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Ectopic overexpression of an *Arabidopsis* monothiol glutaredoxin *AtGRXS17* affects floral development and improves response to heat stress in chrysanthemum (*Chrysanthemum morifolium* Ramat.)

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ABSTRACT

Chrysanthemum is one of the most economically valuable ornamental plants worldwide. However, the high temperature is a hindrance to chrysanthemum cultivation, causing delayed flowering, poor quality of ray florets, and reduced shelf life. Here, we report that ectopic overexpression of an *Arabidopsis* monothiol glutaredoxin *AtGRXS17* in chrysanthemum improved response to heat stress, 35/25 °C (day/night temperatures) and 45/25 °C following 35/25 °C, in terms of flowering time and ray floret quality, respectively. Under heat stress conditions, *AtGRXS17*-overexpressing (*AtGRXS17*-OE) chrysanthemum plants had reduced H_2O_2 production, lower oxidative damage of cellular membranes, and greater photosynthetic ability compared to wild-type controls. Ectopic overexpression of *AtGRXS17* promoted faster flower bud formation, resulting in earlier flowering, and prevented heat stress-caused loss of ray florets shape and wilting compared to wild-type controls. The findings demonstrate an approach to enhance quality flower production in a valuable ornamental crop.

1. Introduction

Chrysanthemums are globally important ornamental flowers, which can be cultivated year-round under either natural or artificially controlled environments (Kjaer and Ottosen, 2011; Khoddamzadeh, 2016). During summer cultivation, high temperatures often cause delayed flowering and deterioration in flower quality, resulting in poor shelf life (Nozaki et al., 2006; Wang et al., 2008). Conventional and molecular breeding efforts have been made to maintain appropriate flowering time and flower quality, under heat stress without success, presumably due to the polygenic nature of the traits (Wang et al., 2008; Hong et al., 2009; da Silva et al., 2013). The transfer of foreign genes has also been used in attempts to improve the characteristics such as tolerance to heat stress and male sterility in chrysanthemum (Shinoyama et al., 2012; da Silva et al., 2013). For example, ectopic overexpression of *Arabidopsis AtDREB1A* in chrysanthemum increases heat tolerance, photosynthetic capacity, RuBisCO biosynthesis, and sucrose-phosphate synthase activities (Hong et al., 2009). The latter study indicates that improvement of heat tolerance in chrysanthemum plants may be associated with photosynthesis ability. The inhibition of photosynthesis by heat stress has been associated with decreased activity of RuBisCO in various plant species (Berry and Bjorkman, 1980; Salvucci and Crafts-Brandner, 2004). Despite progress, none of the studies have been shown to enhance flowering time and flower quality under heat stress conditions, though regulation of a nuclear factor gene, such as *CmNF-YB8*, coordinating aging is able to modulate flowering time under normal temperature condition (Wei et al., 2017).

Glutaredoxins (GRXs) are small ubiquitous oxidoreductases that

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play roles in regulating plant responses to heat stress, flowering time, flower development, and antioxidant metabolism (Xing et al., 2005; Rouhier et al., 2008; Xing and Zachgo, 2008; Li et al., 2009). GRXs belong to the thioredoxin (TRX) superfamily and catalyze the reduction of disulfide bonds of their substrate proteins in a glutathione (GSH)-dependent manner, which is associated with detoxification of reactive oxygen species (ROS) and heavy metal, xenobiotic conjugation, and cellular functions (Rouhier et al., 2007, 2008; Verma et al., 2016; Begas et al., 2017). Plant GRXs are classified into four types based on the motif sequence, namely CPYC, CGFS, CC, and GRL-type (Ziemann et al., 2009; Garg et al., 2010). The AtGRXS17, one of *Arabidopsis thaliana* monothiol GRXs, is grouped into the CGFS-type (class II) based on its predicted amino acid sequence and the composition of active-site motif (Rouhier et al., 2006; Garg et al., 2010). When it was ectopically overexpressed in tomato, the transgenic plants showed tolerance to heat, chilling, and drought stresses (Wu et al., 2012, 2017; Hu et al., 2015). Knocking out *AtGRXS17* in *Arabidopsis* resulted in a reduced heat tolerance and delayed flowering (Cheng et al., 2011; Knuesting et al., 2015). Therefore, we hypothesized that ectopic overexpression of *AtGRXS17* in chrysanthemum would preserve flowering time and improve response to heat stress. Here, we report engineering *AtGRXS17*-overexpressing (*AtGRXS17*-OE) chrysanthemum plants and examine the effect of *AtGRXS17*-OE on flowering time and flower quality under heat stress conditions, which are a critical barrier of quality cut- and pot-flower production.

2. Materials and methods

2.1. Plant materials and growth conditions

Three spray-type and one standard-type chrysanthemum (*Chrysanthemum morifolium* Ramat.) cultivars with high marketability were used for this study: 'Vivid Scarlet', 'Peach ND', and 'Orange ND' (autumn-flowering spray-type cultivars with 13-14-h critical day-length; deep red-, very pale pink-, and yellow-colored ray florets, respectively) and 'Backma' (summer-autumn flowering standard-type cultivar with 12-14-h critical day-length; white-colored ray florets) which frequently suffer from high temperature caused by black-clothing the greenhouse to employ a short-day photoperiod for floral induction in summer season of long-day photoperiod. Plantlets of each cultivar were produced *via* shoot tip culture according to the previous report (Bush et al., 1976). The shoot tip-derived plantlets were propagated by a single-node culture on the MS medium (Murashige and Skoog, 1962) (Duchefa Biochemie, The Netherlands) containing 0.2% of activated charcoal (Avondale Laboratories, England) and 30 g/L sucrose (Duchefa Biochemie), solidified with 8 g/L Plant Agar™ (Duchefa Biochemie) following adjustment of pH to 5.8. Leaf explants for plant regeneration test and *Agrobacterium*-mediated transformation were cut into about 0.3 × 0.5 cm pieces after detaching the fully expanded leaves from the 5-week-old plantlets.

