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Research article

# Transgenic sweetpotato plants overexpressing *tocopherol cyclase* display enhanced α-tocopherol content and abiotic stress tolerance

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ABSTRACT

Oxidative stress caused by reactive oxygen species (ROS) under various environmental stresses significantly reduces plant productivity. Tocopherols (collectively known as vitamin E) are a group of lipophilic antioxidants that protect cellular components against oxidative stress. Previously, we isolated five tocopherol biosynthesis genes from sweetpotato (*Ipomoea batatas* [L.] Lam) plants, including tocopherol cyclase (*IbTC*). In this study, we generated transgenic sweetpotato plants overexpressing *IbTC* under the control of cauliflower mosaic virus (CaMV) *35S* promoter (referred to as TC plants) via *Agrobacterium*-mediated transformation to understand the function of *IbTC* in sweetpotato. Three transgenic lines (TC2, TC9, and TC11) with high transcript levels of *IbTC* were selected for further characterization. High performance liquid chromatography (HPLC) analysis revealed that α-tocopherol was the most predominant form of tocopherol in sweetpotato tissues. The content of α-tocopherol was 1.6–3.3-fold higher in TC leaves than in non-transgenic (NT) leaves. No significant difference was observed in the tocopherol content of storage roots between TC and NT plants. Additionally, compared with NT plants, TC plants showed enhanced tolerance to multiple environmental stresses, including salt, drought, and oxidative stresses, and showed consistently higher levels of photosystem II activity and chlorophyll content, indicating abiotic stress tolerance. These results suggest *IbTC* as a strong candidate gene for the development of sweetpotato cultivars with increased α-tocopherol levels and enhanced abiotic stress tolerance.

## 1. Introduction

Plants are sessile organisms and therefore highly sensitive to various abiotic and biotic stresses during their life cycle. Environmental stresses trigger the production of reactive oxygen species (ROS), inducing oxidative stress and consequently affecting plant quality and productivity. To cope with oxidative stress, plants have developed efficient antioxidant systems such as antioxidant enzymes and low molecular weight antioxidants such as ascorbate, carotenoids, and tocopherols.

Tocopherols (collectively known as vitamin E) are a group of low molecular weight lipophilic antioxidants, first discovered as essential nutrients for reproduction in rats (Evans and Bishop, 1922). The structure of tocopherols comprises a polar chromanol ring with hydrophobic polyprenyl side chains. Depending on the number of methyl groups in the chromanol ring, tocopherols are classified into four types: α-, β-, γ-, and δ-tocopherol (Fritsche et al., 2017). Tocopherols are primarily synthesized in plastids of photosynthetic organisms such as plants, algae, and cyanobacteria, and are localized in chloroplast membranes, plastoglobules, and thylakoid membranes. Additionally, tocopherols are involved in plant growth and physiological pathway and play an important role in enhancing abiotic stress tolerance of plants by their ROS scavenging activity (Munne-Bosch, 2005; Chen et al., 2006; Asensi-Fabado and Munne-Bosch, 2010; Hasanuzzaman et al., 2014).

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*Abbreviations:* TC, Tocopherol cyclase; ROS, Reactive oxygen species; $H_2O_2$, Hydrogen peroxide; MV, Methyl viologen; DAB, 3,3′-diaminobenzidine; qRT-PCR, quantitative reverse transcription polymerase chain reaction

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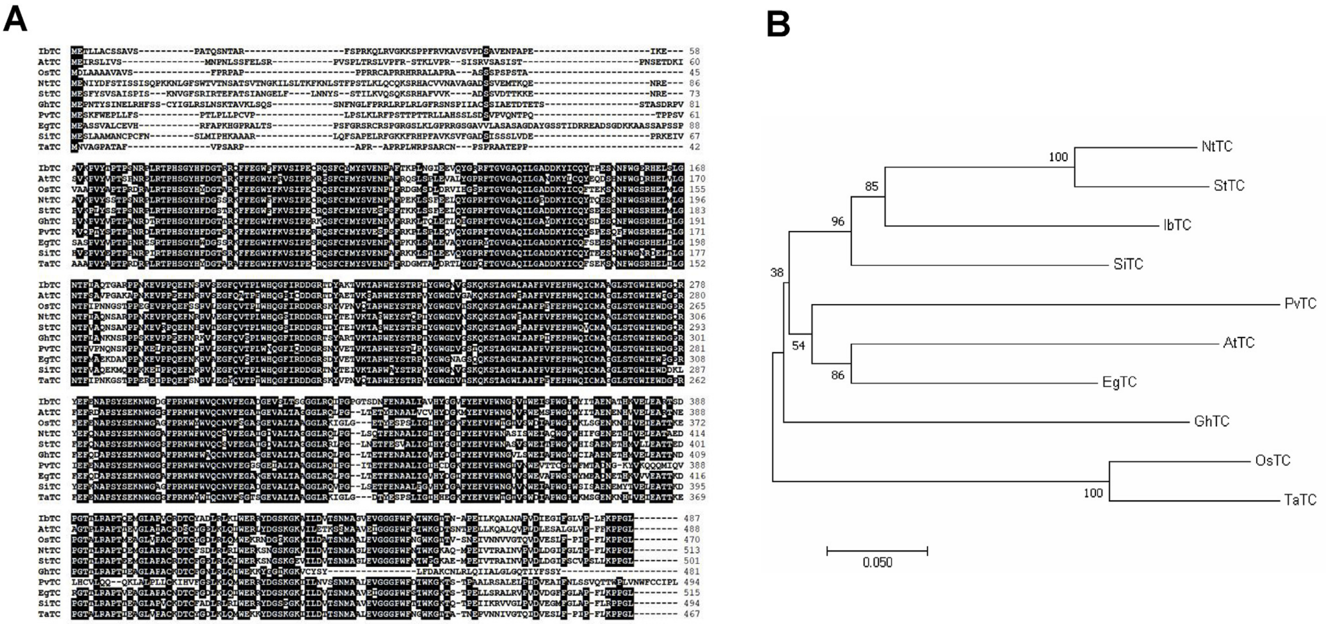

**Fig. 1.** Multiple amino acid sequence alignment and phylogenetic analysis of tocopherol cyclase (TC) enzymes of various plant species. **a** Alignment of the deduced amino acid sequences of IbTC (KP306524) and TCs from other plant species including *Nicotiana tabacum* (NtTC; KJ645980), *Solanum tuberosum* (StTC; AY536918), *Sesamum indicum* (SiTC; EU143248), *Phaseolus vulgaris* (PvTC; EU684954), *Arabidopsis thaliana* (AtTC; NM119430), *Eucalyptus gunnii* (EgTC; AY336944), *Gossypium hirsutum* (GhTC; DQ456881), *Oryza sativa* (OsTC; XM015770843.2), and *Triticum aestivum* (TaTC; DQ456882). Amino acid sequence alignment was performed using ClustalW. **b** Phylogenetic analysis of IbTC and other plant TCs.

