WT plants (Fig. 6c), indicating that PC plants could maintain high photochemical efficiency. Another evaluation was ETR, as it can quantitatively represent the electron transfer from PSII to PSI (Munekage et al. 2004). The ETR values of WT and PC plants were about the same under normal growth conditions. During drought stress, the ETR values of both had decreased significantly, while WT plants had lower ETR than PC plants (Fig. 6b). Collectively, our results showed that the PC plants could maintain higher qP and ETR than WT plants during drought stress, suggesting that GB accumulation in PC plants is involved in protecting PSII from drought stress.

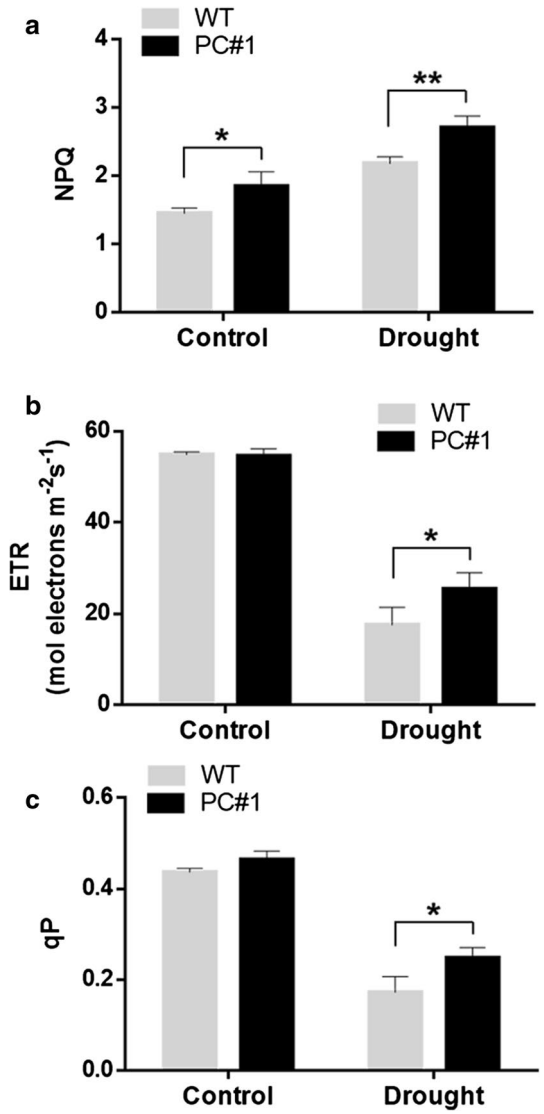

Fig. 6 Changes in non-photochemical quenching (NPQ, **a**), electron transport rates of PSII (ETR, **b**), photochemical quenching (qP, **c**) in the fourth and fifth fully expanded leaf of the potato WT and PC plants under normal conditions and drought stress for 9 days. Significance of differences was analyzed by two-tailed Student's *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$. Error bars indicate SD of eight replicates

Discussion

Potato is one of the major food crops grown worldwide but vulnerable to drought stress, necessitating the breeding for drought tolerance potato varieties. Plastid transformation has developed for almost three decades and showed its unique advantages in improving the agronomic traits of crops. For example using tobacco as model plants, plastid-transformed plants showed increased tolerance to herbicides, biotic stress (insect, disease) and abiotic stress (drought, salt) (Bock 2015). Not like tobacco, only till recently, potato plastid engineering has been conducted to confer insect resistance (Zhang et al. 2015). In the present work, we transformed the bacterial *codA* gene into potato plastid genome plants and obtained the homoplasmic PC plants with enhanced drought tolerance (Figs. 1, 3).

It has already been indicated that most plastid-encoded gene expressions in tuber amyloplasts were markedly lower when compared to those in leaf chloroplasts (Valkov et al. 2009). Consistently, our results indicated that the signal of *codA* mRNA transcripts of PC plants in leaves was around ten times stronger than that in tubers (Fig. 2a). Compared with leaf chloroplasts, gene expression at the posttranscriptional and translational levels was much lower in nongreen plastids (Valkov et al. 2009; Zhang et al. 2012). COD protein expression level was thus assumed to be much lower in tubers compared with that in leaves (less than tenfold). Although with a drastic difference in *codA* gene expression, PC plants could stably accumulate GB in both leaves and tubers at a similar level (Fig. 2b), either under normal conditions or exposed to drought stress (Fig. S2). This suggests that the GB content in PC plants is unrelated to the expression level of *codA* gene and PC plants can generate an active COD enzyme, which is capable of efficiently converting choline into GB. That the GB content (measured by ^{1}H-NMR

