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Research article

Overexpression of *MpCYS4*, a phytocystatin gene from *Malus prunifolia* (Willd.) Borkh., delays natural and stress-induced leaf senescence in apple

Yanxiao Tan, Yingli Yang, Chao Li, Bowen Liang, Mingjun Li, Fengwang Ma*

State Key Laboratory of Crop Stress Biology for Arid Areas, College of Horticulture, Northwest A&F University, Yangling, Shaanxi 712100, PR China

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ABSTRACT

Phytocystatins are a well-characterized class of naturally occurring protease inhibitors that prevent the catalysis of papain-like cysteine proteases. The action of cystatins in stress tolerance has been studied intensively, but relatively little is known about their functions in plants during leaf senescence. Here, we examined the potential roles of the apple cystatin, *MpCYS4*, in leaf photosynthesis as well as the concentrations and composition of leaf proteins when plants encounter natural or stress-induced senescence. Overexpression of this gene in apple rootstock M26 effectively slowed the senescence-related declines in photosynthetic activity and chlorophyll concentrations and prevented the action of cysteine proteinases during the process of degrading proteins (e.g., Rubisco) in senescing leaves. Moreover, *MpCYS4* alleviated the associated oxidative damage and enhanced the capacity of plants to eliminate reactive oxygen species by activating antioxidant enzymes such as ascorbate peroxidase, peroxidase, and catalase. Consequently, plant cells were protected against damage from free radicals during leaf senescence. Based on these results, we conclude that *MpCYS4* functions in delaying natural and stress-induced senescence of apple leaves.

1. Introduction

Leaf senescence is a developmentally programmed degenerative process that constitutes the final step in a leaf lifespan. It is controlled by multiple physiological and environmental factors that trigger a highly regulated, ordered series of events involving cessation of photosynthesis, disintegration of chloroplasts, breakdown of leaf proteins, a loss of chlorophyll (chl), and removal of amino acids (Buchanan-Wollaston, 1997). Among these, the degradation of chloroplasts is one of the most important hydrolytic processes not only because they are the single most important source of re-mobilizable nutrients, especially nitrogen (N), but also because their breakdown causes a decline in the photosynthetic potential of leaves (Krupinska, 2007). Cysteine proteinases (CPs) are involved with a variety of proteolytic functions in higher plants, and are the most abundant enzymes associated with leaf senescence in numerous species (Roberts et al., 2012; Diaz and Martinez, 2013). Changes in the temporal patterns of CP in parallel with senescence are consistent with an increase in proteolytic activities and a reduction in mainly chloroplastic proteins (Breeze et al., 2011; Roberts et al., 2012). Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which represents the major nitrogen investment in crops and the first source of transportable N, is the main target of CPs (Masclaux-Daubresse et al., 2010; Carrión et al., 2013). At higher concentrations, these CPs are potentially damaging even though they are also essential to the maintenance and survival of their host organisms. Therefore, CP activities must be adequately regulated by inhibitors such as cystatins (Benchabane et al., 2010).

Plant cystatins, i.e., phytocystatins, comprise an independent subfamily on the cystatin phylogenetic tree. They lack disulfide bridges and putative glycosylation sites, and can reversibly inhibit the activity of papain-like CPs or C13 legumain peptidases due to

Abbreviations: APX, ascorbate peroxidase; CAB, chlorophyll a/b binding protein; CAT, catalase; chl, chlorophyll; CP, cysteine proteinase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; MV, methyl viologen; PAO, pheophorbide α oxygenase; Pn, net photosynthetic rate; POD, peroxidase; qRT-PCR, quantitative real-time PCR; RBCS, small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SAG, senescence-associated gene; TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid.

* Corresponding author.
E-mail addresses: fwm64@sina.com, fwm64@nwsuaf.edu.cn (F. Ma).

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tight and irreversible interactions (Benchabane et al., 2010). Phytocystatins have dual functions as defense proteins and regulators of protein turnover. They modulate endogenous and heterologous CPs and participate in a variety of physiological processes, such as plant growth and development, programmed cell death, the accumulation and mobilization of storage proteins in seeds and tubers (Benchabane et al., 2010), plant defense responses (Popovic et al., 2013; Quain et al., 2014), and senescence (Sugawara et al., 2002; Prins et al., 2008; Tajima et al., 2011; Diaz-Mendoza et al., 2014). Sugawara et al. (2002) have characterized a CP inhibitor gene, *Dc-CPIn*, that suppresses petal-wilting in senescing flowers of *Dianthus caryophyllus*. Prins et al. (2008) have reported that transgenic tobacco (*Nicotiana tabacum*) plants expressing the cystatin *OC-I* from rice (*Oryza sativa*) grow more slowly than the untransformed controls, are delayed in their leaf senescence, and have increased abundances of leaf proteins, especially two Rubisco activase isoforms. A senescence-related cysteine protease–cystatin complex has been described from the biochemical and molecular characterization of leaf samples from *Spinacea oleracea* (Tajima et al., 2011). This complex comprises the 41-kDa *SoCP* and a 14-kDa cystatin, *CPI*. Purified recombinant *CPI* has strong inhibitory activity against *SoCP*, but their coordinated expression in senescent leaves suggests that both are involved in that process. All of these results demonstrate that phytocystatins have roles in Rubisco turnover in leaves undergoing senescence, and they confirm the importance of protease–inhibitor interactions in leaf senescence. However, the mechanisms by which they function at the molecular level are still largely unknown.

We previously isolated a cystatin gene, *MpCYS4* (GenBank Accession No. KF477275), from the leaves of apple (*Malus prunifolia*) (Tan et al., 2014). This gene is induced by water deficit, heat (40 °C), methyl viologen (MV), or exogenous treatment with abscisic acid, and is rapidly up-regulated in apple leaves during the aging and senescence process. Furthermore, we used *Agrobacterium*-mediated transformation to introduce *MpCYS4* into apple rootstock M26 and found that its overexpression enhanced drought tolerance in those plants (Tan et al., 2017). For the investigation presented here, we examined how constitutive expression of *MpCYS4* might affect leaf photosynthesis and alter the concentration and composition of leaf proteins during natural or stress-induced senescence in apple plants.