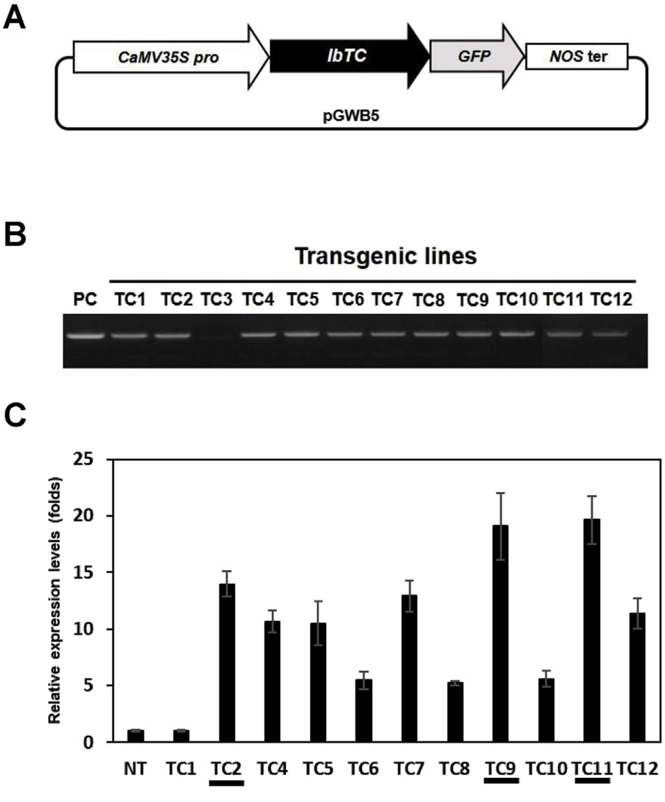

**Fig. 2.** Generation of transgenic sweetpotato plants overexpressing *IbTC*. **a** Schematic representation of the construct used for generating transgenic sweetpotato plants overexpressing *IbTC*. **b** Genomic DNA PCR analysis of transgenic plants using *IbTC*-specific primers. PC, positive control. **c** Quantitative reverse transcription PCR (qRT-PCR) analysis of *IbTC* expression in transgenic (TC) and non-transgenic (NT) sweetpotato plants using *IbTC*-specific primers. Data are the means ± standard deviation (SD) of three technical qRT-PCR replicates from individual plants.

In the tocopherol biosynthetic pathway, p-hydroxyphenylpyruvate (HPP) is converted to α-tocopherol by the action of six enzymes in the following order: 4-HPP dioxygenase (HPPD), homogentisate phytyltransferase (HPT), 2-methyl-6-phytylbenzoquinol methyltransferase (MPBQ), MPBQ methyltransferase (MT), tocopherol cyclase (TC), and γ-tocopherol methyltransferase (TMT) (Jiang et al., 2017). The enzyme HPPD catalyzes the formation of aromatic head group, homogentisic acid (HGA), from HPP. The catalytic activity of HPT converts HGA to MPBQ, which is transformed to 2,3-dimethyl-5-phytyl-benzoquinol (DMPBQ) by the MT enzyme. Then, TC converts two substrates, MPBQ and DMPBQ, into δ-tocopherol and γ-tocopherol, respectively. Finally, TMT converts δ-tocopherol and γ-tocopherol to β-tocopherol and α-tocopherol, respectively (Fritsche et al., 2017).

Over the past few years, several studies have carried out the metabolic engineering of tocopherol biosynthesis genes with the aim to increase the tocopherol content of various plant species. The enzyme HPPD was first identified in carrot (*Daucus carota*) cells, and the overexpression of *Arabidopsis thaliana HPPD* (*AtHPPD*) elevated the tocopherol content to 37% in leaves and 28% in seeds compared with non-transgenic (NT) plants (Tsegaye et al., 2002). In leaves of transgenic lettuce (*Lactuca sativa* L.) plants overexpressing *LsHPT*, α-tocopherol and γ-tocopherol were increased by 4-fold and 2.6-fold, respectively, compared with NT plants (Ren et al., 2011). However, transgenic soybean (*Glycine max*) plants overexpressing *AtMT* did not show an increase in the total tocopherol content because while the content of γ- and α-tocopherol increased in transgenic soybean seeds, that of δ- and β-tocopherol decreased simultaneously (Van Eenennaam et al., 2003).

The TC enzyme was first discovered in plastoglobules of plant cells (Ytterberg et al., 2006). TC catalyzes the penultimate step in the tocopherol biosynthesis pathway synthesizing γ- and δ-tocopherol. Overexpression of *AtTC* increased the total tocopherol content by 4–10-fold in transgenic tobacco (*Nicotiana tabacum*) leaves (Liu et al., 2008) and 7-fold in transgenic *Arabidopsis* leaves (Kanwischer et al., 2005) compared with NT leaves. In rice (*Oryza sativa*), overexpression of *OsTC* enhanced salt stress tolerance (Ouyang et al., 2011), and overexpression of tobacco *TC* (*NtTC*) conferred drought tolerance (Woo et al., 2014). These results indicate that *TC* regulates tocopherol

