The Safflower bHLH Transcription Factor CtbHLH41 Negatively Regulates SA-induced Leaf Senescence Through Interaction with CtCP1

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Research

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Abstract

Background

Salicylic acid (SA) plays an important role in regulating leaf senescence. However, the molecular mechanism of leaf senescence of safflower (Carthamus tinctorius) is still elusive. In this study we found that the bHLH transcription factor (TF) CtbHLH41 in Carthamus tinctorius significantly delayed leaf senescence and inhibited the expression of senescence-related genes.

Results

In order to explore how CtbHLH41 promotes leaf senescence, we carried out yeast two-hybrid screening. In this study, by exploring the mechanism of CtbHLH41 regulating CtCP1, it was found that CtCP1 promoted the hydrolysis of CtbHLH41 protein, accelerated the transcriptional activities of salicylic acid-mediated senescence-related genes CtSAG12 and CtSAG29, chlorophyll degradation genes CtNYC1 and CtNYE1, and accelerated leaf senescence. We found a negative SA regulator CtANS1, which interacts with CtbHLH41 and regulates its stability, thereby inhibiting CtCP1-mediated leaf senescence.

Conclusions

In short, our results provide a new insight into the mechanism of CtbHLH41 actively regulating the senescence of safflower leaves induced by SA.

Introduction

Leaf senescence is a process of programmed degradation of intracellular materials and redistribution of nutrients. During leaf senescence, nutrients such as sugars, amino acids, and nucleotides after the cell contents are hydrolyzed are transported from the senescence parts to the plant growth parts or storage organs to facilitate their better growth and successful reproduction (Lim et al., 2007, Schippers et al., 2015, Woo et al., 2019). Chloroplast contains most of the protein in leaves and stores abundant nitrogen resources. More than 80% of the nitrogen content in plant grains comes from the recycling of chloroplast nitrogen during senescence (Girondé et al., 2015). The reutilization of nutrients during senescence affects the vigor and quality of seeds, such as wheat, corn and other crops(Robinson et al., 2012). From this point of view, the impact of senescence on crop yields is crucial.

So far, there have been many studies on the physiological and molecular functions of plant hormones in the regulation of leaf senescence. In general, cytokinin (CTK) and auxin inhibit leaf senescence, while ethylene, abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and gibberellin (GA) promote leaf senescence (Schippers et al., 2015). Current research shows that SA also plays a role in age-dependent aging. High levels of SA in senescent leaves are involved in regulating the up-regulation of leaf senescence-related genes SAGs. A large number of SAGs such as PRIa, cbitinase and SAG12 in Arabidopsis thaliana lacking the SA signal synthesis pathway (nprl and pad4 mutants, NabG

overexpression plants) have reduced or have not express of SAGs. Transcriptome analysis found that: SA pathway and age-dependent senescence look alike. The SA pathway is specifically involved in age-dependent leaf senescence, and further studies have also shown that the content of SA in NabG overexpressing plants is drastically reduced, and the phenotype of delayed senescence is age-dependent rather than dark-induced leaf senescence(Morris et al., 2000).

Leaf senescence is a complex physiological process, which is finely regulated by many transcription factors, such as bHLH transcription factor. bHLH transcription factors have a DNA binding domain composed of about 60 amino acids. The C-terminal of the binding domain is the HLH region, which is a homodimer or heterodimer formed by the interaction of hydrophobic amino acids. Combines with different parts of the target gene promoter (Atchley et al., 1999). At the N-terminus of the binding domain is a basic region. The basic region can be bind to DNA, and its conserved amino acids can recognize E-box (5'-CANNTG-3') (Massari & Murre, 2000) and G-box (5'-CACGTC-3') (Martínez-García et al., 2000). The Arabidopsis bHLH transcription factor subfamily phytochrome-interacting factors (PIFs) are involved in the process of light signal transmission. For example, PIF5 positively regulates the dark-induced senescence process and promotes the degradation of chlorophyll(Zhang et al., 2015).

Cysteine proteases are also involved in the senescence of plants. During senescence, many cellular metabolic processes are reduced, including transcription, but the expression of genes called senescence-associated (SAG) is induced. It includes genes encoding enzymes for degradation such as proteases and nucleases, and genes for enzymes involved in carbohydrate and lipid metabolism. Although there are many SAG studies on different plants, only certain SAGs are strictly related to plant senescence. For example, Arabidopsis SAG12 (AtSAG12) is one such gene whose expression is strictly related to aging (Miller et al., 1999, Otegui et al., 2005). During leaf senescence, the pepper (Capsicum annuum) PLCP gene (CaCP) is induced to express and is also induced by abiotic and biotic stress treatments(Xiao et al., 2014).