2.2. Plasmid vector, bacterial strain, and Agrobacterium-mediated transformation

AtGRXS17 cDNA (GenBank AT4G04950: monothiol gultaredoxin-S17) was synthesized from the RNA of *Arabidopsis thaliana* Col-0 and inserted into the pBI121 vector (GenBank AF485783.1) to construct pBI121-*AtGRXS17*, replacing the *GUS* gene flanked by *Xba* I and *Sac* I, in which the *AtGRXS17* is driven by the cauliflower mosaic virus (CaMV) 35S promoter (Wu et al., 2012). The plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 using the freeze-thaw method (Holsters et al., 1978). Prior to transformation, cultivar and hormone combinations were optimized for plant regeneration (supplementary Fig. 1). *Agrobacterium*-mediated transformation was performed with a modified procedure (Han et al., 2008). Explants of 'Vivid Scarlet' and 'Peach ND' were immersed in a liquid co-cultivation medium [one-eighth strength MS, 20 g/L sucrose, 1.0 mg/L BA, 0.5 or 1.0 mg/L IAA, 200 μM acetosyringone (Sigma-Aldrich, USA), pH 5.4] with re-suspended *A. tumefaciens* cells containing pBI121-*AtGRXS17* (OD_{600} = 1.0–1.2) for 10 min. After inoculation, explants were transferred to a semi-solid co-cultivation medium (liquid co-cultivation medium with 7 g/L Plant Agar™), followed by incubating at 25 °C in darkness for three days. The explants were then sub-cultured on an shoot induction medium [MS, 30 g/L sucrose, 1.0 mg/L BA, 0.5 or 1.0 mg/L IAA, 400 mg/L cefotaxime sodium salt (Duchefa Biochemie), 7 g/L Plant Agar™, pH 5.7] in the dark for ten days. The explants were cultured on a selection medium containing the selectable antibiotics [MS, 30 g/L sucrose, 1.0 mg/L BA, 0.5 or 1.0 mg/L IAA, 400 mg/L cefotaxime sodium salt, 20 mg/L kanamycin monosulfate (Sigma-Aldrich), 7 g/L Plant Agar™, pH 5.7] in the dark for five weeks. Regenerated shoots were transferred to a rooting medium (half strength MS, 30 g/L sucrose, 10 mg/L kanamycin monosulfate, 7 g/L Plant Agar™, pH 5.7) and cultured under light condition (16-h day-length, 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The concentration of kanamycin monosulfate in the selection and rooting media was decided through a preliminary test (supplementary Fig. 2). Regenerated plantlets were then transferred to 9 cm diameter plastic pots with a commercial compost (Chamsarang™: Nongwoo Bio Co., Korea). Potted plantlets were acclimatized according to the method by the previous report (Han et al., 2004) and cultivated in a rain shelter.

2.3. Nucleic acid analyses

Acclimatized T_0 plants were subjected to polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) analyses to verify the integration of foreign genes into chrysanthemum genome and the transcription of *AtGRXS17*, respectively. Primer sets were designed to amplify *AtGRXS17* and *NPTII*: forward primer 5′-ATGAGCGGTACGGTGAAGGATA-3′ and reverse primer 5′-TCAACC ACTTTCCCACTGAA-3′ for *AtGRXS17*, forward primer 5′- ACAAGATG GATTGCACGCAGG-3′ and reverse primer 5′-AACTCGTCAAGAAGGCG ATAG-3′ for *NPTII* (Flachowsky et al., 2011). Genomic DNA was extracted from the fully expanded upper leaf of the completely acclimatized plantlet at about eight- to ten-leaf stage using the Plant DNeasy Mini kit (QIAGEN, USA). PCR was carried out using 2x Hot Taq Master Mix (PhileKorea, Korea) with each gene-specific primer set. The transformation efficiency was determined as the percentage of the number of PCR-positive plants/number of *Agrobacterium*-inoculated leaf explants. Total RNA was extracted from lately expanded young upper leaves of preferentially obtained nine PCR-positive plants using Plant RNeasy Mini kit (QIAGEN). First-strand cDNAs were synthesized from 80 ng of total RNA with random hexamers using SuperScript III First-strand cDNA Synthesis Kit (Invitrogen, USA) according to the manufacturer's instructions. The primer set mentioned above for detection of *AtGRXS17* gene was used for cDNA amplification. All gel images were taken using a molecular imaging system (Kodak Image Station 4000 MM Pro: Carestream Health Inc., Rochester, NY, USA) driven by an imaging software (Carestream Ver. 5.0: Carestream Health Inc.).

2.4. Ploidy analysis

Ploidy levels of the transgenic plants were examined to exclude false analysis of the phenotypes due to altered ploidy levels. Leaf disks at about 5 mm^2 were cut from *in vitro* plantlets and chopped with a sharp razor blade in the nuclei extraction buffer (Partec, Germany). One minute later, each sample was filtered through a 30 μm nylon mesh supported by a tube (Patec). Two milliliter of staining buffer (Partec) was added to each tube containing the filtrate. After inserting the tube at the probe of ploidy analyzer (PAII: Partec), the ploidy of sample was measured.

2.5. Multiplication of stock plants by cutting

The distal parts at 5 ± 1 cm length from main shoots of wild type and *AtGRXS17*-OE chrysanthemum plants were detached, planted in 9 cm diameter plastic pots containing a commercial compost (Nongwoo Bio Co.), and cultivated in a growth room (16-h day-length, 75 μmol·m^{-2}·s^{-1}, 25 ± 2 °C) for thirty-five or forty days.

2.6. Flowering time measurements under natural and artificial heat stress conditions

For comparison of flowering time between wild type and *AtGRXS17*-OE chrysanthemum plants under a natural condition, especially with natural temperature and photoperiod changes in a rain shelter at 36°22′45.95″N/128°8′46.30″E (supplementary Fig. 3), forty-day-old stock plants with about twelve leaves were transplanted into 30 cm diameter plastic pots containing the commercial compost mixed with Osmocote™ Pro 3–4 M (30 g/pot; Everris, Geldermalsen, The Netherlands) and cultivated in a rain shelter on April 25th in spring 2014. In addition, on April 28th in spring 2015, the newly propagated forty-day-old stock plants were directly transferred into the growth chambers with a short-day photoperiod (daytime: 10 a.m. to 6 p.m., 150 μmol·m^{-2}·s^{-1}; nighttime: 6 p.m. to 10 a.m. of the following day) and temperatures of continuous 35 °C or 35/25 °C (day/night temperatures) to investigate flowering time under mild heat stress conditions. The flowering time was determined as the date when at least 50% of the flower buds of an individual plant completely opened, and the period taken from stem cutting date to flowering date was calculated.