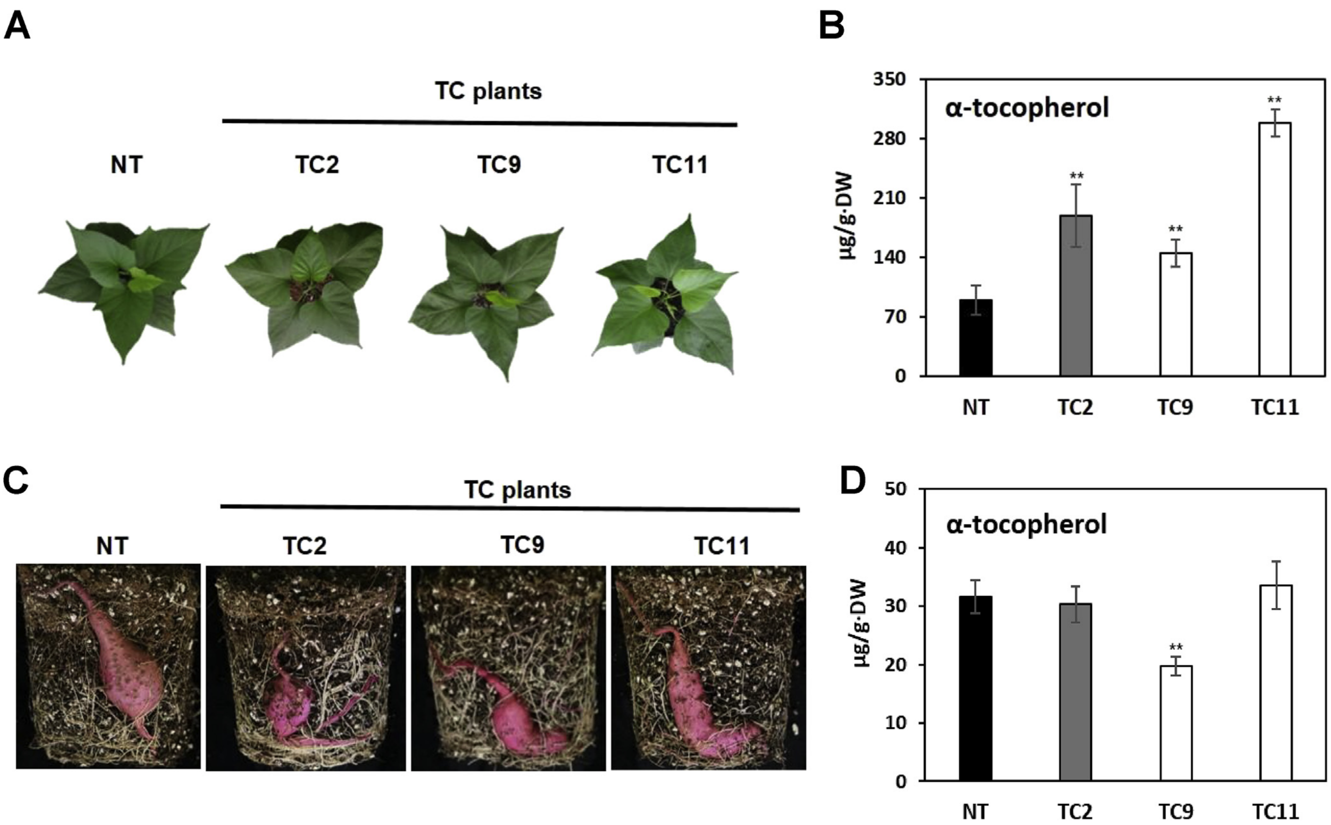

**Fig. 3.** Content of α-tocopherol in leaves and storage roots of TC and NT sweetpotato plants. **a** Photographs of aerial parts of 3-week-old TC and NT plants. **b** Quantification of α-tocopherol content in leaves using fluorescence high performance liquid chromatography (HPLC). **c** Photographs of storage roots of 4-month-old TC and NT plants. **d** Quantification of α-tocopherol in storage roots using fluorescence HPLC. Levels of β-, γ-, and δ-tocopherol were negligible. Data represent mean ± SD of three independent replicates. Significant differences between transgenic and NT plants are indicated with asterisks ($*P < 0.05$; $**P < 0.01$; One way ANOVA with Tukey's HSD post hoc test).

accumulation, leading to abiotic stress tolerance in plants. However, to date, there is no report on the effect of *TC* overexpression in sweetpotato (*Ipomoea batatas* [L.] Lam) plants.

Sweetpotato is one of the seven major food crops in the world and a prospective industrial starch crop, with higher carbohydrate productivity than other starch crops grown on marginal lands (FAO, 2009; Mukhopadhyay et al., 2011; Ziska et al., 2009). Sweetpotato shows relatively high adaptability to harsh environmental conditions because of its high antioxidant activity. Moreover, sweetpotato is considered a nutritionally valuable crop, as it is rich in dietary fiber, potassium, minerals, and various antioxidants including anthocyanins, carotenoids, vitamin C, and tocopherols (Ishida et al., 2000; Wang et al., 2016). The content of antioxidants is abundant in leaves and storage roots of sweetpotato plants (Teow et al., 2007). Among antioxidants, the tocopherol content of storage roots is 0.26 mg per 100 g (USDA, 2018).

Previously, we isolated five tocopherol biosynthetic genes (*IbHPPD*, *IbHPT*, *IbMT*, *IbTC*, and *IbTMT*) in sweetpotato cultivar Yulmi and investigated their expression levels in leaves under drought, salt, and oxidative stresses (Ji et al., 2016). *IbHPPD* was highly expressed under drought and oxidative stresses; *IbHPT* was strongly induced under drought stress; and *IbMT* and *IbTC* were dramatically induced in response to salt stress treatment. Additionally, transient overexpression of these five tocopherol biosynthetic genes in tobacco leaves via agroinfiltration up-regulated the level of α-tocopherol (Ji et al., 2016). Therefore, transgenic sweetpotato plants overexpressing each tocopherol biosynthesis gene are currently being generated to understand the function of these genes. In this study, we successfully generated transgenic sweetpotato plants (referred to as TC plants) to demonstrate the function of overexpressing *IbTC* in sweetpotato. These TC plants show increased α-tocopherol content and enhanced tolerance to various abiotic stresses including salt, drought, and oxidative stresses.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Plants of sweetpotato cultivar Xushu 29 were grown in a growth chamber under controlled conditions (25 ± 3 °C temperature and 16 h light/8 h dark photoperiod). To analyze tocopherol content, leaves and storage roots of sweetpotato plants were harvested 3 weeks and 4 months after planting, respectively.

### 2.2. Phylogenetic analysis

Sequences of TC family members were obtained using BLAST searches in the GenBank database. Predicted amino acid sequences were determined from published nucleotide sequences and aligned using BioEdit and BoxShade server. Phylogenetic trees were constructed using the neighbor-joining method with 1000 bootstrap replicates with MEGA6.