In the course of our research on CtbHLH41, we found that the overexpression of CtbHLH41 slows down leaf senescence, but the regulation of the molecular mechanism is not very clear. CtCP1 was included in the results of CtbHLH41 screening by yeast two-hybrid, which may promote leaf senescence of plants. At present, there is no study on the regulation of CtCP1 protein by CtbHLH41. In this study, by exploring the mechanism of CtbHLH41 regulating CtCP1, it was found that CtCP1 promoted the hydrolysis of CtbHLH41 protein, accelerated the transcription and expression of senescence-related genes mediated by salicylic acid, and accelerated leaf senescence. We also found a gene CtANS1 that negatively regulates SA, which also interacts with CtbHLH41 and regulates its protein stability, thereby delaying leaf senescence caused by CtCP1. In general, the relationship among CtbHLH41, CtCP1 and CtANS1 during safflower leaf senescence was described by protein-protein interaction. This study expounded the molecular mechanism of CtbHLH41, CtCP1 and CtANS1 regulating leaf senescence, provided a theoretical basis for the further study of safflower nutrition and nutrient reflux, and provided a new idea to better solve the problem of nutrient transport in production in the future.

Results

CtbHLH41 negatively regulates SA-mediated Senescence of Carthamus tinctorius leaves

A large amount of bHLH TF expression was significantly induced in the process of natural senescence. CtbHLH41 gene is homologous to Arabidopsis thaliana AT5G48560 (light signal bHLH). We used the safflower genome database(https://safflower.scuec.edu.cn/index.html) BLAST to search for a bHLH gene and identified it as CtbHLH41 (CCG019190.1). Then a phylogenetic tree was constructed based on the amino acid sequences of 13 different plant species including safflower to identify bHLH homologous proteins. All proteins contain a basic region and a HLH region, and CtbHLH41 is highly homologous to CsBHLH094 from Cynara cardunculus var. scolymus (Supplementary Figure S1).

The developmental stages of safflower leaves can be divided into three stages: non-senescence (NS), premature senescence (ES) and late senescence (LS). We measured the expression of CtbHLH41 in these three stages. It was found that the expression of CtbHLH41 in NS stage was lower than that in ES and LS stages, and the expression trend was increasing (Fig. 1A). BHLH has previously been found to play an important role as a regulator in JA-mediated defense against necrotizing pathogens and SA-induced cell death (Liu et al., 2020, Yan et al., 2020). In order to study the possible involvement of CtbHLH41 in SA-mediated response, we first examined the induction and time dynamics of CtbHLH41 expression after SA treatment. As shown in figure 1B, the expression of CtbHLH41 is induced by SA. We constructed CtbHLH41 overexpression vector and transformed it into Arabidopsis thaliana, and produced 9 independent Arabidopsis lines to verify the function of CtbHLH41 in leaf senescence (supplementary figure S2). The isolated leaves and whole plants of wild type, mutant and CtbHLH41 overexpression lines were used to determine leaf senescence induced by SA. After SA treatment, the leaves of mutant Arabidopsis thaliana seedlings were more yellowed than those of wild type (WT) and transgenic seedlings (Fig. 1C). The chlorophyll content and the maximum quantum yield of photosystem II (Fv/Fm) in the leaves of mutant plants decreased significantly, which was the same as the color difference between them (figure 1DE). We used transient system to transform CtbHLH41 overexpression and antisense inhibition vectors into isolated safflower leaves to further prove the senescence phenotype of Arabidopsis thaliana leaves (supplementary figure S1). As before, safflower showed delayed senescence due to overexpression of CtbHLH41, while safflower showed early senescence due to antisense inhibition of CtbHLH41 expression (figure 1F). In addition, the CtbHLH41 overexpression and antisense inhibition vectors of Carthamus tinctorius also showed corresponding differences in chlorophyll content (Fig. 1G). Consistent with these findings, chlorophyll content is lower, which represents the expression of senescence-related genes (SAGs); for example, SAG12 and SAG29 are stronger in mutants than in transgenic plants (figure 1H-I). We found that the increased expression of CtNYC1 and CtNYE1 in safflower leaves was due to the inhibition of CtbHLH41 expression (supplementary figure S3). The above results show that CtbHLH41 plays a negative regulatory role in leaf senescence induced by SA.