2.7. Heat treatment of plants

To determine a heat stress condition capable of distinguishing a phenotypic difference without simultaneous extreme damage in both wild type and *AtGRXS17*-OE chrysanthemum plants in 2014, wild type 'Vivid Scarlet' plants were first subjected to three different heat treatments under a long-day photoperiod (daytime: 6 a.m. to 10 p.m., 150 μmol·m^{-2}·s^{-1}; nighttime: 10 p.m. to 6 a.m. of the following day): constant 35/25 °C (day/night temperatures) for 6 days, 45/25 °C for 3 days after 35/25 °C for 3 days, and constant 45/25 °C for 2 days. Of them, the treatment elevating temperature (45/25 °C for 3 days after 35/25 °C 3 days) by the results from the chlorophyll fluorescence quenching analysis was selected for further heat stress condition treatment. In 2015, to examine heat-response of wild type and *AtGRXS17*-OE chrysanthemum plants at vegetative phase, the thirty-five-day-old stock plants were transferred into a growth chamber with the long-day photoperiod (daytime: 6 a.m. to 10 p.m., 150 μmol·m^{-2}·s^{-1}; nighttime: 10 p.m. to 6 a.m. of the following day) and treated by the selected heat stress condition. Meanwhile, to examine heat-response of the flowers in wild type and *AtGRXS17*-OE chrysanthemum plants, the short-day photoperiod (daytime: 10 a.m. to 6 p.m., 150 μmol·m^{-2}·s^{-1}; nighttime: 6 p.m. to 10 a.m. of the following day) at 25 ± 2 °C was used for wild type stock plants seven days earlier based on the result of the last year experiment under a natural condition, which allowed synchronization of flowering time between wild type and *AtGRXS17*-OE plants of 'Vivid Scarlet'. Blooming plants were treated at 35/25 °C for one week and subsequently at 45/25 °C for one week.

2.8. Chlorophyll fluorescence quenching analysis

Chlorophyll fluorescence quenching analysis was performed every day throughout the duration of the heat treatment for thirty-five-day old stock plants at vegetative phase using a pulse-amplitude modulated chlorophyll fluorometer (Mini PAM: Walz, Germany) according to manufacturer's instructions and the method for *Arabidopsis* (Brooks and Niyogi, 2011): Briefly, the chlorophyll fluorescence from the adaxial side of leaf that adapted in the dark for more than 30 min was measured during the periodic irradiation of saturation pulse to draw an induction curve. Then, maximum photochemical efficiency (Fv/Fm) of photosystem II (PSII), electron transport rate (ETR) through PSII, PSII operating efficiency (Φ_{PSII}), and coefficient of photochemical quenching (qP) were collected.

2.9. Electrolyte leakage measurement

Leaf disks, including main vein at 1 cm diameter from the second and third fully expanded leaves of each plant, were punched out using a cork borer after heat treatment for six days. The two disks were immersed together in a 15 mL de-ionized water, subjected to reduce pressure in a vacuum chamber, and incubated at room temperature for 2 h. The primary electrolyte leakage (Eo) was measured using an electrical conductivity meter (Orion Star A325: Thermo Fisher Scientific Inc., USA). After measuring the Eo, the samples were autoclaved and held at room temperature for 12 h to cool off. The total electrolytes (Et) was measured, and percentage of primary electrolyte leakage was calculated using the formula, EL (%) = (Eo/Et) × 100 (Oh et al., 2009).

2.10. Histochemical detection of H_2O_2

Endogenous H_2O_2 of leaves was visualized by staining with 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich) as described previously (Wu et al., 2012; Hu et al., 2015). The first and fourth fully expanded leaves from a plant were sampled and completely submerged in a 0.1% DAB solution (pH 3.8). The samples were incubated overnight in darkness and then bleached in boiling ethanol (94%) three times. Quantitative analysis of DAB staining was conducted using Image J App. (http://imagej.nih.gov/ij/index.html).

2.11. Statistical analysis

All experiments were conducted with at least three replicates: The means for transgenic (*AtGRXS17*) and wild type treatments represent the values measured from at least twelve and three plants, respectively. The transgenic (*AtGRXS17*) treatment also contained at least four plants each from the examined *AtGRXS17*-OE lines, unless otherwise indicated. Data were presented as mean ± standard deviation (S.D.), of which separation was performed using the *t*-test or least significant difference (LSD) test. The collected raw data were subjected to one-way analysis of variance (ANOVA) before each statistic test using SAS software version 9.3 (SAS Institute, USA).

3. Results

3.1. Generation of AtGRXS17-OE chrysanthemum plants

in vitro regeneration ability of four cultivars ('Vivid Scarlet', 'Peach ND', 'Orange ND', and 'Baekma') and the response to kanamycin monosulfate in two cultivars ('Vivid Scarlet' and 'Peach ND') were examined prior to the *Agrobacterium*-mediated transformation of chrysanthemum. Under the present tissue culture conditions, 'Vivid Scarlet' and 'Peach ND' displayed an excellent performance in terms of shoot regeneration percentage and the number of regenerated shoots per explant while 'Orange ND' and 'Baekma' showed low frequency of shoot regeneration with rare shoots per explant and gradual necrosis of regenerated shoots, respectively, which pushed through with 'Vivid Scarlet' and 'Peach ND' as the cultivars for rapid approach to the *Agrobacterium*-mediated transformation (supplementary Fig. 1). Effective shoot regeneration media contained 1.0 mg/L BA plus 1.0 mg/L IAA for 'Vivid Scarlet' and 1.0 mg/L BA plus 0.5 mg/L IAA for 'Peach ND' (supplementary Fig. 1). In addition, 20 mg/L kanamycin was used for selection of transgenic shoots (supplementary Fig. 2). A total of 2540 explants (1595 for 'Vivid Scarlet' and 945 for 'Peach ND') were co-

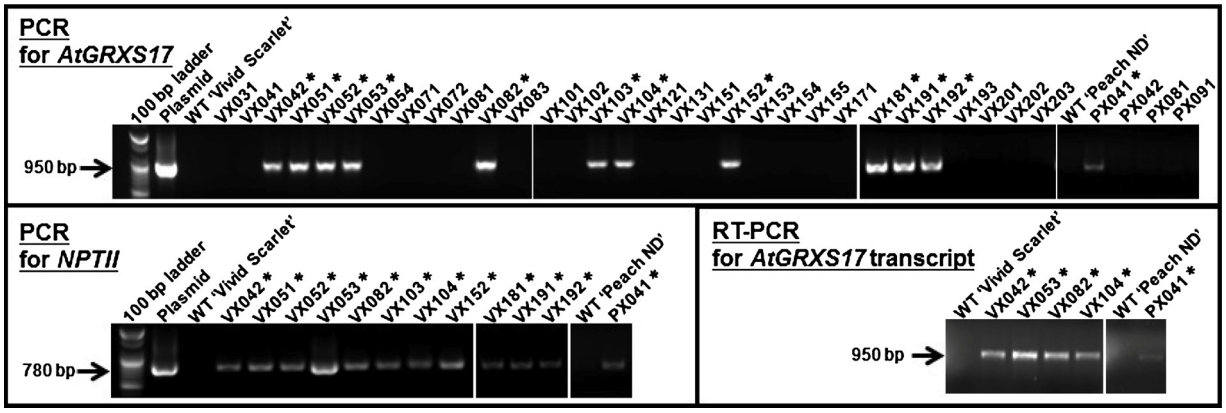

Fig. 1. Detection of transgenes and *AtGRXS17* transcripts in acclimatized plants by PCR and RT-PCR analyses, respectively. Asterisks indicate positive plants. All images were taken using a molecular imaging system (Kodak Image Station 4000 MM Pro: Carestream Health Inc., Rochester, NY, USA) driven by an imaging software (Carestream Ver. 5.0: Carestream Health Inc.). Full-length gels are presented in supplementary Fig. 5.