spectroscopy) in our PC plants was not higher than that in previously reported transgenic potato lines was probably due to the different GB measurement and calculation methods used (Ahmad et al. 2008). Nevertheless, it has been demonstrated that choline import into chloroplast is a constraint step and is essential for GB synthesis (Nuccio et al. 1998; McNeil et al. 2000; Nuccio et al. 2000; Zhang et al. 2008). Moderate increase in GB content in the PC plants may be partially due to the limited availability of choline in plastids. Taken together, we speculate that activity of COD can maintain at a considerably high level in PC plants either in tuber amyloplasts or leaf chloroplasts despite their different *codA* gene expression levels and efficiently convert choline into GB; however, a limited capacity of transporting endogenous choline into the plastids may be attributed to a moderately increased level of GB in PC plants. It has been proposed that the choline content in chloroplasts could be increased by over-expressing a high affinity choline transporter (BetT) of *E. coli* (Lamark et al. 1991; Nuccio et al. 2000). Whether this strategy could enhance the choline content in plastids remained to be elucidated.

It has been proven that even slight GB accumulation by introducing the *codA* gene into nuclear genome of transgenic plants is enough to improve the abiotic tolerance. For example, slight GB accumulation (0.28 ± 0.03 μmol/g FW) in transgenic Japanese persimmon enhanced its tolerance to salt stress (Gao et al. 2000). Transgenic tomato plants with chloroplast-targeted COD accumulated GB at 0.3 ± 0.02 μmol/g FW and conferred significant tolerance to salt and water stresses (Goel et al. 2011) and chilling stress (Park et al. 2004). Interestingly, the degree of chilling tolerance was similar in both lowest GB-accumulating line (as low as 0.09 μmol/g FW) and higher GB-accumulated line (0.3 μmol/g FW), suggesting that GB at low level could adequately confer a high level of tolerance (Park et al. 2004). Moreover, the chloroplast-targeted COD tomato plants with low leaf GB content were more tolerant to chilling stress than were cytosol- or both (chloroplast and cytosol)-targeted plants (Sakamoto and Murata 1998; Park et al. 2007). This indicated that GB accumulation was more effective in the chloroplasts than in the cytosol in protecting transgenic plants from abiotic stress. In agreement with previous studies, although containing a moderate GB in our PC plants, the plants developed very well compared to the WT controls during a water deficit assay and exhibited less suffering from drought and better recovery rate after re-watering (Fig. 3a). Moreover, PC plants also had higher leaf RWC (Fig. 3b) and leaf chlorophyll content (Fig. 3c) compared to WT plants under drought stress.

ROS levels rise excessively in response to diverse stresses, potentially leading to oxidative stress and damage to macromolecules such as proteins, lipids and nucleic acids (Apel and Hirt 2004). During drought stress, chloroplastic ROS production has long been proposed as a major driver of redox signal or damage in plant cells (Noctor et al. 2014). In order to cope with excessive ROS production under stress, plants have evolved efficient enzymatic and non-enzymatic mechanisms to regulate ROS levels (Noctor and Foyer 1998). A plethora of studies have reported effects of drought on activities of the major antioxidant enzymes such as SOD, CAT and APX (Cruz de Carvalho and Contour-Ansel 2008). Our results showed that three antioxidant enzyme activities SOD, POD and APX were enhanced in PC plants compared with WT plants under drought stress (Fig. 4a–c), suggesting that accumulated GB in plastids of PC plants could help to quench ROS and alleviate its damage to cellular components (Demiral and Türkan 2004). SODs, PODs and APXs are located throughout different compartments of the plant cell, while CATs are exclusively located in the peroxisomes. In addition, APX has a higher affinity for H_2O_2 than CAT, though both APX and CAT are major enzymatic cellular scavengers of H_2O_2 (Mittler 2002). This might explain why the CAT activity did not increase significantly during drought in our results. PC plants had lower MDA level (Fig. 4e) compared to WT plants when exposed to water deficit. The lower MDA level indicates less cell membrane damage in PC plants (Gorham 1995; Chen et al. 2000). Moreover, high proline accumulation was observed in PC plants (Fig. 4f), which is consistent with previous studies that reported the enhanced proline content in *codA*-transgenic tomato (Goel et al. 2011) in response to salt stress. High proline synthesis in stressed plants can favor a better recovery of these plants (De Ronde et al. 2004), protect photosynthetic apparatus and increase grain yield (Vendruscolo et al. 2007).