Physical interaction between CtbHLH41 and CtCP1

During the screening of potential interaction partners of CtbHLH41 using yeast two-hybrid system (Y2H), we found that CtbHLH41 could interact with CtCP1 (supplement figure S2). In addition, the biological significance of their interaction remains to be determined. Interestingly, cysteine proteases (CP) have been revealed to be involved in a variety of physiological processes through the SA pathway (Kovács et al., 2016). Therefore, we speculate that CtbHLH41 may form a complex with CtCP1 to regulate the SA-mediated response. The interaction between Y2H and bimolecular fluorescence complementarity (BiFC) was further confirmed by Y2H and bimolecular fluorescence complementarity (BiFC) measurements. As shown in Fig. 2A and B, CtbHLH41 interacts with CtCP1, and the Basic at the N-terminal of CtbHLH41 gene is essential for their interaction. We also confirmed their interaction by pull-down analysis in vitro. The results showed that CtbHLH41 with GST fusion could retain CtCP1-His, while GST alone could not (Fig. 2C). Finally, in order to further confirm the direct regulation of CtCP1 expression by CtbHLH41, we also used the CtCP1 promoter (2.0kb) fused with LUC gene (CtCP1p:LUC) to determine the transient expression in tobacco leaves. The effector plasmid contains CtbHLH41 gene driven by cauliflower mosaic virus (CaMV) 35s promoter (35S:CtbHLH41). As shown in Fig. 4D and F the co-expression of CtbHLH41 gene and a reporter plasmid resulted in a significant up-regulation of LUC signal compared with the control. To sum up, these results suggest that there is a physical interaction between CtbHLH41 and CtCP1, and they may form a complex to regulate safflower leaf senescence mediated by SA.

CtCP1 is regulating SA-mediated leaf senescence of Carthamus tinctorius.

Since CtbHLH41 participates in SA-mediated safflower leaf senescence and interacts with CtCP1, we speculate that CtCP1 may also participate in SA response through the interaction with CtbHLH41. In order to study the possible involvement of CtCP1 in SA-mediated response, we determined the induced expression of CtCP1 after SA treatment at first. As shown in figure 3B, CtCP1 expression is induced by SA at the mRNA levels. Combined with its strong expression in senescent leaves (Fig. 3A), we concluded that CtCP1 may also play a role in SA-induced leaf senescence. Therefore, the exfoliated leaves of plants with WT, cp1 mutants or CtCP1 overexpression were used for the determination of SA-induced leaf senescence. After SA treatment, cp1 mutants showed delayed leaf senescence compared with WT plants (figure 3C). Compared with wild-type plants, mutant plants also showed less cell death, higher chlorophyll content and lower SAG expression (figure 3D-F and supplementary figure S4). In contrast, 35S:CtCP1 transgenic plants showed accelerated leaf senescence after SA treatment, accompanied by an increase in cell death and a decrease in chlorophyll content, but higher SAG expression (figure 3D-F and supplementary figure S4). The same results were found in safflower leaves (figure 3G-J and supplementary figure S4). Therefore, these observations suggest that CtCP1 positively regulates safflower leaf senescence induced by SA.

Safflower leaf senescence induced by CtbHLH41 delayed SA is CtCP1 dependent

We cloned the promoter of 2kb upstream of CtCP1 gene and constructed a β-glucuronidase (GUS) vector driven by this region, and then transferred it into Arabidopsis thaliana to further prove the function of CtCP1 in response to SA. The results showed that the GUS activity of transgenic Arabidopsis thaliana treated with SA was enhanced under histochemical staining (figure4A-B). In view of the interaction between CtCP1 and CtbHLH41, we suspect that CtbHLH41 is involved in safflower leaf senescence mediated by CtCP1. Arabidopsis lines overexpressing CtbHLH41 were hybridized with Arabidopsis lines overexpressing CtCP1 or mutant cp1, respectively. Arabidopsis thaliana plants overexpressing CtbHLH41/cp1 turn yellow more slowly and contain more chlorophyll than plants overexpressing CtbHLH41/CP1 or overexpressing CtCP1 or mutant cp1 and overexpressing CtbHLH41 or bhlh41 alone (figure 4C-E and supplementary figure S5). Consistent with these findings, the chlorophyll content is lower, which means that the expression of senescence-related genes (SAGs) is stronger in mutants than in transgenic plants (figure 4F-G and supplementary figure S5). Therefore, the senescence of safflower leaves induced by CtbHLH41-delayed SA is CtCP1-dependent.

CtCP1 promotes the hydrolysis of CtbHLH41 protein

Recent studies have shown that cysteine proteases are usually involved in plant senescence by hydrolyzing substrates (Deng et al., 2019). CtbHLH41-His fusion protein and some reported conventional protease substrates (including casein, azo casein, protein, gelatin, collagen and l.leucine.p.nitroaniline) were used to verify the effects of different substrates on CtCP1 activity. The enzyme had the highest activity against CtbHLH41-His fusion protein, with a relative activity of 165% respectively, and was further improved under SA treatment (figure 5A-B).