### 2.3. RNA extraction and gene expression analysis

Total RNA was extracted from sweetpotato leaves using GeneAll Ribospin Plant™ kit (GeneAll, Seoul, Korea), according to the manufacturer's instructions. Subsequently, cDNA was synthesized using TopScript™ RT DryMIX (dT18) (Enzynomics, Daejeon, Korea). To analyze gene expression, quantitative reverse transcription PCR (qRT-PCR) was performed using gene-specific primers and Ever-Green 20

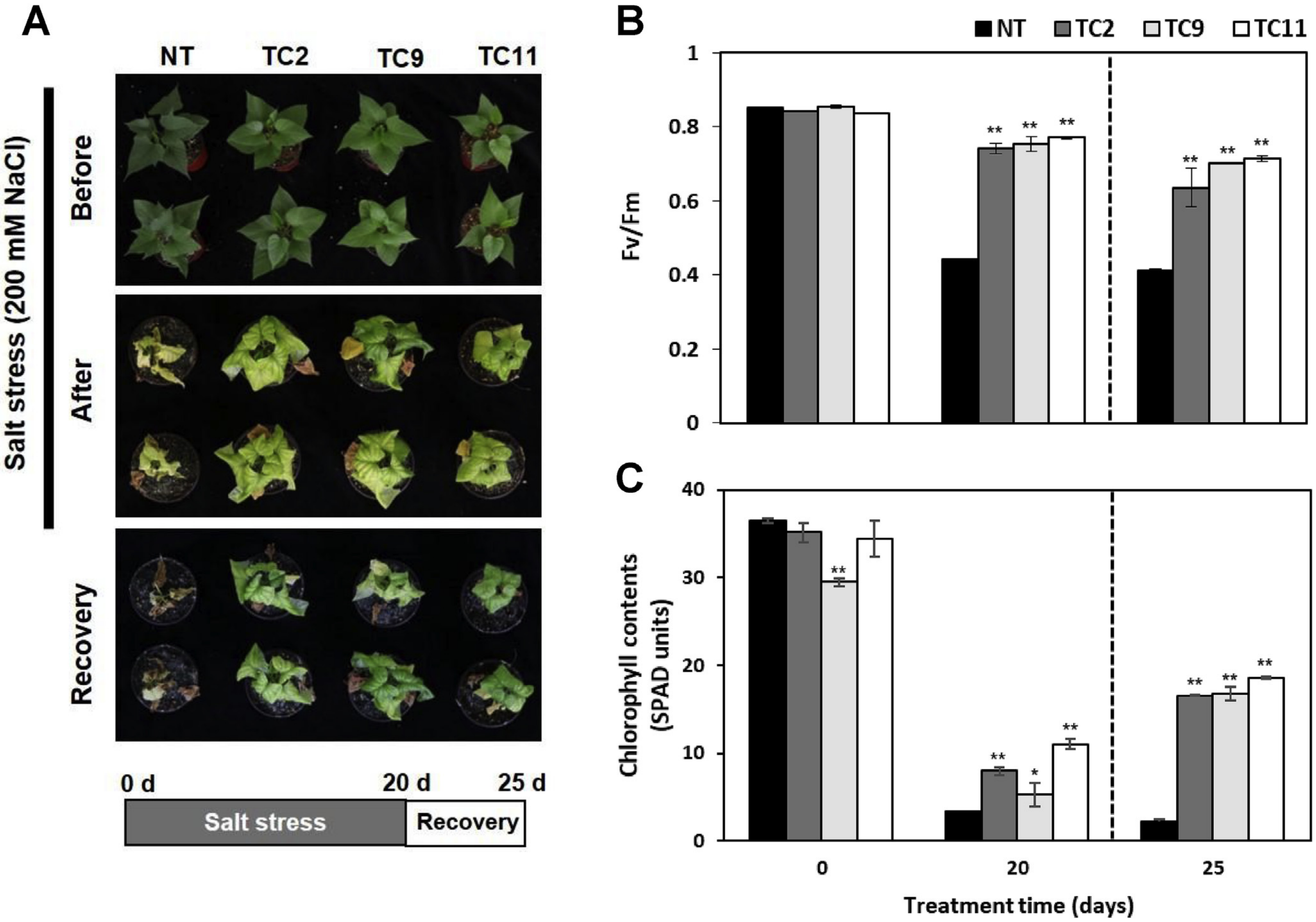

**Fig. 4.** Analysis of salt stress tolerance in TC and NT sweetpotato plants. **a** Images of plants treated with 200 mM NaCl for 20 days, followed by recovery for 5 days. **b** Photosynthetic efficiency (Fv/Fm) of photosystem II (PSII). **c** Relative chlorophyll content of sweetpotato plants. Data represent mean ± SD of three independent replicates. Significant differences between TC and NT plants are indicated with asterisks (*$P < 0.05$; **$P < 0.01$; One way ANOVA with Tukey's HSD post hoc test).

fluorescent dye (BioFact, Daejeon, Korea) on a CFX real-time PCR system with CFX system software (Bio-Rad, Hercules, CA, USA). Sweetpotato *ubiquitin* (*IbUbi*) gene was used as an internal control, and the gene expression level was assessed based on the CT values of three replicates.

### 2.4. Plasmid construction and generation of transgenic sweetpotato plants

The pGWB5 vector expressing a translation fusion of *IbTC* with the *green fluorescent protein* (*GFP*) gene under the control of cauliflower mosaic virus (CaMV) *35S* promoter (*35S*:*IbTC-GFP*), constructed previously (Ji et al., 2016), was transformed into embryogenic callus of sweetpotato (cv. Xushu 29) using *Agrobacterium tumefaciens* strain EHA105 (Lim et al., 2004). Transgenic calli were first screened on hygromycin-containing medium and then confirmed by genomic DNA PCR analysis of leaf samples using the following primers: 5′-CGCACA ATCCCACTATCCTT-3′ and 5′-AAATGGTATCCGCTGTGAGG-3'. To select *IbTC* overexpressing transgenic plants for the physiological function analysis of *IbTC*, qRT-PCR was performed using the following primers: 5′-CCGCTATGATGGCAGTAAAG-3′ and 5′-ATCCCTTCGATGTCAAC TGG-3'.

### 2.5. Analysis of tocopherol content

Leaves of 3-week-old plants and storage roots of 4-month-old plants were used for the analysis of tocopherol content. Plants were cultivated in plastic pots filled with soil in a growth room. Conditions used for the extraction of tocopherols and high performance liquid chromatography (HPLC) analysis were the same as those used for carotenoid analysis. The detection of tocopherols was conducted using a fluorescence detector at 450 nm (excitation) and 325 nm (emission) (Knecht et al., 2015). Tocopherol contents were expressed as the mean μg $g^{-1}$ dry weight (DW).