2. Materials and methods

2.1. Plant materials and growing conditions

All experiments were conducted at Northwest A & F University, Yangling, China (34°20′N, 108°24′E). To prepare our plant materials, we used tissue culture to propagate three transgenic M26 (*M. domestica*) lines (#1, #3, and #4) as described by Tan et al. (2017), that over-express *MpCYS4* as well as plants of the non-transformed wild type (WT). The plantlets were rooted and transferred to plastic pots (cube-shaped: 4 cm × 5 cm × 5 cm) that contained a mixture of forest soil, sand, vermiculite, and perlite (5:1:1:1, v:v:v:v). Culture conditions included a 16-h photoperiod (flux density approximately 100 μmol photons m^{-2} s^{-1}), 24 ± 2 °C, and 70 ± 5% relative humidity. At the eight-leaf stage, individual plants of uniform size (about 5 cm tall) were moved to plastic pots (30 cm × 26 cm × 22 cm) filled with a 5:1:1 (v:v:v) mixture of forest soil:sand:organic substrate, and placed in a greenhouse under ambient light, with a 23–25°C/15–17 °C (day/night) temperature cycle, and a relative humidity of 65–70% at the experimental field of Northwest A & F University. Before the experiments began, plants were thoroughly watered each day and supplied once a week with half-strength Hoagland nutrient solution (pH 6.0). Standard horticultural practices were followed for disease and pest control. After three months of growth under these conditions, healthy and uniform plants of each line were assigned to two watering regimes: well-watered (control), 80 ± 5% field capacity; or drought, 50 ± 5% field capacity. Irrigation was withheld from the drought-stressed plants beginning on 10 July 2015, while normal watering continued for the control plants. At 9:00 h on each sampling day, the net photosynthetic rate (Pn) was recorded. Afterward, the ninth to twelfth leaves from the base of a stem were removed from five plants per treatment, then frozen in liquid nitrogen and stored at −80 °C. The experiments were terminated after 70 d, on 20 September 2015. After this long period of drought stress was completed, plants in the well-watered group were used for observing natural leaf senescence between 26 October and 26 November 2015.

2.2. Measurement of photosynthetic rates

Net photosynthesis was evaluated with a portable system (Li-6400; LiCor, Huntington Beach, CA, USA). All measurements were performed at 500 μmol photons m^{-2} s^{-1}, with the cuvette CO_2 concentration set at 400 μmol CO_2 mol^{-1} air, and vapor pressure deficit at 2.0–3.4 kPa. Data were recorded from fully expanded, fully light-exposed leaves from the same position on five plants.

2.3. Quantification of chlorophyll, soluble proteins, and Rubisco protein

Chlorophyll was extracted with 80% (v/v) acetone (10 mL) from leaf samples (0.1 g) that were incubated in the dark at 25 °C for 24 h, then the concentrations were determined spectrophotometrically according to the method of Lichtenthaler and Wellburn (1983).

For protein assays, frozen leaves (0.1 g) were homogenized with a chilled mortar and pestle, using 50 mM Hepes-KOH buffer (pH 7.5) that contained 10 mM $MgCl_2$, 2 mM EDTA, 10 mM dithiothreitol, 1% (v/v) Triton X-100, and 10% (v/v) glycerol. The homogenate was centrifuged at 13,000 g for 5 min at 4 °C, and the supernatant was used for determining soluble proteins according to the method of Bradford (1976). This supernatant was mixed with NuPAGE LDS sample buffer, boiled for 3 min, and placed on a NuPAGE 12% Bis-Tris gel. The Rubisco concentration in the supernatant was determined spectrophotometrically via formamide extraction of the CBB R250-stained bands that corresponded to the large (55-kDa) and small (13-kDa) subunits of Rubisco, as separated by NuPAGE (Wang et al., 2013, 2014). Calibration curves were produced with BSA (Izumi et al., 2010).

2.4. Measurement of cysteine proteinase activity

Cysteine proteinase activity was measured in leaf discs as described previously (Salvesen and Nagase, 1989) using benzoyl-L-arginine-*p*-nitroanilide (Sigma, Steinheim, Germany) as the reaction substrate. For the extraction of proteases, leaf samples (0.1 g) were homogenized in 1 mL citrate phosphate buffer (100 mM; pH 6.5) that contained 30 mM cysteine and 30 mM EDTA. The homogenate was placed on ice for 2 h with periodic vortexing. Following centrifugation at 13,000 g for 30 min at 4 °C, the supernatant was used for the following assay. Reaction was performed with 700 μL supernatant and 200 mL of 1 mM benzoyl-L-arginine-*p*-nitroanilide, incubated at 37 °C for 30 min, then terminated with 1 mL 3% (v/v) HCl/ethanol. Absorbance was read at 405 nm, and the cysteine proteinase activity was calculated by reference to a standard curve produced with papain (Sigma).

2.5. Measurement of Rubisco degradation using in vitro assays

The effect of *MpCYS4* expression on protein degradation *in vitro* was determined according to the method of Yoshida and Minamikawa (1996). Soluble protein extracts (100 μg) from leaves of either WT or *MpCYS4*-transformed plants were incubated at 37 °C for 4 h in the presence or absence of 100 μM E64 (a CP inhibitor) in 50 mM sodium acetate (pH 5.4) that contained 10 mM β-mercaptoethanol. Immediately after incubation, the samples were loaded onto a NuPAGE 12% Bis-Tris gel for detecting and separating Rubisco protein bands. Formamide was used to extract the CBB R250-stained bands that corresponded to the large and small subunits of Rubisco (Wang et al., 2013, 2014), and the protein concentration was determined according to the procedure described above.

2.6. Assay of oxidative stress tolerance

Fully mature leaves from WT and transgenic plants were sliced into 7 mm × 10 mm pieces and incubated in either hydrogen peroxide (H_2O_2; 0.59, 0.88, or 1.18 M) or water for 24 h. The leaf pieces were collected at the end of the treatment and used for calculating total chl concentrations and levels of H_2O_2.

2.7. Evaluation of H_2O_2, malondialdehyde (MDA), and activities of H_2O_2-scavenging enzymes

The H_2O_2 was extracted and measured as described by Patterson et al. (1984). Frozen leaf samples (0.1 g) were homogenized in 5 mL of pre-cooled acetone and centrifuged at 1500 g for 10 min. Titanium chloride (0.1%, w/v) and concentrated ammonia (0.1 mL) were added to the supernatant (1 mL), after reacted at 25 °C for 10 min, the mixture was centrifuged at 1500 g for 10 min. Absorbance was read at 410 nm, and the H_2O_2 concentration was calculated according to the standard curve.