cultivated with *A. tumefaciens* harboring pBI121-*AtGRXS17*. Fourteen weeks later, a total of 320 shoots (229 in 'Vivid Scarlet' and 91 in 'Peach ND') were regenerated, and a total 130 shoots (96 in 'Vivid Scarlet' and 34 in 'Peach ND') of them were rooted on a rooting medium containing 10 mg/L kanamycin monosulfate (supplementary Fig. 4 and supplementary Table 1).

PCR analyses for the acclimatized plants revealed that twelve plants (eleven in 'Vivid Scarlet' and one in 'Peach ND') contained both *AtGRXS17* and *NPTII* genes (Fig. 1 and supplementary Fig. 5a–f), showing transformation efficiency to approximately 0.69% in 'Vivid Scarlet' and 0.11% in 'Peach ND' (supplementary Table 1). To examine the transcription of the *AtGRXS17* in the PCR-positive plants, RT-PCR analysis for the preferentially obtained nine T_0 plants including eight 'Vivid scarlet' and one 'Peach ND' T_0 plants was carried out and showed the presence of expected 950 bp *AtGRXS17* transcripts in all the examined T_0 plants (Fig. 1 and supplementary Fig. 5g–h).

in vitro culturing conditions with different growth regulators and long culturing periods can affect the ploidy of plant cells (Ochatt et al., 2011; Niedz and Evens, 2016). Therefore, all regenerants including twelve transgenic plant lines were subjected to ploidy analysis. Thirteen out of 130 regenerants showed an abnormality in ploidy level. Regardless, all twelve transgenic plants showed normal ploidy (supplementary Fig. 6). Preferentially obtained three transgenic lines (mainly VX042, VX053, and VX082) were randomly selected for further analyses of heat stress response, flowering time, and ray floret quality after multiplication by cutting.

3.2. Phenotype of AtGRXS17-OE chrysanthemum plants under natural growth conditions

The wild type and *AtGRXS17*-OE chrysanthemum plants propagated by cutting were cultivated, and the phenotypes were compared during the same period under natural growth conditions in a rain shelter. Overall growth and morphology of *AtGRXS17*-OE plants were visually indistinguishable from those of wild type plants (Fig. 2). However, it took 201 (October 3rd in 2014) and 207 (October 9th in 2014) days on average ($p < 0.001$) up to developing floral buds after cutting in *AtGRXS17*-OE and wild type 'Vivid Scarlet' plants, respectively (Fig. 3 and Suppl. Fig. 7). The earlier formation of floral buds in the transgenic plants was subsequently connected with earlier flowering, which took 215 (October 17th in 2014) days on average that is about seven days shorter ($p < 0.001$) than 222 (October 24th in 2014) days of wild type plants (Fig. 2). Meanwhile, it took 222 (October 24th in 2014) and 229 (October 31st in 2014) days ($p < 0.001$) up to flowering after cutting in *AtGRXS17*-OE and wild type 'Peach ND' plants, respectively (Fig. 2).

3.3. Response and adaptation to heat stress in AtGRXS17-OE chrysanthemum plants

Four *AtGRXS17*-OE plants derived from each of three transgenic lines (VX042, VX053, and VX082) were analyzed under the selected heat stress condition, 45 °C for 3 days following 35 °C for 3 days which causing a drastic decline of photosynthetic ability in wild type plants (supplementary table 2). The visual phenotypes of the *AtGRXS17*-OE plants encountered with heat stress appeared healthier than those of severely stunted and wilted wild type controls (Fig. 4a). The newly developing leaves of *AtGRXS17*-OE plants were green and heathy, while those of wild type plants were necrotized under the heat stress conditions (Fig. 4b).

3.4. Photosynthetic ability, electrolyte leakage, and H_2O_2 accumulation in AtGRXS17-OE chrysanthemum plants

The *AtGRXS17*-OE 'Vivid Scarlet' chrysanthemum lines showed relatively higher level of photosynthesis than that of wild type controls after the heat treatment [45/25 °C for 3 days following 35/25 °C for 3 days]. Fv/Fm (potential quantum yield), Φ_{PSII} (quantum yield of photosystem II), and ETR (electron transport rate) values of *AtGRXS17*-OE 'Vivid Scarlet' plants were not statistically different from those of wild type plants up to five days after heat treatment (Fig. 5a). However, the four parameters including qP (coefficient of photochemical quenching) on six days after heat treatment obviously revealed the differences ($p < 0.01$) in the values: 0.746/0.674 ($LSD_{0.01} = 0.025$) (*AtGRXS17*-OE/wild type 'Vivid Scarlet' plants) in Fv/Fm, 0.523/0.396 ($LSD_{0.01} = 0.061$) in Φ_{PSII}, 8.357/6.273 ($LSD_{0.01} = 1.001$) in ETR, and 0.780/0.709 ($LSD_{0.01} = 0.020$) in qP (Fig. 5a).

In addition to the photosynthetic performance, the *AtGRXS17*-OE 'Vivid Scarlet' and 'Peach ND' plants were compared with the wild type plants by H_2O_2-DAB staining and electrolyte leakage after heat stress treatment (Fig. 5b and c, and supplementary Fig. 8). On sixth day after heat treatment, electrolyte leakage of the detached leaves from the wild type plants was higher than the *AtGRXS17*-OE plants: 11.5% (wild type) and 9.6% (*AtGRXS17*-OE) in 'Vivid Scarlet' ($p < 0.001$), 82.0% (wild type) and 66.3% (*AtGRXS17*-OE) in 'Peach ND' ($p < 0.05$) (Fig. 5c and supplementary Fig. 8b). Moreover, the leaves of *AtGRXS17*-OE plants displayed brighter brown intensity than those of wild type plants when stained by DAB after heat stress treatment (Fig. 5b and supplementary Fig. 8a), which indicates that the amount of H_2O_2 in *AtGRXS17*-OE plants is significantly less ($p < 0.05$) than that in wild type plants after heat stress.