### 2.6. Salt and drought stress treatments

3-week-old sweetpotato plants were used for salt and drought stress treatments. To induce salt stress, plants were irrigated with 200 mM NaCl solution every 3 days for 20 days. To induce drought stress, plants were dehydrated for 18 days. After salt and drought stress treatments, plants were re-watered to examine recovery ability.

### 2.7. MV-mediated oxidative stress treatment, DAB staining, and ion leakage analysis

To induce oxidative stress, leaf discs (12 mm in diameter) excised from the third to fifth leaves of 1-month-old plants. The leaf discs were incubated in 3 μM methyl viologen (MV)-containing medium at 25 °C under dark for 12 h followed by incubation under light intensity at 150 mmol $m^{-2}$ $s^{-1}$. To visualize the degree of damage caused by oxidative stress, MV-treated leaf discs were floated on 3,3′-diaminobenzidine (DAB) solution (1 mg $ml^{-1}$ in water; pH 3.8). To measure the extent of cellular damage, ion leakage was quantified using an ion conductivity meter (MTD, Schwerzenbach, Switzerland).

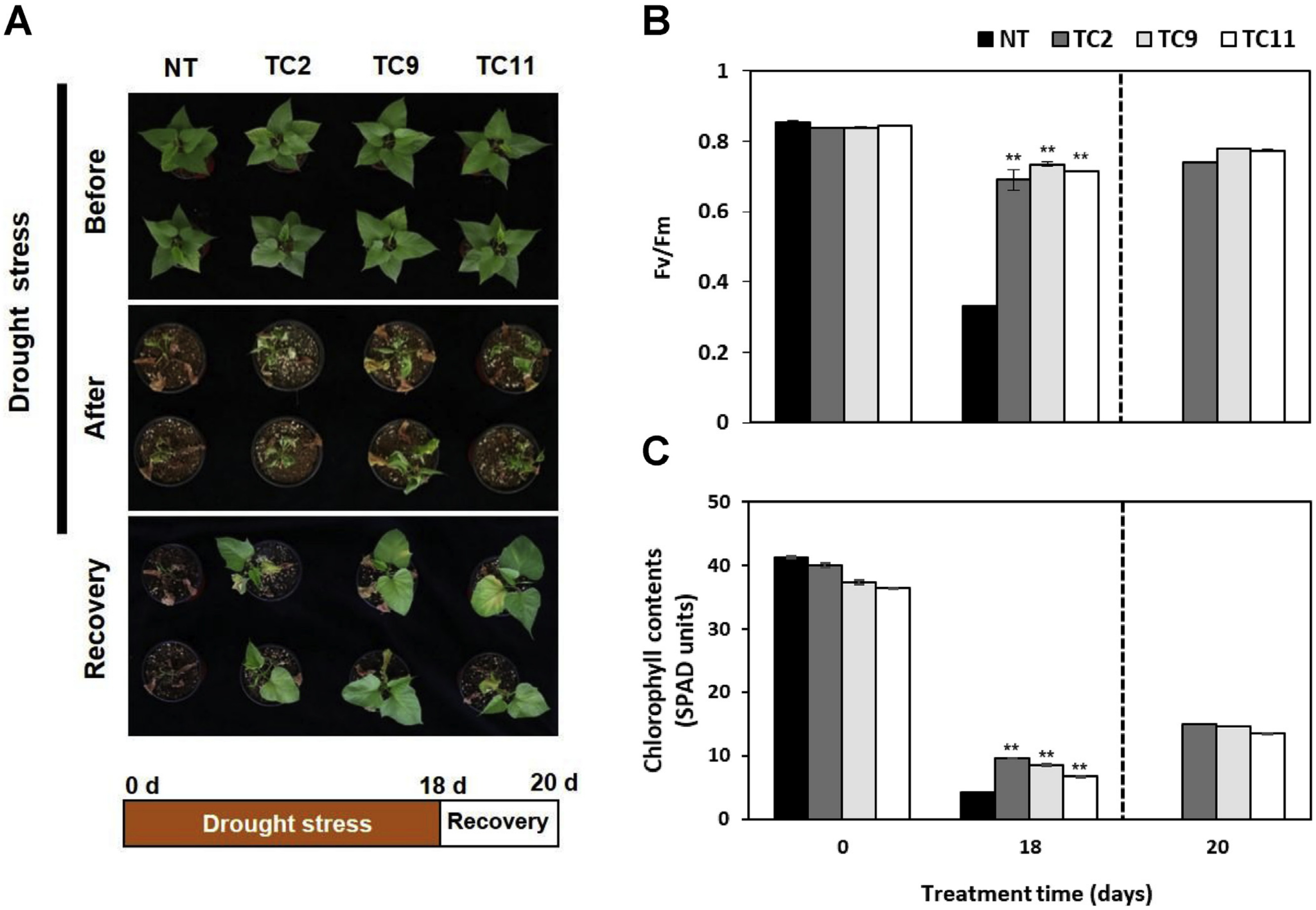

**Fig. 5.** Analysis of drought stress tolerance in TC and NT sweetpotato plants. **a** Images of plants subjected to drought stress for 18 days, followed by recovery for 2 days. **b** PSII photosynthetic efficiency (Fv/Fm). **c** Relative chlorophyll content of sweetpotato plants. Data represent mean ± SD of three independent replicates. Significant differences between TC and NT plants are indicated with asterisks ($^*P < 0.05$; $^{**}P < 0.01$; One way ANOVA with Tukey's HSD post hoc test).

### 2.8. Analysis of photosynthetic activity and chlorophyll content

To determine the photosynthetic efficiency of photosystem II (PSII), maximum quantum yield (Fv/Fm) of the third and fourth leaves from the top of plants was measured using a portable chlorophyll fluorescence meter (Handy pEA, Hansatech, England) after 30 min of dark adaption. Chlorophyll content of the third and fourth leaves from the top of plants was measured using a portable chlorophyll meter (SPAD-502, Konica Minolta, Japan).

### 2.9. Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA). The subsequent multiple comparisons of means were examined based on the Tukey's honestly significant differences (HSD) post hoc test. Statistical significance was set at $^*P < 0.05$ and $^{**}P < 0.01$.