Lipid peroxidation was estimated as the concentration of total 2-thiobarbituric acid (TBA) reactive substances, and was expressed as equivalents of MDA. Frozen leaf (0.1 g) was homogenized in 0.3% (w/v) TBA and 10% (w/v) trichloroacetic acid (TCA). After boiled for 20 min, it was cooled quickly on ice. The mixture was centrifuged at 13,000 g for 15 min, and the supernatant was used for determining the MDA concentration as reported by Heath and Packer (1968).

For monitoring H_2O_2-scavenging enzymes, leaf samples (0.1 g) were ground in a chilled mortar with 1% (w/v) polyvinylpolypyrrolidone, then homogenized with 1.2 mL of 50 mM Hepes-KOH buffer (pH 7.8) containing 1 mM EDTA and 0.3% (v/v) Triton X-100. For only the assay of ascorbate peroxidase (APX), 1 mM ascorbate was added to this mixture. Each homogenate was centrifuged at 13,000 g for 20 min at 4 °C. The supernatant was used for the following assays. Catalase (CAT) activity was determined by monitoring the decrease in absorbance at 240 nm due to the decomposition of H_2O_2 (extinction coefficient of 39.4 mM^{-1} cm^{-1}) (Chance and Maehly, 1955). Peroxidase (POD) was assayed at 470 nm (extinction coefficient 25.2 mM^{-1} cm^{-1}) by using H_2O_2 and guaiacol as the reaction substrates (Chance and Maehly, 1955). Activity of APX was monitored as the decrease in absorbance at 290 nm when reduced ascorbate was oxidized (extinction coefficient of 2.8 mM^{-1} cm^{-1}) (Nakano and Asada, 1981).

2.8. Gene expression analysis by quantitative real-time PCR

Total RNA was extracted according to the CTAB method (Chang et al., 1993), and residual DNA was removed by treating with RNase-free DNase I (Invitrogen, USA). Integrity of the RNA was checked via electrophoresis on a 1.2% agarose gel. The first-strand cDNAs were synthesized with 2 μg of total RNA in a 20-μL volume, using a SYBR Prime Script RT-PCR Kit II (TaKaRa).

Quantitative real-time PCR (qRT-PCR) was performed on an iQ5.0 instrument (Bio-Rad, USA), using SYBR Green qPCR kits (TaKaRa) according to the manufacturer's instructions. Each reaction was conducted in triplicate with a final volume of 20 μL, and apple *EF-1α* was amplified as the internal control. The relative quantities of target gene transcripts were determined per the $2^{-\triangle CT}$ method (Livak and Schmittgen, 2001). Specific primers for genes coding for the Rubisco small subunit (RBCS), light-harvesting chl a/b binding protein (CAB), pheide α oxygenase (PAO), and a senescence-associated protein are listed in Table 1. All reactions were repeated three times to minimize inherent errors, and were performed on three separate RNA extracts from three independent samples. Values for mean expression and standard deviation (SD) were calculated from the results of three independent replicates.

2.9. Statistical analysis

The experiments of drought-induced and natural leaf senescence were repeated thrice with consistent results. Data from one representative experiment are shown here. All statistical analyses were conducted with SigmaPlot software (Systat Software). The data were evaluated via one-way analysis of variance (ANOVA), using the SPSS-11 for Windows statistical software package. Statistical differences were compared by Student's *t*-tests at significance levels of $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***).

3. Results

3.1. MpCYS4 overexpression delays drought-induced leaf senescence in apple

Leaf senescence is typically accompanied by declines in photosynthetic activity and chl concentrations. Here, under control conditions, the photosynthetic rate in all genotypes began to drop, but only slightly, at Day 40. In response to long-term drought stress, Pn decreased significantly in both WT and the three lines of *MpCYS4*-transformed plants throughout the experimental period, with rates being much lower for the WT (Fig. 1A). A similar trend was noted for total chl concentrations (Fig. 1B). Likewise, the concentrations of total soluble protein and Rubisco protein were reduced progressively in all genotypes under drought conditions, with that decline being more rapid in the WT (Fig. 1C and D).

Table 1
Sequences for primers used in quantitative real-time PCR.

Gene	Accession no.	Primer sequence (5′–3′)
RBCS[a]	L24497	F: AAACTGGGTTCCCTGCTTGGA
		R: GGCAGCTTCCACATTGTCCAG
CAB[b]	X17697	F: ATCTTGATGGGTGCCGTGGAG
		R: AGCCTCTGTGTCTTCTGCAAGG
PAO[c]	CN877341	F: ACCCGAGTGGTTTGGTACTTGTGA
		R: TACACGAGGAGCATTTGAGGGTGT
SAG12[d]	EB122814	F: GAAGGAAGCCATCATTGCAGCCAA
		R: ACCATGGTCAAGACTCGTTCCACA
EF-1α[e]	DQ341381	F: ATTCAAGTATGCCTGGGTGC
		R: CAGTCAGCCTGTGATGTTCC

[a] *RBCS*, gene coding for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase.
[b] *CAB*, gene coding for the light-harvesting chl a/b binding protein.
[c] *PAO*, gene coding for the pheide α oxygenase.
[d] *SAG12*, senescence-associated gene 12.
[e] *EF-1α*, served as internal control.