The photosynthetic rate of PC plants is higher than that of WT plants under drought stress (Fig. 5a). Previous study showed that there was a linear correlation between CO_2 assimilation rate and stomatal conductance in control and GB-fed plants (Yang and Lu 2006). Our results indicated that under drought stress, the stomatal conductance of potato leaves decreased; however, the PC plant maintained higher stomatal conductance than WT plants (Fig. 5b). The increased photosynthetic rate of PC plants was due to an increase in stomatal conductance.

PSII is a key part of various stress injuries such as drought, high temperature and strong light. When the plant is exposed to severe abiotic stress, the damage rate of PSII will exceed its repair rate, and the photoinhibition caused by the irreversible inactivation of PSII will lead to the decrease of photosynthetic activity of plants, thereby reducing the photosynthetic rate (Murata et al. 2007; Nixon et al. 2010; Umena et al. 2011; Nishiyama and Murata 2014). Drought stress also reduces the activity of PSII, causing electron transfer blocked and increasing the excess light energy in plants. Excess light energy

is highly susceptible to the generation of ROS (singlet oxygen), which causes degradation of photosynthetic pigments and destruction of photosynthetic mechanisms, causing photooxidation or photobleaching. NPQ is positively correlated with the heat dissipation of the lutein-dependent cycle which plays an important role in protecting the plant photosynthetic apparatus from reducing the excess excitation energy. The increase in NPQ is beneficial to dissipate excess excitation energy and protect plants from excessive light energy. qP reflects the share of light energy absorbed by the PSII antenna for photochemical electron transport, reflecting to some extent the openness of the PSII reaction center (Krause et al. 1990). ETR refers to the electron transfer rate of plant leaves and can express the patency of electron transport from PSII to PSI (Schreiber et al. 1995). The NPQ, qP and ETR of PC plants were higher than WT plants under drought stress, which indicates that PC plants can dissipate more excess light energy and protect plant photosynthetic apparatus to maintain high photochemical efficiency (Fig. 6a–c). Under drought stress, GB accumulated in vivo in PC plants might have certain protective effects on various components in the electron transport chain, thereby increasing the electron transport activity and enhancing the photochemical efficiency of PC plants leaves. At the same time, GB accumulating in vivo could enhance the dissipation ability of plants and decrease the damage of photosystem caused by drought stress. Our results indicated that GB accumulated in PC plants can protect the photosynthesis machinery and improve drought tolerance, while GB may not function as osmoprotectant because of its low accumulation level in PC plants which was lower than the osmotically effective concentration (Fig. 2b).

In conclusion, for the first time, by using plastid transformation technology, the GB synthesis gene *codA* from *A. globiformis* was successfully transformed into potato plastid genome. Although high expression of *codA* in chloroplast does not lead to a high GB content that may be due to the limitation of choline in plastids, compartmentation of GB in plastids conferred considerably high tolerance to drought stress. These findings suggest that biosynthesis of GB by plastid engineering is an effective method to increase drought tolerance and transportation of choline from cytosol into chloroplast entailing the next effort for GB engineering.

Author contribution statement JZ and XY designed the experiments. LY, QS and YW performed the experiments. LY, SL, CJ, LC, XY and JZ analyzed the data. JZ, LY and XY wrote the paper with the input from other authors.

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References

Ahmad R, Kim MD, Back KH, Kim HS, Lee HS, Kwon SY, Murata N, Chung WI, Kwak SS (2008) Stress-induced expression of choline oxidase in potato plant chloroplasts confers enhanced tolerance to oxidative, salt, and drought stresses. Plant Cell Rep 27(4):687–698

Ahmad R, Kim YH, Kim MD, Kwon SY, Cho K, Lee HS, Kwak SS (2010) Simultaneous expression of choline oxidase, superoxide dismutase and ascorbate peroxidase in potato plant chloroplasts provides synergistically enhanced protection against various abiotic stresses. Physiol Plant 138(4):520–533

Al Hassan M, Chaura J, López-Gresa MP, Borsai O, Daniso E, Donat-Torres MP, Mayoral O, Vicente O, Boscaiu M (2016) Native-invasive plants vs. halophytes in Mediterranean salt marshes: stress tolerance mechanisms in two related species. Front Plant Sci 7:473