## 3. Results

### 3.1. Phylogenetic analysis of TC

The *IbTC* has an open reading frame (ORF) of 1461 bp, and the deduced protein sequence of IbTC comprises 487 amino acids (Genbank accession number KP306524). To perform phylogenetic analysis, the amino acid sequence of IbTC was compared with that of TC enzymes in nine other plant species: tobacco (*Nicotiana tabacum*; NtTC), potato (*Solanum tuberosum*; StTC), sesame (*Sesamum indicum*; SiTC), common bean (*Phaseolus vulgaris*; PvTC), *Arabidopsis* (AtTC), eucalyptus (*Eucalyptus gunnii*; EgTC), cotton (*Gossypium hirsutum*; GhTC), rice (*Oryza sativa*; OsTC), and wheat (*Triticum aestivum*; TaTC) (Fig. 1a). Results of amino acid sequence alignment indicated that IbTC shares 85% sequence similarity with NtTC and StTC (Fig. 1b).

### 3.2. Generation of transgenic sweetpotato plants overexpressing IbTC

The *35S:IbTC-GFP* construct overexpressing the *IbTC-GFP* fusion under the control of the constitutive CaMV *35S* promoter was introduced into sweetpotato embryogenic callus (cv. Xushu 29) using *Agrobacterium*-mediated transformation (Fig. 2a). Eleven independent transgenic lines (referred to as TC plants) were generated and confirmed by genomic DNA PCR using cassette-specific primers (Fig. 2b). The expression level of *IbTC* was evaluated in all 11 TC plants using qRT-PCR, and three lines (TC2, TC9, and TC11) showing higher *IbTC* transcript levels than other lines were selected for further characterization (Fig. 2c).

### 3.3. TC plants display enhanced α-tocopherol content in leaves

To determine the effect of *IbTC* overexpression on the accumulation of tocopherol in transgenic sweetpotato plants, we analyzed the tocopherol content of 3-week-old leaves and 4-month-old storage roots of TC and NT plants (Fig. 3a and c). The predominant tocopherol in the sweetpotato tissues was determined as α-tocopherol, whereas β-, γ-, and δ-tocopherols were rarely detected. The contents of α-tocopherol in photosynthetic leaves of TC2 ($188.9 \pm 36.8\,\mu g\,g^{-1}$ DW), TC9 ($144.9 \pm 15.6\,\mu g\,g^{-1}$ DW), and TC11 ($298.9 \pm 16.0\,\mu g\,g^{-1}$ DW) were

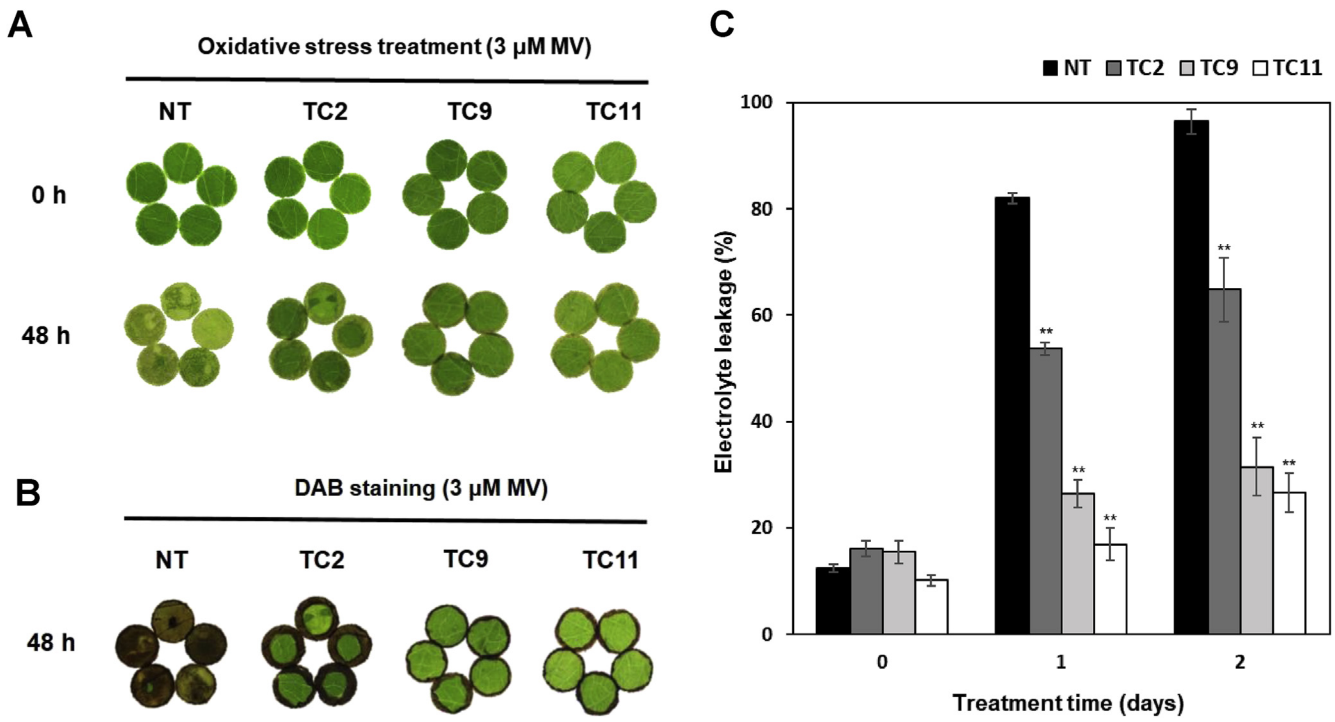

**Fig. 6.** Analysis of methyl viologen (MV)-induced oxidative stress tolerance in TC and NT sweetpotato plants. **a** Images of leaf discs treated with 3 μM MV for 48 h **b** Detection of reactive oxygen species (ROS) in leaves using DAB staining. **c** Ion leakage in detached leaves treated with 3 μM MV for 0, 1, and 2 days. Data represent mean ± SD of three independent replicates. Significant differences between TC and NT plants are indicated with asterisks ($^*P < 0.05$; $^{**}P < 0.01$; One way ANOVA with Tukey's HSD post hoc test).

1.6–3.3-fold higher than that of NT leaves (90.3 ± 17.2 $\mu g\, g^{-1}$ DW) (Fig. 3b). However, in storage roots, α-tocopherol content was only 10% higher in TC11 (33.5 ± 4.1 $\mu g\, g^{-1}$ DW) than in NT plants (31.6 ± 2.8 $\mu g\, g^{-1}$ DW) (Fig. 3d), and the α-tocopherol content of TC2 were similar to those of NT plants, while TC9 had lower α-tocopherol content.