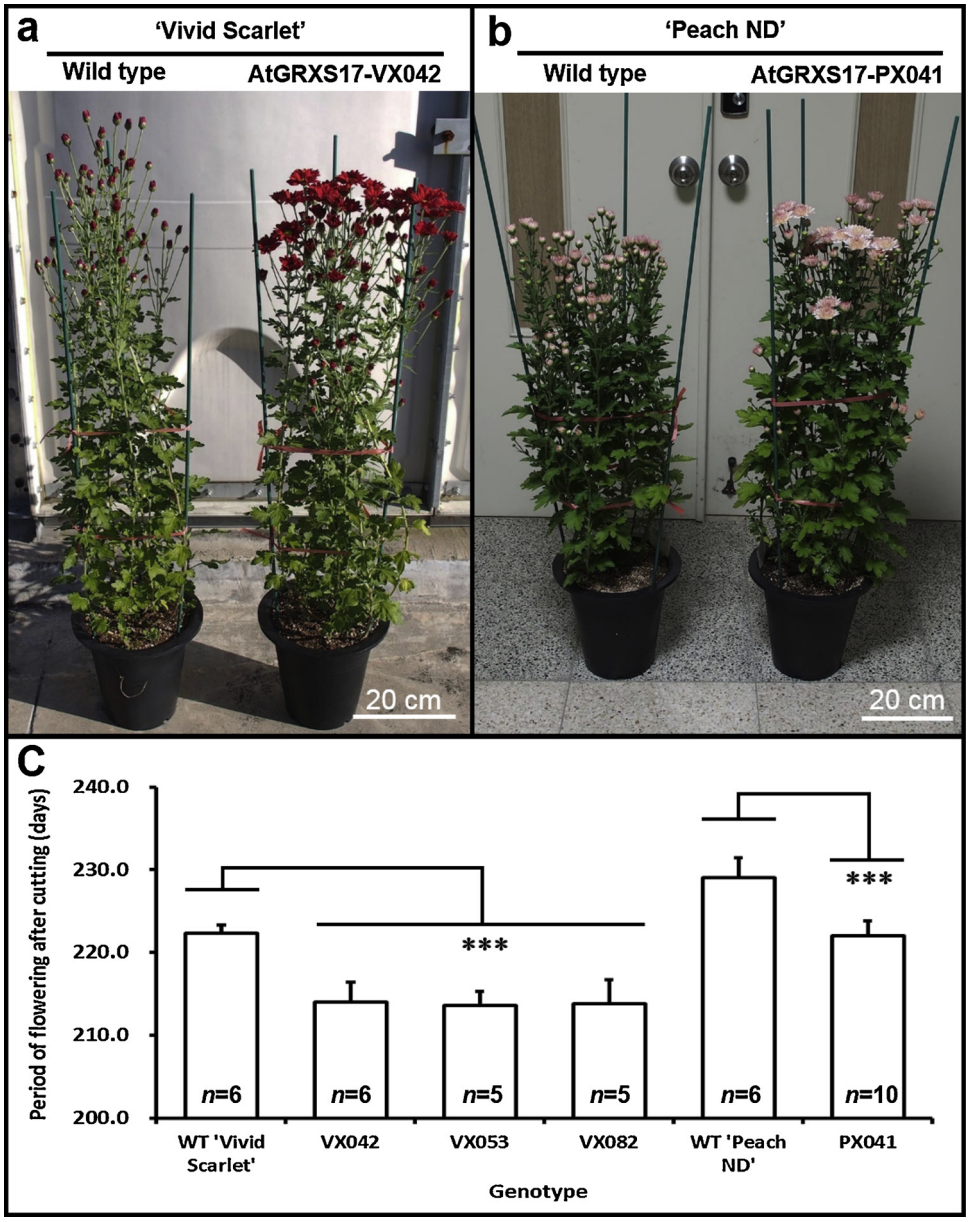

Fig. 2. Phenotypes during the flowering period under natural growth conditions. Data represent means ± S.D. from five to ten independent biological replicates and were analyzed using Student *t* test, $^{***}p < 0.001$.

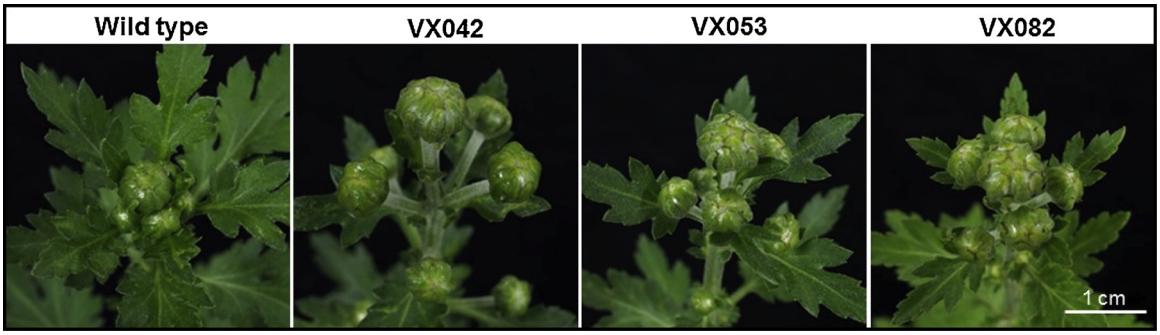

Fig. 3. Ectopic overexpression of *AtGRXS17* in chrysanthemum 'Vivid Scarlet' plants promotes faster flower bud formation than wild type plants under natural growth conditions.