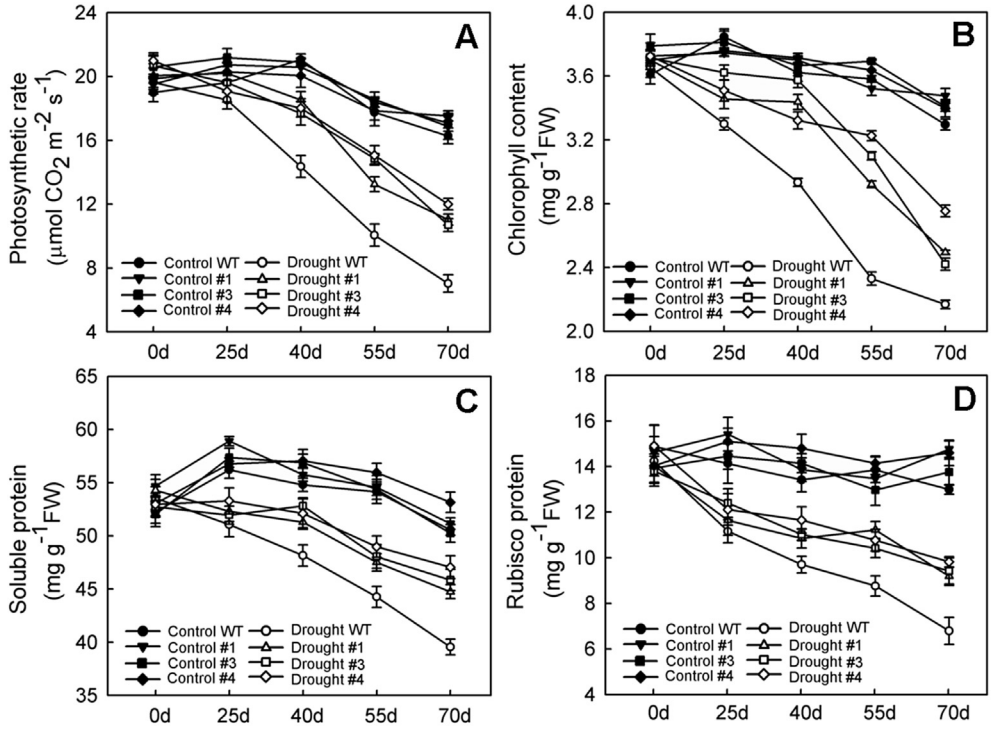

Fig. 1. Changes in net photosynthetic rates (A), chlorophyll concentrations (B), levels of soluble protein (C), and Rubisco protein concentrations (D) in wild-type (WT) and 35S:*MpCYS4* transgenic apples (Lines #1, #3, and #4) under long-term drought stress. Data are means ± SD of 5 replicate samples.

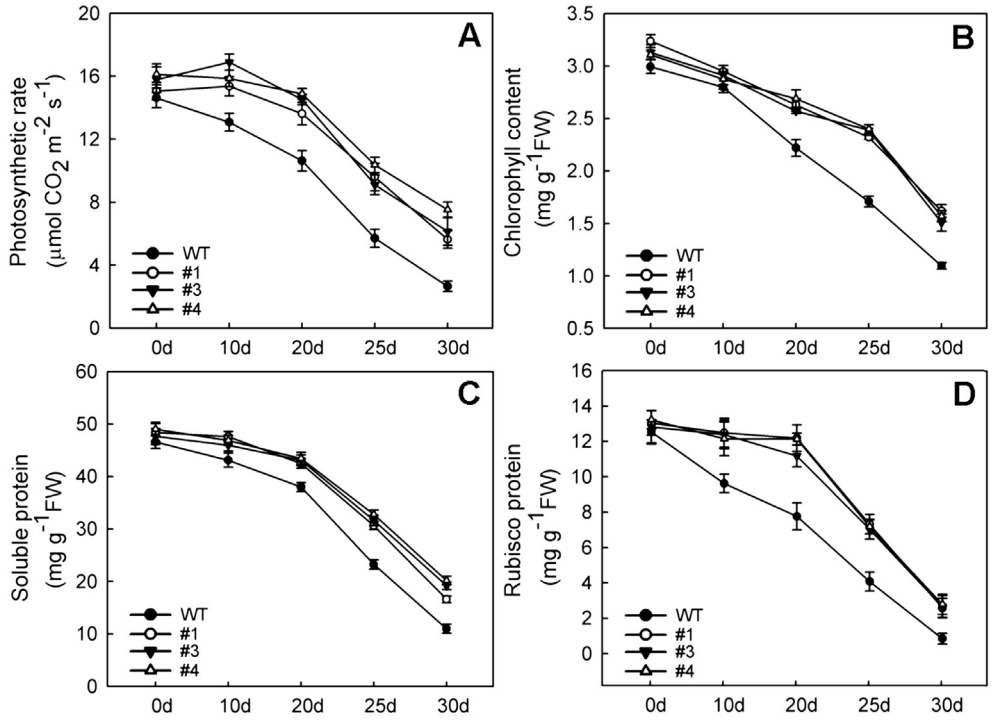

Fig. 2. Changes in net photosynthetic rates (A), chlorophyll concentrations (B), levels of soluble protein (C), and Rubisco protein concentrations (D) in wild-type (WT) and 35S:*MpCYS4* transgenic apples (Lines #1, #3, and #4) during natural leaf senescence. Data are means ± SD of 5 replicate samples.

3.2. MpCYS4 overexpression delays natural leaf senescence in apple

During natural leaf senescence, values for Pn and total chl concentrations decreased rapidly over the observation period. However, overexpression of *MpCYS4* appeared to slowed that drop (Fig. 2A and B). Concentrations of total soluble protein and Rubisco protein also declined as aging progressed, although this degradation was less dramatic for the transgenics than for the

corresponding WT (Fig. 2C and D). In particular, levels of Rubisco protein remained relatively constant in the transgenics in the first 20 d while that protein in the WT had already begun to degrade from Day 0.

Over time, expression of two photosynthesis-related nuclear genes – *RBCS* and *CAB* –was preferentially down-regulated, even though transcripts were maintained at higher levels in the transgenic plants (Fig. 3A and B). One good indicator of senescence, *PAO*, is a key chl-degradation gene (Wang et al., 2013). Here, it was greatly up-regulated in WT plants from Day 25, but its expression was obviously lower in the transgenics (Fig. 3C). In addition, expression of *SAG12*, a senescence-associated gene (SAG, as termed by Lim et al., 2003) on Day 30 was up-regulated by 14.6-fold in the WT plants but by only 7.9-, 3.9-, and 5.5-fold in Lines #1, #3, and #4, respectively (Fig. 3D).

3.3. Cysteine proteinase activity and Rubisco degradation in apple leaves

In both petals and leaves, CP activity typically increases during their senescence (Sugawara et al., 2002; Prins et al., 2008). Therefore, in our experiments, we measured and quantified endogenous CP activity in the leaves of WT and *MpCYS4*-transformed plants during natural leaf senescence or when plants were exposed to long-term drought stress. As shown in Fig. 4A, activity rose in all genotypes, but most dramatically in the WT. This suggested that overexpression of *MpCYS4* can deter CP activity during the phase of leaf senescence.