Alia Kondo Y, Sakamoto A, Nonaka H, Hayashi H, Saradhi PP, Chen TH, Murata N (1999) Enhanced tolerance to light stress of transgenic plants that express the *codA* gene for a bacterial choline oxidase. Plant Mol Biol 40(2):279–288

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373–399

Bartoli CG, Simontacchi M, Tambussi E, Beltrano J, Montaldi E, Puntarulo S (1999) Drought and watering-dependent oxidative stress: effect on antioxidant content in *Triticum aestivum* L. leaves. J Exp Bot 50(332):375–383

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39(1):205–207

Bock R (2015) Engineering plastid genomes: methods, tools, and applications in basic research and biotechnology. Annu Rev Plant Biol 66:211–241

Camire ME, Kubow S, Donnelly DJ (2009) Potatoes and human health. Crit Rev Food Sci Nutr 49:823–840

Chen THH, Murata N (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Curr Opin Plant Biol 5(3):250–257

Chen THH, Murata N (2008) Glycinebetaine: an effective protectant against abiotic stress in plants. Trends Plant Sci 13(9):499–505

Chen WP, Li PH, Chen THH (2000) Glycinebetaine increases chilling tolerance and reduces chilling-induced lipid peroxidation in *Zea mays* L. Plant Cell Environ 23(6):609–618

Cheng YJ, Deng XP, Kwak SS, Chen W, Eneji AE (2013) Enhanced tolerance of transgenic potato plants expressing choline oxidase in chloroplasts against water stress. Bot Stud 54:30

Cho KS, Han EH, Kwak SS, Cho JH, Im JS, Hong SY, Sohn HB, Kim YH, Lee SW (2016) Expressing the sweet potato orange gene in transgenic potato improves drought tolerance and marketable tuber production. C R Biol 339(5–6):207–213

Cruz de Carvalho MH, Contour-Ansel D (2008) (h)GR, beans and drought stress. Plant Signal Behav 3(10):834–835

De Ronde JA, Cress WA, Krüger GH, Strasser RJ, Van Staden J (2004) Photosynthetic response of transgenic soybean plants, containing an *Arabidopsis P5CR* gene, during heat and drought stress. J Plant Physiol 161(11):1211–1224

Demiral T, Türkan I (2004) Does exogenous glycinebetaine affect antioxidative system of rice seedlings under NaCl treatment? J Plant Physiol 161(10):1089–1100

Demmig-Adams B, Adams WW (1996) Xanthophyll cycle and light stress in nature: uniform response to excess direct sunlight among higher plant species. Planta 198(3):460–470

Gao M, Sakamoto A, Miura K, Murata N, Sugiura A, Tao R (2000) Transformation of Japanese persimmon (*Diospyros kaki* Thunb.) with a bacterial gene for choline oxidase. Mol Breed 6(5):501–510

Gerbens-Leenes W, Hoekstra AY, van der Meer TH (2009) The water footprint of bioenergy. Proc Natl Acad Sci USA 106(25):10219–10223

Giannopolitis CN, Ries SK (1977) Superoxide dismutases. Plant Physiol 59:315–318

Goel D, Singh AK, Yadav V, Babbar SB, Murata N, Bansal KC (2011) Transformation of tomato with a bacterial *codA* gene enhances tolerance to salt and water stresses. J Plant Physiol 168(11):1286–1294

Gorham J (1995) Betaines in higher plants—biosynthesis and role in stress metabolism. In: Wallsgrove RM (ed) Amino acids and their derivatives in higher plants. Cambridge University, Cambridge, pp 173–204

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol 51(1):463–499

Hayashi H, Alia Mustardy L, Deshnium P, Ida M, Murata N (1997) Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase; accumulation of glycinebetaine and enhanced tolerance to salt and cold stress. Plant J 12(1):133–142

Hayashi H, Sakamoto A, Nonaka H, Chen TH, Murata N (1998) Enhanced germination under high-salt conditions of seeds of transgenic *Arabidopsis* with a bacterial gene (*codA*) for choline oxidase. J Plant Res 111(2):357–362

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125(1):189–198

Hijmans RJ (2003) The effect of climate change on global potato production. Am J Potato Res 80(4):271–279

Ikuta S, Imamura S, Misaki H, Horiuti Y (1977) Purification and characterization of choline oxidase from *Arthrobacter globiformis*. J Biochem 82(6):1741–1749