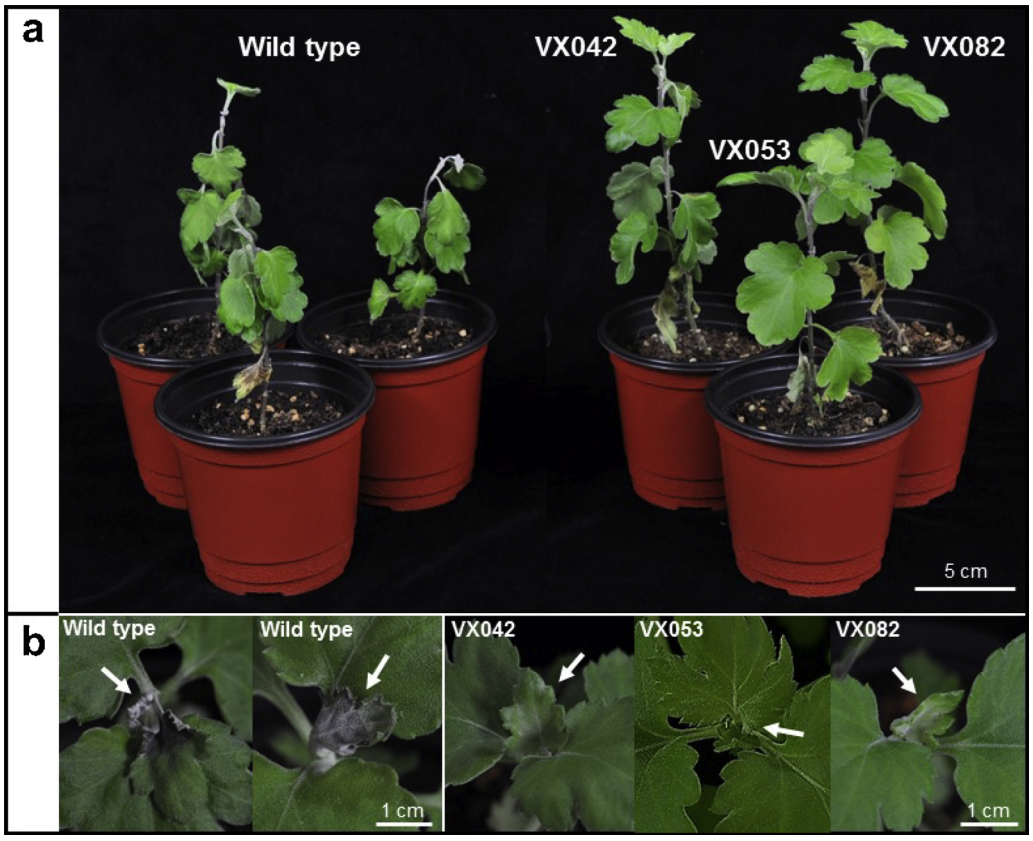

Fig. 4. Performance of *AtGRXS17*-OE chrysanthemum 'Vivid Scarlet' plants under heat stress conditions [45/25 °C (day/night temperatures) for 3 days following 35/25 °C for 3 days]. *AtGRXS17*-OE and wild type plants after heat stress (a) and the newly developing leaves of *AtGRXS17*-OE and wild type plants after heat stress (b). The arrows indicate the newly developing leaves.

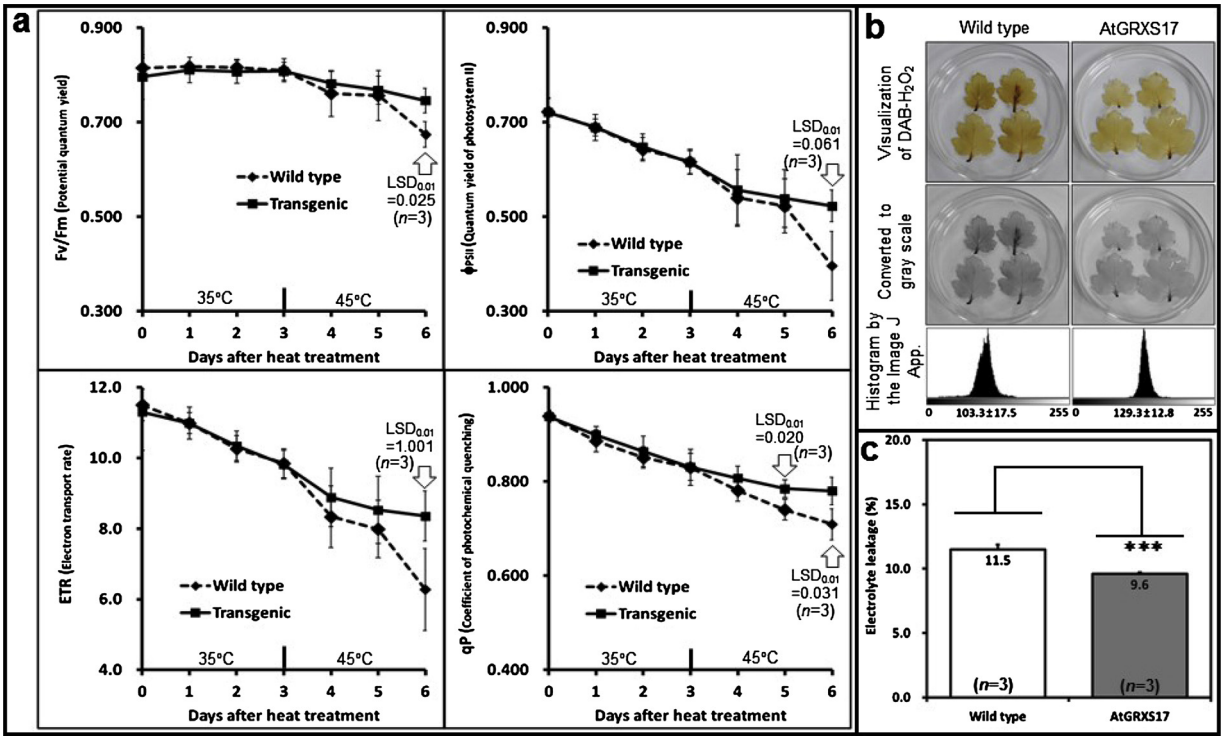

Fig. 5. Change of photochemical parameters relevant to photosynthetic ability (a), H_2O_2 accumulation (b), and electrolyte leakage (c) in *AtGRXS17*-OE and wild type chrysanthemum 'Vivid Scarlet' plants under heat stress conditions. The values under each histogram of panel b present the means ± S.D. The bars in panel c also indicate ± S.D. Student *t* test, *** $p < 0.001$.