To determine whether apple Rubisco is susceptible to degradation by endogenous CP, we conducted *in vitro* assays using extracts of transgenic and WT plants that were incubated in the absence or presence of the CP inhibitor E64 (Fig. 4B). When compared with the WT response, Rubisco was protected from degradation by endogenous CP in the transgenic samples. This effect could be mimicked by including E64 in the assay of WT extracts.

3.4. MpCYS4-overexpressing plants are more tolerant to oxidative stress

To assess how tolerant these transgenic plants were to oxidative stress, we treated leaf samples with 0.59, 0.88, or 1.18 M H_2O_2. After 24 h of exposure, most of the WT pieces were bleached while leaves from the overexpressing lines retained more of their normal color, regardless of the H_2O_2 concentration tested. Although an increase in H_2O_2 concentration led to more severe chl loss for all genotypes, the transgenic lines displayed much higher chl concents than the WT all the time (Fig. 5A). Although the endogenous level of H_2O_2 was enhanced with the increase in exogenous H_2O_2 concentration, the transgenic lines still contained significantly less H_2O_2 than did the WT (Fig. 5B). These results indicated that overexpression of *MpCYS4* alleviates the negative phenotypic effects associated with oxidative stress.

3.5. H_2O_2 accumulation and lipid peroxidation during leaf senescence in apple

The production of H_2O_2 is a characteristic, symptomatic indicator of senescence. In our well-watered control plants, H_2O_2 concentrations remained at a relatively stable level throughout the monitoring period. However, a significant H_2O_2 burst (181%) occurred in drought-stressed WT leaves during the first 25 d while *MpCYS4* overexpression reduced that burst by approximately 40% under the same treatment conditions (Fig. 6A). Levels of H_2O_2 in WT leaves also increased quickly and significantly as natural senescence progressed, and those concentrations were 1.3-fold higher on Day 30 than on Day 0. By comparison, the leaves from transgenic plants accumulated much less H_2O_2 over the same time

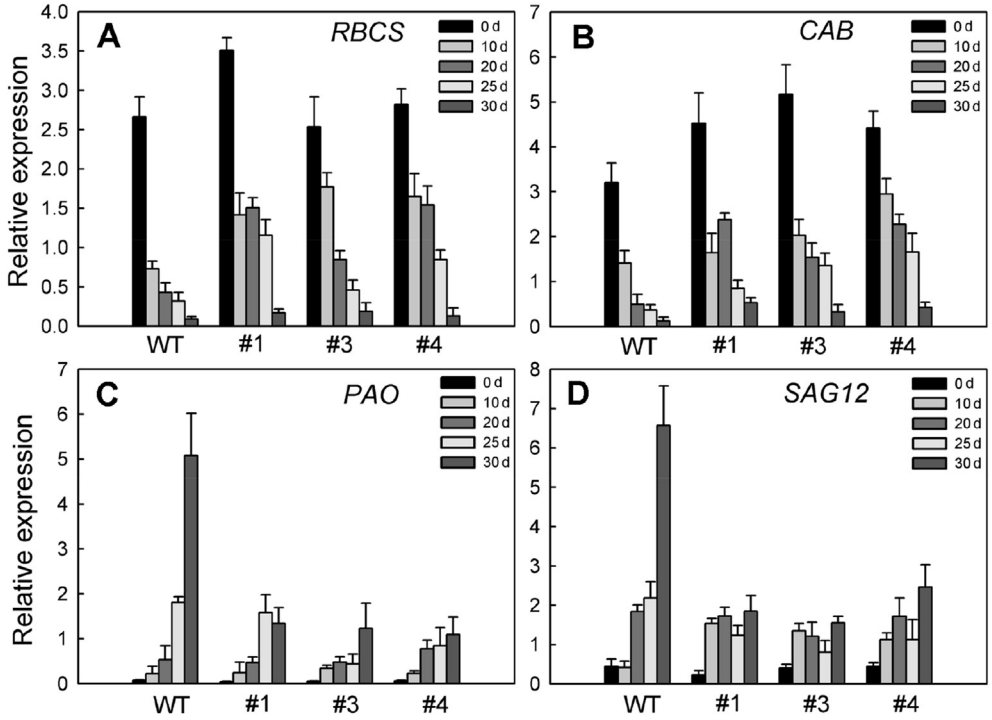

Fig. 3. Quantitative real-time PCR analysis of expression of genes coding for the Rubisco small subunit (RBCS) (A), light-harvesting chl a/b binding protein (CAB) (B), pheide α oxygenase (PAO) (C), and a senescence-associated protein 12 (SAG12) (D) in wild-type (WT), and *MpCYS4*-transformed apples during natural leaf senescence. Apple elongation factor-1α (*EF-1α*) served as reference gene. Data are means ± SD of 3 replicate samples.