Jefferies RA (2010) Responses of potato genotypes to drought. I. Expansion of individual leaves and osmotic adjustment. Ann Appl Biol 122(1):93–104

Kanamoto H, Yamashita A, Asao H, Okumura S, Takase H, Hattori M, Yokota A, Tomizawa K (2006) Efficient and stable transformation of *Lactuca sativa* L. cv. Cisco (lettuce) plastids. Transgenic Res 15(2):205–217

Kathuria H, Giri J, Nataraja KN, Murata N, Udayakumar M, Tyagi AK (2009) Glycinebetaine-induced water-stress tolerance in *codA*-expressing transgenic indica rice is associated with up-regulation of several stress responsive genes. Plant Biotechnol J 7(6):512–526

Ke Q, Wang Z, Ji CY, Jeong JC, Lee HS, Li H, Xu B, Deng X, Kwak SS (2016) Transgenic poplar expressing *codA* exhibits enhanced growth and abiotic stress tolerance. Plant Physiol Biochem 100:75–84

Krause GH, Somersalo S, Zumbusch E, Weyers B, Laasch H (1990) On the mechanism of photoinhibition in chloroplasts. Relationship between changes in fluorescence and activity of photosystem II. J Plant Physiol 136(4):472–479

Lamark T, Kaasen I, Eshoo MW, Falkenberg P, McDougall J, Strøm AR (1991) DNA sequence and analysis of the bet genes encoding the osmoregulatory choline—glycine betaine pathway of *Escherichia coli*. Mol Microbiol 5(5):1049–1064

Maehly AC, Chance B (1954) The assay of catalases and peroxidases. In: Glick D (ed) Methods of biochemical analysis. Interscience Publisher, New York, pp 357–424

Mansour M (2000) Nitrogen containing compounds and adaptation of plants to salinity stress. Biol Plant 43(4):491–500

McNeil SD, Rhodes D, Russell BL, Nuccio ML, Shachar-Hill Y, Hanson AD (2000) Metabolic modeling identifies key constraints on an engineered glycine betaine synthesis pathway in tobacco. Plant Physiol 124(1):153–162

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7(9):405–410

Mohanty A, Kathuria H, Ferjani A, Sakamoto A, Mohanty P, Murata N, Tyagi AK (2002) Transgenics of an elite indica rice variety *Pusa Basmati* 1 harbouring the *codA* gene are highly tolerant to salt stress. Theor Appl Genet 106(1):51–57

Munekage Y, Hashimoto M, Miyake C, Tomizawa K, Endo T, Tasaka M, Shikanai T (2004) Cyclic electron flow around photosystem I is essential for photosynthesis. Nature 429(6991):579–582

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15(3):473–497

Murata N, Takahashi S, Nishiyama Y, Allakhverdiev SI (2007) Photoinhibition of photosystem II under environmental stress. Biochim Biophys Acta 1767(6):414–421

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8(19):4321–4325

Nishiyama Y, Murata N (2014) Revised scheme for the mechanism of photoinhibition and its application to enhance the abiotic stress tolerance of the photosynthetic machinery. Appl Microbiol Biotechnol 98(21):8777–8796

Nixon PJ, Michoux F, Yu J, Boehm M, Komenda J (2010) Recent advances in understanding the assembly and repair of photosystem II. Ann Bot 106(1):1–16

Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49:249–279

Noctor G, Mhamdi A, Foyer CH (2014) The roles of reactive oxygen metabolism in drought: not so cut and dried. Plant Physiol 164(4):1636–1648

Nuccio ML, Russell BL, Nolte KD, Rathinasabapathi B, Gage DA, Hanson AD (1998) The endogenous choline supply limits glycine betaine synthesis in transgenic tobacco expressing choline monooxygenase. Plant J 16(4):487–496

Nuccio ML, McNeil SD, Ziemak MJ, Hanson AD, Jain RK, Selvaraj G (2000) Choline import into chloroplasts limits glycine betaine synthesis in tobacco: analysis of plants engineered with a chloroplastic or a cytosolic pathway. Metab Eng 2(4):300–311

Ort DR (2001) When there is too much light. Plant Physiol 125:29–32

Papageorgiou GC, Murata N (1995) The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem II complex. Photosynth Res 44(3):243–252