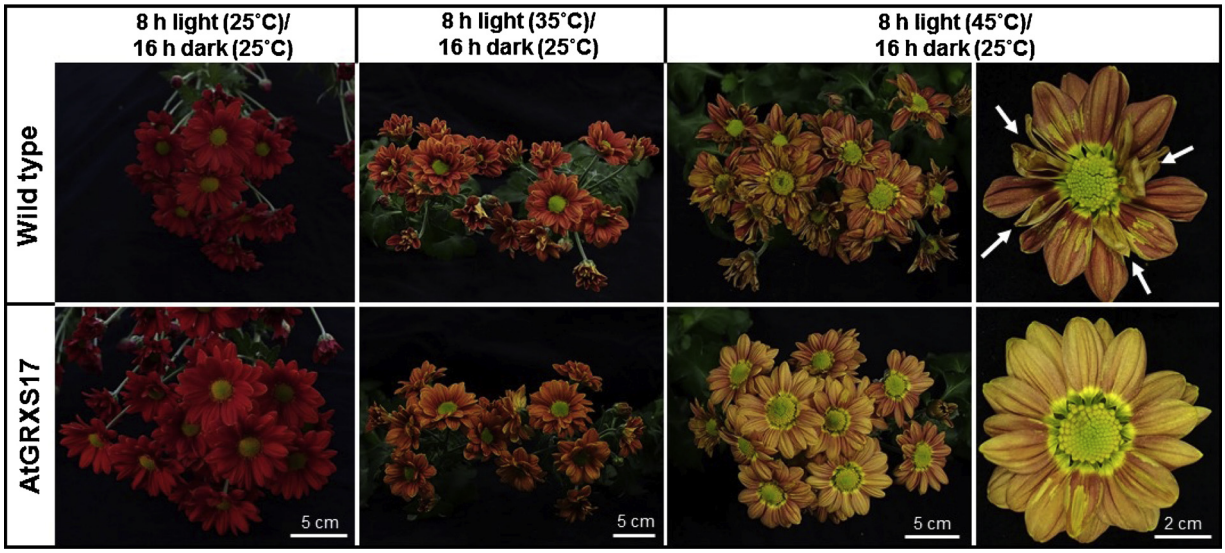

Fig. 6. The ray florets of *AtGRXS17*-OE and wild type 'Vivid Scarlet' plants under heat stress conditions. The ray florets of *AtGRXS17*-OE and wild type plants before heat treatment (left panel), after 7 d of treatment at 35/25 °C (middle panel), and after 7 d of treatment at 35/25 °C followed by additional 7 d of treatment at 45/25 °C (right panel). Each picture for *AtGRXS17* except the last capitulums consists of the mixed flowers from VX042, VX053, and VX082 lines. The arrows indicate the damaged ray florets.

3.5. AtGRXS17-OE 'Vivid Scarlet' plants preserve the ray floret development without wilting under heat stress conditions

The flowering of wild type and *AtGRXS17*-OE 'Vivid Scarlet' plants, when synchronized in flowering time by adjusting day-length (Fig. 6, left panel), were subjected to heat treatment to examine the heat-response of their flowers. The ray florets of wild type and *AtGRXS17*-OE 'Vivid Scarlet' plants were gradually decolorized as the heat stress period progressed (Fig. 6, middle and right panels). However, the ray florets of wild type plants were severely wilted and damaged, while the ray florets of *AtGRXS17*-OE 'Vivid Scarlet' plants maintained their shapes. More decoloration compared to that of wild type plants under 45/25 °C heat stress conditions was observed (Fig. 6, right panel).

3.6. Alteration of flowering time in AtGRXS17-OE chrysanthemum under heat stress conditions

Severe heat stress (45/25 °C for 3 d following 35/25 °C for 3 d) to 'Vivid Scarlet' young plants causes necrosis of newly developing leaves from shoot tips in wild type plants (Fig. 4b). To avoid necrosis in wild type controls, two mild heat stress conditions, continuous 35 °C in 8-h photoperiod or daytime 35 °C /night 25 °C in 8-h photoperiod, were employed to compare the flowering time of wild type and *AtGRXS17*-OE 'Vivid Scarlet' plants under each heat stress condition. After 6 weeks (4 weeks for floral bud formation and development followed by 2 weeks for flowering from the bud) under both mild heat stress treatments, the *AtGRXS17*-OE 'Vivid Scarlet' plants promoted about seven days earlier flowering ($p < 0.001$) than wild type plants in both treatments (Fig. 7). Earlier flowering performance in the *AtGRXS17*-OE 'Vivid Scarlet' plants coincided with that under natural conditions (Fig. 2).

4. Discussion

AtGRXS17-OE in tomatoes enhanced tolerance to heat, chilling, and further drought stresses, implying that the overexpression of *AtGRXS17* may be a promising way for improving the multi-tolerance to various abiotic stresses in ornamental flowering plants (Wu et al., 2012, 2017; Hu et al., 2015). Indeed, ectopic overexpression of *AtGRXS17* in chrysanthemum conferred improved response to heat stress that is one of the hindrances in quality flower production under high temperature conditions, possibly by reducing the oxidative damage of cell membrane systems and maintaining photosynthetic ability under heat stress. Our results obtained from electrolyte leakage measurement and DAB-staining showed less electrolyte leakage and reduced H_2O_2 accumulation in the *AtGRXS17*-OE chrysanthemum encountering heat stress. Further, in the current study investigating expanded photochemical parameters (Φ_{PSII}, ETR, and qP in addition to Fv/Fm) related to photosynthetic efficiency, the *AtGRXS17*-OE lines also revealed relatively superior photosynthetic ability against heat stress, suggesting that AtGRXS17 has conserved functions in anti-oxidative stress and heat-response across multiple plant species.

The enhancement of floral development by AtGRXS17 implicates ROS in heat-related floret decline. Reactive oxygen species are known toxic by-products during heat stress, resulting in significant oxidative damage to plant tissues and organs (Gill and Tuteja, 2010; Wu et al., 2012). In addition, a feature of petal senescence is a rise in ROS and a change in redox balance (Rogers, 2012). Indeed, under heat stress conditions, not only newly developing leaves but also ray florets of *AtGRXS17*-OE plants retained heathy shapes, while those of wild type plants displayed rapid senescence and necrotic symptoms, indicating that the overexpression of *AtGRXS17* suppresses the senescence and necrosis of the floral tissues during heat stress.