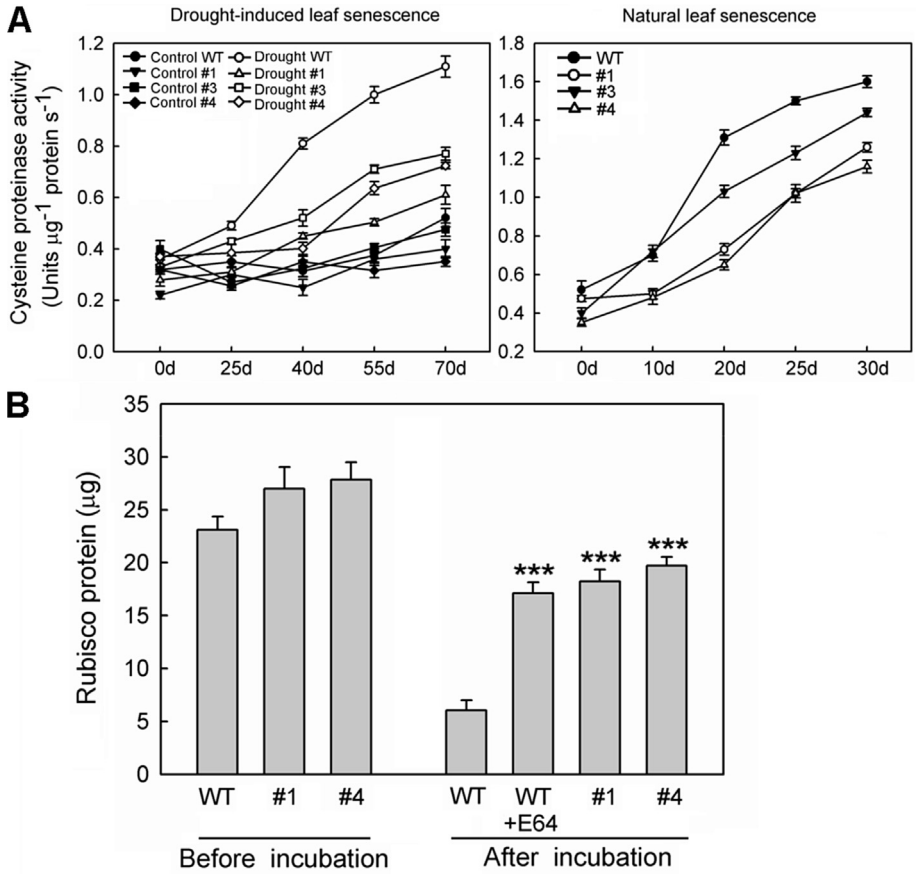

Fig. 4. Measurement of leaf cysteine proteinase activity and Rubisco degradation using *in vitro* assays. (A) Comparison of cysteine proteinase activity in leaves from wild-type (WT) and *MpCYS4*-transformed plants (Lines #1, #3, and #4) during long-term drought stress (left) or natural leaf senescence (right). Data are means ± SD of 5 replicate samples. (B) *In vitro* assays showing Rubisco is protected by *MpCYS4* in transgenic plants and by E64 in wild-type (WT) plants against degradation. Abundance of Rubisco holoenzyme protein was detected in soluble protein extracts of WT and transgenic Lines #1 and #4 before and after incubation for 4 h at 37 °C. Data are means ± SD from 3 independent experiments. Values were significantly different between transgenics and WT at $P < 0.001$ (***), based on Student's *t*-tests.

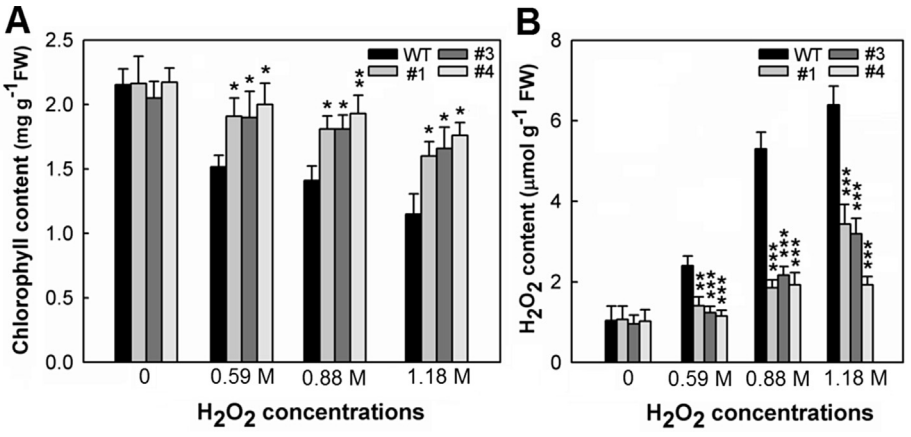

Fig. 5. Concentrations of chlorophyll (A) and endogenous H_2O_2 (B) in leaf pieces from transgenic lines #1, #3, and #4 and wild-type (WT) plants after H_2O_2 treatment. Data are means ± SD from 3 independent experiments. Values were significantly different between transgenics and WT at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***), based on Student's *t*-tests.

span (Fig. 6B).

Reactive oxygen species (ROS) can directly react with proteins, amino acids, and nucleic acids, and cause lipid peroxidation, which can then be estimated based on the amount of MDA produced. Here, the levels of MDA found in the leaves of both WT and *MpCYS4*-expressing plants increased substantially during long-term drought stress or natural leaf senescence, although concentrations were much lower in the transgenic lines (Fig. 6C and D). These results suggested that *MpCYS4* overexpression enhances tolerance to oxidative stress-induced membrane hydroperoxidation in the transformed plants.

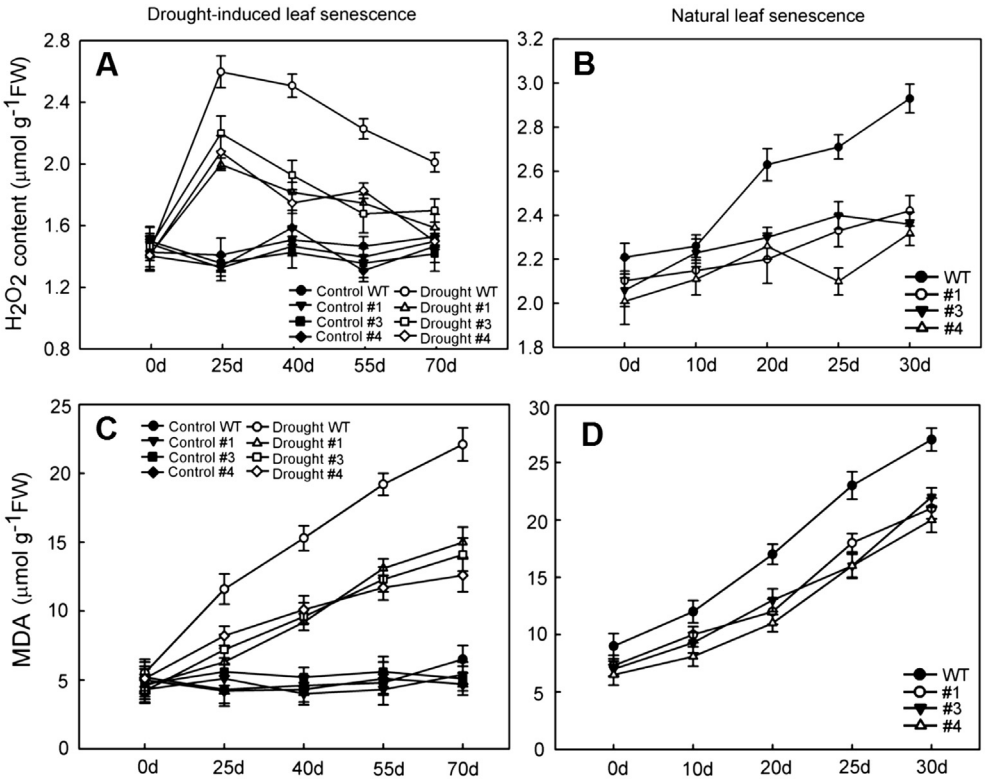

Fig. 6. Changes in H_2O_2 concentrations (A, B) and malondialdehyde (MDA) accumulations (C, D) in leaves of wild-type (WT) and *MpCYS4*-transformed plants (Lines #1, #3, and #4) during long-term drought stress (left) or natural leaf senescence (right). Data are means ± SD of 5 replicate samples.