### 3.4. *TC plants exhibit increased tolerance to salt stress*

To evaluate the effect of *IbTC* overexpression on salt stress tolerance, 3-week-old TC and NT plants were irrigated with 200 mM NaCl every 3 days for 20 days and then recovered for 5 days by re-watering. After salt stress treatment, NT plants showed symptoms of severe growth inhibition such as wilting and chlorosis compared with TC plants. This difference between NT and TC plants became more pronounced after recovery (Fig. 4a). Chlorophyll content and PSII efficiency (Fv/Fm) vary under environmental stresses and are used as indicators of the physiological status and photosynthetic activity of plants (Ouyang et al., 2011). During salt stress treatment and recovery period, all TC plants maintained higher levels of chlorophyll and photosynthetic activity than NT plants (Fig. 4b and c). Collectively, these data suggest that overexpression of *IbTC* increases salt tolerance of sweetpotato plants.

### 3.5. *TC plants show increased drought tolerance*

Under water deficient conditions, plants primary close stomatal apparatus and then produce antioxidants such as α-tocopherol to scavenge ROS. To determine the correlation between *IbTC* overexpression and drought stress tolerance in sweetpotato, 3-week-old TC and NT plants were dehydrated for 18 days and then recovered by re-watering for 2 days. Prior to drought stress, no difference was observed between TC and NT plants. However, after drought stress, NT plants showed severe wilting and failed to recover after re-watering (Fig. 5a). By contrast, TC plants showed consistently higher Fv/Fm values and chlorophyll content than NT plants throughout the experiment, which represent drought resistant phenotypes (Fig. 5b and c). Thus, our data suggest that TC plants are resistant to drought stress.

### 3.6. *TC plants exhibit enhanced tolerance to MV-mediated oxidative stress*

To investigate oxidative stress tolerance of TC plants, the overproduction of ROS in plant cells was evaluated following MV treatment. After MV-mediated oxidative stress treatment, the membrane damage can be confirmed by DAB staining and ion leakage analysis (Abbasi et al., 2007). Leaf discs excised from leaves of 1-month-old TC and NT plants were incubated in a medium containing 3 μM MV (Fig. 6a) and stained with DAB to determine the accumulation of hydrogen peroxide ($H_2O_2$). The intensity of DAB staining indicated that MV-induced damage in NT plants was more severe than that in TC plants (Fig. 6b). Additionally, ion leakage in NT plants was 81% at 24 h and 96% at 48 h post-treatment, indicating critical damage. By contrast, TC plants exhibited significantly less ion leakage (26–64%) at 48 h following MV treatment (Fig. 6c). Thus, TC plants showed greater resistance to MV-mediated oxidative stress than NT plants.

## 4. Discussion

In situations of lacking micro-nutrients such as vitamins and minerals for over 2 billion people worldwide and increasing of malnutrition in children, new nutrient-rich crop cultivars and stable food supply are needed to solve these problems (FAO, 2014). Tocopherols are one of the many nutrients essential for humans and animals, and daily consumption of tocopherols is required to maintain optimal health. However, tocopherols are synthesized only by photosynthetic organisms and therefore must be obtained from plant sources. Several studies have been conducted on the accumulation of tocopherols in plants (Schneider, 2005; Chen et al., 2006). Foods are biofortified with tocopehrol to improve their tocopherol content, while simultaneously converting β-, γ-, and δ-tocopherol to α-tocopherol, which shows the highest activity in the human body (Traber, 2007; Jiang et al., 2017). Tocopherols, especially α-tocopherol, play a key role in maintaining a proper redox state of chloroplastic antioxidant network in plants under environmental stresses (Munne-Bosch, 2005). Therefore, increasing the tocopherol content of plants by metabolic pathway engineering is necessary for improving plant growth under harsh conditions and for ensuring an optimum supply of tocopherol-rich foods (Vinocur and Altman, 2005; Lin, 2011).

Sweetpotato is considered an important health food ranked number one in 10 best foods by Center for Science in the Public Interest (CSPI) (CSPI, 2016). In many studies, contents of carotenoids and anthocyanins have been increased in sweetpotato plants by metabolic engineering (Kang et al., 2017; Park et al., 2015). However, research on the metabolic engineering of tocopherols in sweetpotato has not yet been reported. To enhance the tocopherol content and environmental stress tolerance of sweetpotato, we previously isolated five tocopherol biosynthetic genes (*IbHPPD*, *IbHPT*, *IbMT*, *IbTMT*, and *IbTC*) from sweetpotato (cv. Yulmi). The *IbTC* gene catalyzes the penultimate reaction of the tocopherol biosynthetic pathway to produce δ- and γ-tocopherol (Ji et al., 2016). In this study, we constitutively overexpressed the *IbTC* gene in sweetpotato and analyzed the physiological role of *IbTC* in tocopherol accumulation and abiotic stress tolerance.

In most plant species, α-tocopherol is mainly contained in leaves, and γ-tocopherol is the major form of tocopherol in seeds; however, β- and δ-tocopherol are rarely detected in plants (Munne-Bosch and Alegre, 2002; Szymanska and Kruk, 2008a, 2008b). In this study, TC plants showed 1.6–3.3-fold higher α-tocopherol content in leaves than in NT plants. However, in storage roots, the level of α-tocopherol accumulation was only 1.1-fold higher in TC plants than in NT plants (Fig. 3). A number of tocopherol forms are equally distributed in the envelope and thylakoid membranes of chloroplasts. Plastoglobules are lipoprotein particles in chloroplasts and storage subcompartments of thylakoid membranes. Plastoglobules contain the enzyme TC and approximately one-third of the total tocopherol content, thus representing an important site of tocopherol accumulation (Vidi et al., 2006; Austin et al., 2006; Maeda and DellaPenna, 2007). We speculated that the different results of α-tocopherol content changes in leaves and storage roots of TC plants might be attributed to plastid types depending on plant organs.

In TC and NT plants, β-, γ-, and δ-tocopherols were hardly detected (data not shown). In case of transgenic *Arabidopsis* plants overexpressing *AtTC* showed higher total tocopherol content but lower α-tocopherol content than NT plants because of an extreme shift from α-to γ-tocopherol (Kanwischer et al., 2005). It is possible that all four forms of tocopherols are converted to α-tocopherol in sweetpotato via an unknown mechanism. Previous report showed that α-tocopherol is mainly detected in several sweetpotato cultivars (Oki et al., 2006).