Notably, *AtGRXS17*-OE chrysanthemum plants displayed accelerated floral buds built up and subsequently flowered earlier than wild type plants under both natural and artificial heat stress conditions. This observation suggests that the overexpression of *AtGRXS17* in chrysanthemum facilitates the transition toward reproductive phase in response to natural short-day condition, and/or faster floral development after shifting to reproductive phase. Indeed, several reports indicate that GRX contributes to the development of flower organs and to a photoperiod-signaling. For example, ROXY1 and ROXY2, two members of *Arabidopsis* GRXs, appear to be crucial for petal and anther development (Xing et al., 2005; Xing and Zachgo, 2008; Hofmann, 2009; Li et al., 2009). In addition, AtGRXS17 has been reported to affect the flowering time in a photoperiod-sensitive manner via interacting with a nuclear transcriptional regulator, the Nuclear Factor Y subunit C11 (NF-YC11) (Knuesting et al., 2015). In chrysanthemum, silencing *CmNF-YB8* induces early flowering phenotype (Wei et al., 2017). Since the transcriptional activity of NF-Y proteins relies on the heterotrimeric complex formation of NF-YA, -YB and -YC subunits, AtGRXS17 may

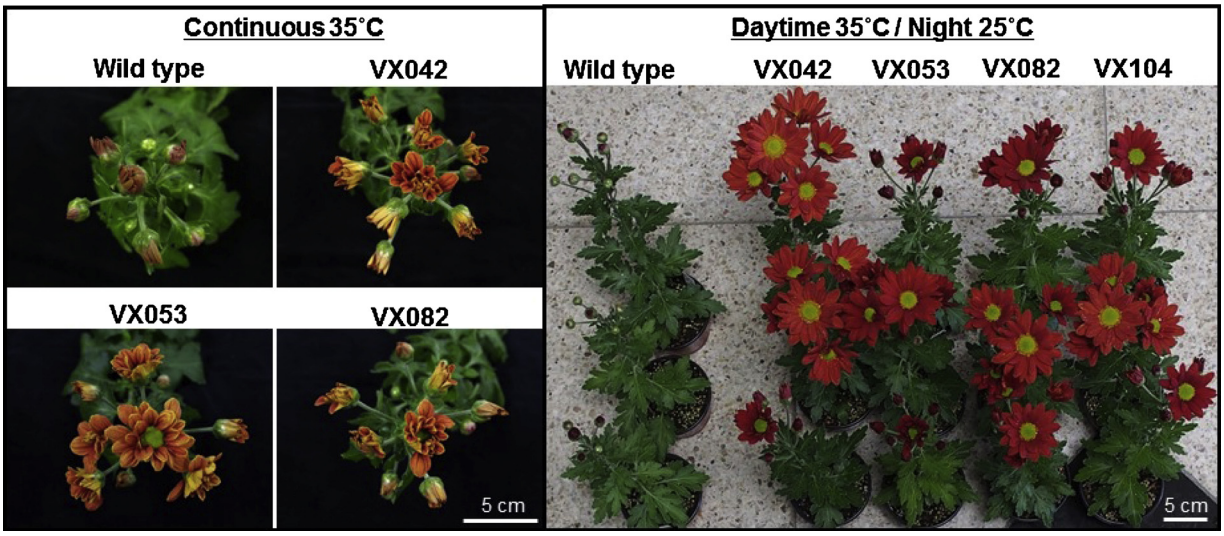

Fig. 7. Flowering time of *AtGRXS17*-OE and wild type 'Vivid Scarlet' plants under two mild heat stress conditions. Forty-day-old *AtGRXS17*-OE and wild type plants were subjected to continuous 35 °C (8-h photoperiod; left panel) or 35/25 °C (day/night temperatures, 8-h photoperiod; right panel). The pictures were taken on six weeks after heat stress and photoperiod treatments.

regulate the activity of NF-Y proteins via stabilizing the complex (Petroni et al., 2012). However, further studies to address how ectopic overexpression of *AtGRXS17* affects the expression levels of a set of flowering integrators such as *CmNF-YB8* coordinating aging as well as SQUAMOSA PROMOTER BINDING-LIKE (SPL) family members, *FTL3*, *SOC1*, *AP1*, *LFY*, or *FUL* are needed (Wei et al., 2017).

While *AtGRXS17*-OE affected flowering time in both natural and artificial heat stress conditions, we did not observe that the heat treatments further hastened flowering time in the *AtGRXS17*-OE plants. Both heat stress and *AtGRXS17* factors could ultimately affect the expression of *FLOWERING LOCUS T* (*FT*) (Wei et al., 2017). One explanation is that the induction of *FT* by two factors might reach a threshold and thus no further early-flowering phenotype was observed. However, the mechanism could be more complicated, and further study will be required to understand how AtGRXS17 and heat affect flowering time. In *Arabidopsis*, AtGRXS17 controls flowering time partially due to its interaction with NF-YC11 (Knuesting et al., 2015). Another NF-Y protein, NF-YB8, also regulates flowering time in chrysanthemum (Wei et al., 2017), suggesting that similar mechanism might be involved in AtGRXS17-associated flowering time regulation. Unfortunately, the molecular mechanism underlying temperature-induced flowering time alteration remains unclear in chrysanthemum, though physiology studies have been conducted previously (Karlsson et al., 1989; Van Der Ploeg and Heuvelink, 2006; Nakano et al., 2013). However, the temperature of our heat stress treatment is out of the previously reported range, precluding us from direct comparison of the results. Therefore, how temperature affects flowering time in chrysanthemum will be also worth of further study. Given that major phytohormones and sugars are particularly associated with heat-tolerance and flowering as well (Asensi-Fabado et al., 2013; Jagadish et al., 2016; Lawas et al., 2018), whether and how *AtGRXS17*-OE affects the metabolic processes of phytohormones and sugars needs to be further explored.

Meanwhile, the chroma of ray florets in the wild type 'Vivid Scarlet' plants was raised under extreme high temperatures (45/25 °C) compared to those of *AtGRXS17*-OE plants (Fig. 6, right panel). Given that flower color is influenced by many factors such as pigment, tissue structure, co-pigmentation, temperature, light, and pH (Nozaki et al., 2006; Zhao and Tao, 2015), further studies to address how the *AtGRXS17*-OE affects flower color during extreme high temperature treatment are needed. However, this result conversely corresponds with previous reports, indicating that AtGRXS17 loss-of-function (*atgrxs17* KO) *Arabidopsis* plants accumulate higher amount of anthocyanin compared to wild type controls under heat stress while the *atgrxs17* KO plants are hypersensitive to high temperature (Cheng et al., 2011). We expect that our results contribute to the production of chrysanthemum flowers under high temperature condition, especially in the summer season at temperate zone.

In conclusion, we showed that ectopic overexpression of *AtGRXS17* in chrysanthemum, an economically important ornamental plant, improved response to heat stress and significantly prevented loss of ray floret shape and wilting caused by heat stress. We additionally found that overexpression of *AtGRXS17* altered the flowering time in chrysanthemum. These findings suggest an approach to enhance flower production under high temperature conditions. Further studies are needed to elucidate the details and mechanism between temperature and *AtGRXS17*-overexprssion in chrysanthemum to provide insights to fine-tune the flowering time and flower quality under heat stress conditions.

Author contributions

J.-S. H. and S. P. conceived and designed the experiments. B.-C. K., Q. W., and S.-J. B. performed most of the experiments. S. S. and F. F. W. provided conceptual advice and analyzed the data. All co-authors participated together in writing the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2019.103864.

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