3.6. Antioxidant enzymes activities during leaf senescence in apple

The activities of antioxidant enzymes involved in scavenging H_2O_2 changed significantly in response to long-term drought. For APX, POD, and CAT, patterns were similar among WT and *MpCYS4*-transformed plants, with activity initially increasing before gradually decreasing as the treatment period was prolonged. However, those activities throughout the experiment were always relatively higher in leaves from the overexpressing lines than from the WT (Fig. 7A–C). We also assayed APX, POD, and CAT during the process of natural leaf senescence and found that patterns of activity differed among plant types (Fig. 7D–F). For example, as the leaves aged, CAT activity gradually decreased in all genotypes, while POD activity initially increased slightly before an irreversible loss occurred. Although these changes varied according to the enzyme being monitored, all levels were always higher in the transgenic leaf samples. These results indicated that *MpCYS4* is involved in H_2O_2 homeostasis, regulating antioxidant enzymes and being indirectly responsible for leaf senescence.

4. Discussion

Leaf senescence is a genetically programmed physiological process in all living organisms. A leaf has a characteristic lifespan of photosynthetic productivity before the senescence program is initiated. Senescence can also occur prematurely if triggered by external factors such as phytohormone applications, shading, temperature, or pathogen attack (Zhao et al., 2016). Drought, the most prominent threat to agricultural production worldwide, also accelerates leaf senescence, which then leads to a decrease in canopy size, reduced photosynthesis, and lower crop yields (Chen et al., 2015).

In C3 plants, 75%–80% of the total leaf N is distributed to the mesophyll chloroplasts, with most of this nitrogen being allocated to protein production. Rubisco accounts for 12–30% of the total leaf protein in such species, catalyzing photosynthetic CO_2 fixation and photorespiratory carbon oxidation (Wada et al., 2015). The degradation of Rubisco and most other stromal proteins begins at an early stage of senescence, so that the released N can be remobilized to other growing leaves or to storage organs (Masclaux-Daubresse et al., 2010). As leaf senescence continued during our observation period, we noted that concentrations of soluble protein and Rubisco protein gradually declined. However, *MpCYS4* overexpression significantly slowed this degradation process, especially with regard to Rubisco levels. Meanwhile, expression of *RBCS* and *CAB* was suppressed less rapidly in transgenic plants than in the WT during the aging process. These results were directly associated with relatively higher photosynthesis rates and greater concentrations of chl in the transgenic apple leaves during natural or drought-induced leaf senescence.

Leaf-yellowing due to chl degradation is often considered the main marker for leaf senescence. The pathway of degradation has been elucidated and several genes in the pathway have been cloned (Takamiya et al., 2000). We found that one key chl-degradation gene, *PAO*, was dramatically up-regulated during natural leaf senescence in WT leaves. However, *MpCYS4* overexpression markedly inhibited *PAO* expression in the transgenic plants. During leaf senescence, most genes are down-regulated while some in a subset are up-regulated. Among these SAGs, the *SAG12* CP is one of the very few that is very senescence-specific (Lohman et al., 1994). Therefore, it is often referred to as the marker gene of senescence. Our examination showed that overexpression of *MpCYS4* significantly inhibited *SAG12* transcripts, and mRNA levels for the latter remained relatively low during natural leaf senescence.