In several studies, tissue-specific promoters are preferred more than the CaMV *35S* promoter to prevent potential negative effects of constitutive gene expression and to increase efficiency (Cai et al., 2007; Gago et al., 2011; Torre et al., 2014). In many studies, tissue-specific promoters have been used to up-regulate the expression of tocopherol biosynthesis genes and consequently increase tocopherol content (Kumar et al., 2005; Lee et al., 2008; Arun et al., 2014). Overexpression of maize (*Zea mays*) *TC* (*ZmTC*) and *AtTC* in rapeseed (*Brassica napus*) plants under the control of a seed-specific promoter increased the content of δ-tocopherol in seeds by 3.2-fold and 6.7-fold, respectively, compared with NT seeds (Kumar et al., 2005). Moreover, overexpression of *IbMYB1* in sweetpotato plants using the storage root-specific *sporamin 1* (*SPO1*) promoter increased the anthocyanin content of storage roots to much higher levels than that using the stress-inducible sweetpotato *peroxidase anionic 2* (*SWPA2*) promoter, although opposite results were obtained for total carotenoid content (Park et al., 2015). Therefore, we speculated that the tocopherol content of transgenic plants may increase further if *IbTC* is expressed under the control of the tissue specific promoter including *SPO1* promoter.

Under stress conditions, antioxidants including α-tocopherol function to scavenge ROS in plants. Consequently, stress tolerant plant species have relatively higher α-tocopherol content than stress sensitive species (Munne-Bosch and Alegre, 2002; Munne-Bosch, 2005). Tomato (*Solanum lycopersicum*) plants with salt stress treatment (50 and 150 mM NaCl) increased α-tocopherol levels as antioxidant (Skłodowska et al., 2009). Transgenic rice (*Oryza sativa*) plants overexpressing tobacco (*Nicotiana tabacum*) *TC* (*NtTC*) and transgenic tobacco plants overexpressing *AtTC* exhibit greater drought stress tolerance and higher tocopherol content than NT plants (Woo et al., 2014; Liu et al., 2008; Mahajan and Tuteja, 2005). A study on ten grass species showed that drought stress induces tocopherol accumulation; tocopherol content increased by 3-fold in nine out of ten grass species under drought stress (Shao et al., 2007). In this study, we observed that TC plants were tolerant to high salt conditions, resulting in higher photosynthetic efficiency and chlorophyll content than NT plants. Additionally, leaves of transgenic plants showed enhanced recovery compared with NT plants after salt stress treatment (Fig. 4). Furthermore, unlike NT plants, TC plants restored normal phenotype upon rehydration after drought stress (Fig. 5). Additionally, at 48 h under MV-mediated oxidative stress, transgenic plants showed lower $H_2O_2$ accumulation and less ion leakage (26–64%) than NT (96%) (Fig. 6). However, when exposed to heat (45 °C for 1 d) and cold (4 °C for 2 d) stresses, no significant differences were observed between 3-week-old TC and NT plants (data not shown).

Tocopherols located in chloroplasts deactivate photosynthesis-derived ROS and inhibit lipid peroxidation by lipid peroxyl radicals in thylakoid membranes. Among the different forms of tocopherols, α-tocopherol plays an important role in protecting the photosynthetic apparatus via the network of chloroplastic antioxidants (Munne-Bosch, 2005). The content of α-tocopherol is regulated by abiotic stresses; under abiotic stresses, stress tolerant plants show higher α-tocopherol content than stress sensitive plants (Munne-Bosch and Alegre, 2002). Moreover, deficiency of α-tocopherol also increases the susceptibility of plants to photo-oxidative stresses (Porfirova et al., 2002; Kanwischer et al., 2005). In this study, accumulation of α-tocopherol in TC plants enabled the maintenance of high photosynthetic efficiency and chlorophyll content under salt and drought stress, and reduction of ion leakages under oxidative stresses. Thus, our results support the correlation between environmental stress tolerance and α-tocopherol accumulation in plants.

Our results suggest that *IbTC* overexpression will be useful for enhancing both abiotic stress tolerance and α-tocopherol content. In plant cells, tocopherols engage in interactive cooperation with antioxidants such as carotenoids, glutathione, and ascorbate to maintain redox homeostasis under abiotic stresses (Munne-Bosch, 2005; Szarka et al., 2012). Previously, transgenic sweetpotato plants overexpressing *IbOr-Ins* which is responsible for the accumulation of carotenoids showed enhanced accumulation of carotenoids and anthocyanins simultaneously (Park et al., 2015). Introduction of *IbTC* into *IbOr-Ins* transgenic sweetpotato plants containing high levels of anthocyanins and carotenoids could be used to enhance the level of various antioxidants. This represents a promising alternative strategy for gene stacking manner in molecular breeding, where multiple genes would be introduced for engineering complex traits and developing novel traits in transgenic crops (Halpin, 2005). Re-transformation of transgenic sweetpotato plants with additional *IbTC* gene copies could improve abiotic stress tolerance and facilitate the development of sweetpotato cultivars rich in antioxidants including anthocyanins, carotenoids, and tocopherols.

Overall, in this study, we successfully generated transgenic sweetpotato plants overexpressing *IbTC*. TC plants showed higher α-tocopherol content in leaves than NT plants. Additionally, TC plants exhibited increased tolerance to MV-mediated oxidative stress and resistance to abiotic stresses, such as high salt and drought, than NT plants. These results suggest that accumulation of α-tocopherol enables plants to overcome various environmental stresses.

## Author contributions

S.E. Kim, C.J. Lee and S.S. Kwak were responsible for the conception, planning, and organization of experiments. S.E. Kim, C.J. Lee, C.Y. Ji, H.S. Kim, S.U. Park, Y.H. Lim., and W.S. Park carried out plant transformation, quantitative reverse transcription PCR, abiotic stress analysis, and tocopherol content analysis. M.J. Ahn, X. Bian, Y. Xie, X. Guo and S.S. Kwak analyzed the data. S.E. Kim, C.J. Lee, and S.S. Kwak wrote the manuscript. S.E. Kim and C.J. Lee prepared the figures, with support from S.S. Kwak.

## Declaration of competing interest

The authors declare that they have no conflict of interest.

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