Park EJ, Jeknić Z, Sakamoto A, DeNoma J, Yuwansiri R, Murata N, Chen TH (2004) Genetic engineering of glycinebetaine synthesis in tomato protects seeds, plants, and flowers from chilling damage. Plant J 40(4):474–487

Park EJ, Jeknić Z, Pino MT, Murata N, Chen TH (2007) Glycinebetaine accumulation is more effective in chloroplasts than in the cytosol for protecting transgenic tomato plants against abiotic stress. Plant Cell Environ 30(8):994–1005

Pinhero RG, Rao MV, Paliyath G, Murr DP, Fletcher RA (1997) Changes in activities of antioxidant enzymes and their relationship to genetic and paclobutrazol-induced chilling tolerance of maize seedlings. Plant Physiol 114(2):695–704

Porra RJ (2002) The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynth Res 73(1):149–156

Rhodes D, Hanson A (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. Annu Rev Plant Biol 44(1):357–384

Sakamoto A, Murata AN (1998) Metabolic engineering of rice leading to biosynthesis of glycinebetaine and tolerance to salt and cold. Plant Mol Biol 38(6):1011–1019

Sakamoto A, Murata N (2000) Genetic engineering of glycinebetaine synthesis in plants: current status and implications for enhancement of stress tolerance. J Exp Bot 51(342):81–88

Schreiber U, Bilger W, Neubauer C (1995) Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of in vivo photosynthesis. In: Schulze ED (ed) Ecophysiology of photosynthesis. Springer, Berlin, pp 49–70

Scott SE, Wilkinson MJ (1999) Low probability of chloroplast movement from oilseed rape (*Brassica napus*) into wild *Brassica rapa*. Nat Biotechnol 17(4):390–392

Staub JM, Maliga P (1995) Expression of a chimeric *uidA* gene indicates that polycistronic mRNAs are efficiently translated in tobacco plastids. Plant J 7(5):845–848

Umena Y, Kawakami K, Shen JR, Kamiya N (2011) Crystal structure of oxygen-evolving photosystem II at a resolution of 1.9 Å. Nature 473(7345):55–60

Ünlü M, Kanber R, Şenyigit U, Onaran H, Diker K (2006) Trickle and sprinkler irrigation of potato (*Solanum tuberosum* L.) in the Middle Anatolian Region in Turkey. Agric Water Manag 79(1):43–71

Valkov VT, Scotti N, Kahlau S, Maclean D, Grillo S, Gray JC, Bock R, Cardi T (2009) Genome-wide analysis of plastid gene expression in potato leaf chloroplasts and tuber amyloplasts: transcriptional and posttranscriptional control. Plant Physiol 150(4):2030–2044

van Loon CD (1981) The effect of water stress on potato growth, development, and yield. Am J Potato Res 58(1):51–69

Vendruscolo EC, Schuster I, Pileggi M, Scapim CA, Molinari HB, Marur CJ, Vieira LG (2007) Stress-induced synthesis of proline confers tolerance to water deficit in transgenic wheat. J Plant Physiol 164(10):1367–1376

Wei DD, Zhang W, Wang CC, Meng QW, Li G, Chen THH, Yang XH (2017) Genetic engineering of the biosynthesis of glycinebetaine leads to alleviate salt-induced potassium efflux and enhances salt tolerance in tomato plants. Plant Sci 257:74–83

Wu Y, You L, Li S, Ma M, Wu M, Ma L, Bock R, Chang L, Zhang J (2017) In vivo assembly in *Escherichia coli* of transformation vectors for plastid genome engineering. Front Plant Sci 8:1454

Yang XH, Lu CM (2006) Effects of exogenous glycinebetaine on growth, CO_2 assimilation, and photosystem II photochemistry of maize plants. Physiol Plant 127(4):593–602

Zhang J, Tan W, Yang XH, Zhang HX (2008) Plastid-expressed choline monooxygenase gene improves salt and drought tolerance through accumulation of glycine betaine in tobacco. Plant Cell Rep 27(6):1113–1124

Zhang J, Ruf S, Hasse C, Childs L, Scharff LB, Bock R (2012) Identification of cis-elements conferring high levels of gene expression in non-green plastids. Plant J 72(1):115–128

Zhang J, Khan SA, Hasse C, Ruf S, Heckel DG, Bock R (2015) Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids. Science 347(6225):991–994

Zhou F, Karcher D, Bock R (2007) Identification of a plastid intercistronic expression element (IEE) facilitating the expression of stable translatable monocistronic mRNAs from operons. Plant J 52(5):961–972