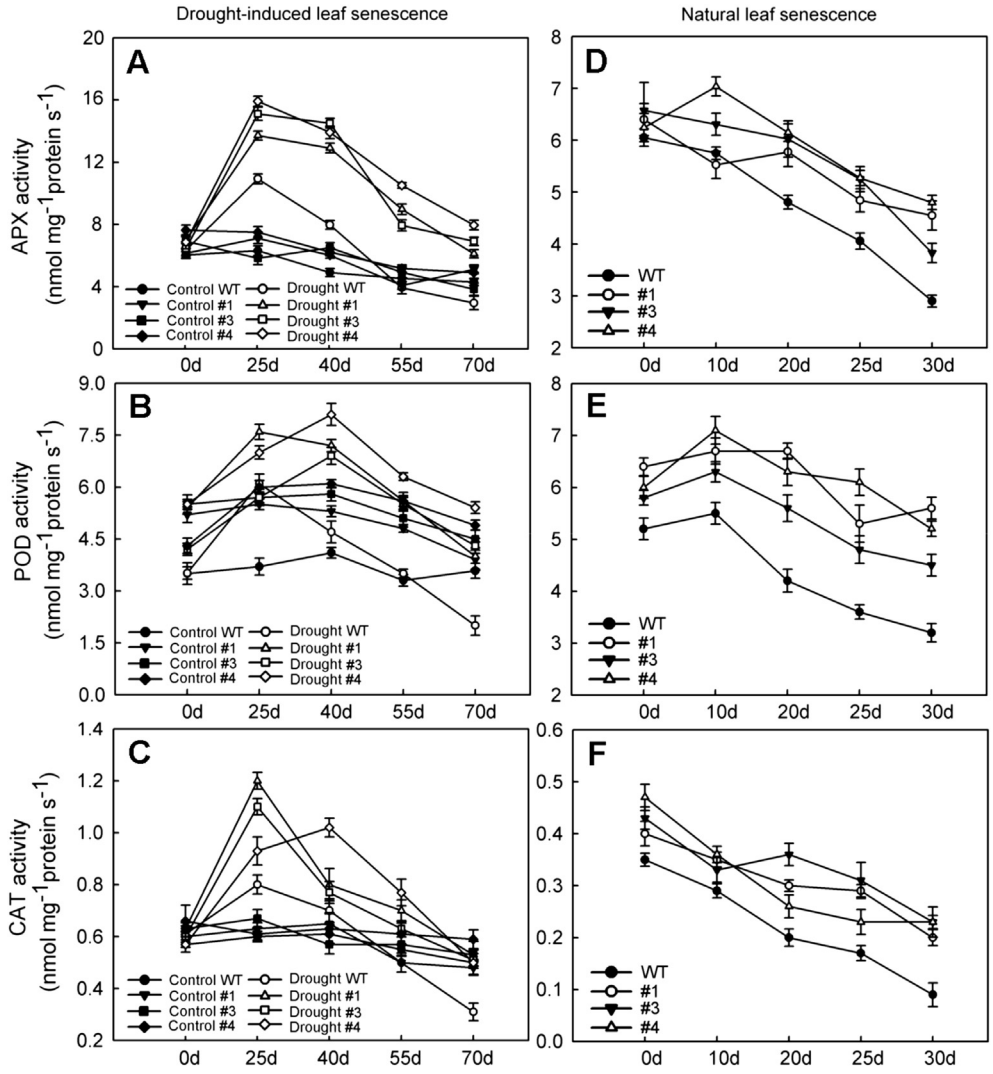

Fig. 7. Activities of main antioxidative enzymes involved in scavenging H_2O_2 measured in leaves of wild-type (WT) and *MpCYS4*-transformed plants (Lines #1, #3 and #4) during long-term drought stress (left) or natural leaf senescence (right). (A, D) APX, (B, E) POD, and (C, F) CAT. Data are means ± SD of 5 replicate samples.

Nevertheless, the mechanism by which *MpCYS4* regulates the expression of *PAO* and *SAG12* needs more in-depth study.

Chloroplast proteins, particularly Rubisco and Rubisco activase, are major targets of leaf CPs. Furthermore, the degradation of Rubisco involves interactions with the cytosol, and can be blocked by endogenous cytosolic CP inhibitors (Prins et al., 2008). In our experiments, we noted that CP activities were sharply up-regulated in leaves in which senescence was either natural or drought-induced. This response is indicative of the massive process of protein degradation. Nevertheless, CP activities were greatly suppressed when *MpCYS4* was over-expressed, evidence that this gene obviously delays such degradation. Our *in vitro* assays also showed that, when compared with WT extracts, Rubisco was protected from degradation by endogenous CP in the *MpCYS4*-transgenic extracts. Therefore, we propose that phytocystatins play a vital role in protecting photosynthesis processes by delaying Rubisco degradation in plants that are undergoing natural or stress-induced senescence.

One hypothesis states that senescence results from the generation of an excess of harmful free radicals, and that the onset of senescence is mainly caused by strong, uncontrolled enhancement in the generation of ROS (Mittler et al., 2011). For many plant species, concentrations of H_2O_2 tend to increase during the senescence period. Our study revealed that, whether senescence was natural or drought-induced, leaves from the WT accumulated more H_2O_2 when compared with the relatively low amount in the transgenic leaves. Consistently, as the main H_2O_2-scavenging enzymes (Mittler, 2002), activities of APX, POD, and CAT were maintained at higher levels in our transgenic lines. Zhang et al. (2008) have shown that overexpression of two cystatins, *AtCYSa* and *AtCYSb*, in *Arabidopsis thaliana* could enhance tolerance to salt, drought, cold, and oxidative stresses, and the amounts of APX protein and NADP-malic enzyme are much higher in transgenic lines. Similarly, overexpression of the rice cystatin *OC-I* in tobacco plants leads to enhanced tolerance to drought, heat, and light stress, which is associated with higher activities of POD and superoxide dismutase in transgenic plants under non-stress and stress conditions (Demirevska et al., 2010). Thus, *MpCYS4* may have important functions in modulating H_2O_2 homeostasis and the activities of antioxidant enzymes, both of which are important components in alleviating oxidative stress-induced damage in senescing plants.

Oxidative stress arises from either excessive ROS accumulations under other stresses or in response to treatment with MV or H_2O_2

(Miller et al., 2010). Here, we found that H_2O_2-induced damage was less extensive in the *MpCYS4*-transgenic plants, which suggested that they were more resistant than the WT to oxidative stress. The transgenics also accumulated lower levels of H_2O_2, indicating that these ROS were rapidly detoxified, as evidenced by the higher activities of antioxidant enzymes assayed here. These results demonstrated that tolerance to oxidative stress is enhanced in transgenic plants that over-express *MpCYS4*. In this regard, better functioning of the photosynthesis apparatus in those over-expressing plants during natural leaf senescence or when exposed to long-term drought stress might be ascribed, at least in part, to their capacity to maintain low intracellular levels of H_2O_2.

In summary, our results lead us to conclude that *MpCYS4* functions in delaying natural and stress-induced senescence of apple leaves. Overexpression of this gene effectively slows the senescence-related declines in photosynthesis and chl concentrations, and prevents cysteine proteinases from acting during the process of protein degradation in senescing leaves. Moreover, *MpCYS4* alleviates associated oxidative damage and improves the capacity for plants to eliminate ROS by activating the antioxidant APX, POD, and CAT enzymes. This then protects plant cells against damage from free radicals. We also found that overexpression of *MpCYS4*, as a conserved plant cystatin, could affect the stomatal behavior and the responses of apple to drought stress (Tan et al., 2017). Based on these results, we propose that *MpCYS4* is potentially useful as part of a research platform for enhancing plant agronomic traits, including drought tolerance and delayed leaf senescence. Such findings open up new possibilities for exploring how cystatins function in multi plant processes and in plant stress responses in addition to their general role as inhibitors of cysteine proteinase. Further investigations are necessary to identify the target proteins and to elucidate the potential mechanisms that support *MpCYS4*-mediated physiological responses.

Author contributions

Y.X. Tan performed and analyzed most of the experiments in this study, with assistance from Y.L. Yang, C. Li, B.W. Liang, and M.J. Li. F.W. Ma provided financial support and critical intellectual input in the design of this study and preparation of the manuscript. All authors discussed the results and commented on the manuscript. The authors declare no competing financial interests.

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