We speculate that CtCP1 may affect the stability of CtbHLH41 protein. We obtained CtCP1 overexpression and antisense safflower leaves (CtCP1-OE and CtCP1-Anti) by transient transformation. Next, we carried out the cell-free CtbHLH41 degradation test, and incubated the total proteins extracted from wild-type, CtCP1-OE and CtCP1-Anti leaves with CtbHLH41-His. As shown in Fig. 5C, the degradation rate of CtbHLH41-His protein in CtCP1-OE leaves was much higher than that in wild-type leaves, while the degradation rate of CtbHLH41-His in CtCP1-Anti leaves was slower. However, the degradation of CtbHLH41-His protein was significantly inhibited by proteasome inhibitor E-64 (Fig. 5C). These results showed that CtCP1 protease hydrolyzed CtbHLH41 protein. In order to further prove that CtCP1 can also promote the hydrolysis of CtbHLH41 in plants, we also used instantaneous system to obtain 35S:CtbHLH41-GFP and 35S:CtbHLH41-GFP + 35S:CtCP1-OE transgenic safflower leaves. Finally, by Western blotting with anti-GFP antibody, we found that the abundance of CtbHLH41-GFP protein in 35S:CtbHLH41-GFP + 35S:CtCP1-OE transgenic leaves was lower (Fig. 5D). In summary, these results suggest that CtCP1 mediates the hydrolysis of CtbHLH41 protein.

CtbHLH41 delays SA-mediated leaf senescence in a CtANS1-dependent manner

BHLH transcription factors usually perform their biological functions by specifically binding to G-box (CACGTG), which exists in the promoter of its target gene (An et al., 2019). Our Y2H results suggest that CtbHLH41 may play a role in safflower leaf senescence mediated by SA by regulating the expression of related genes in the flavonoid pathway (supplement figure S2). Interestingly, a putative G-box element was found in the CtANS1 promoter. In order to confirm this result, we determined the transient expression in tobacco leaves. We cloned the CtANS1 promoter fragment of 2.0kb and fused with pGreenII0800-LUC reporter gene into pCtANS1-LUC, CtbHLH41 to construct the effector vector pGreenII62-SK to produce CtbHLH41-SK. As a result, we found that the promoter of CtANS1 was activated by CtbHLH41 (figure 6D-F). Yeast one-hybrid test showed that the pGADT7-CtbHLH41 fusion protein binds to the four tandem repeats of the G-box motif from the CtANS1 promoter rather than to pGADT7 alone, which strongly activates the expression of the HIS3 reporter gene (Fig. 6C), indicating that CtbHLH41 binds to the G-box motif in the CtANS1 promoter. In order to further study the possible response of CtANS1 to SA, we stained transgenic Arabidopsis thaliana with or without SA treatment and found that SA treatment significantly increased GUS activity. Therefore, we speculate that the promoter of CtANS1 may directly bind to CtbHLH41 and increase its own transcriptional expression to regulate the SA response.

In order to further confirm this conclusion, the genetic relationship between CtbHLH41 and CtANS1 was studied. The bhlh41 mutant hybridized with the ans1 mutant to produce bhlh41/ans1 double mutant, and the 35S:CtbHLH41 mutant hybridized with the ans1 mutant to produce 35S:CtbHH41/ans1, 35S:CtANS1 hybridized with the 35S:CtbHLH41 or bhlh41 mutant to produce 35S:ANS1/CtbHLH41 and 35S:ANS1/bhlh41, respectively (supplementary figure S6). Then the leaf senescence mediated by SA in these transgenic lines was examined. As shown in figure 6F, the overexpression of CtbHLH41 can delay the senescence of ans1, and the overexpression of CtANS1 can also delay the senescence of bhlh41. Consistent with these aging phenotypes, the chlorophyll content and SAG expression between 35S:bHLH41/ans1 and 35S:ANS1/bhlh41 or between 35S:ANS1 and 35S:ANS1/CtbHLH41 were similar (figure 6G-H and supplementary figure S6). Therefore, genetic analysis showed that CtbHLH41 acted on the upstream of CtANS1 and acted as a negative regulator of SA-mediated leaf senescence in a CtANS1-dependent manner.

Discussion

Leaf senescence is not only a passive degradation process of cell structure metabolism, but also the result of a series of fine regulation of gene expression. With the cloning of a large number of senescence-related genes, it has laid a solid foundation for the analysis of the molecular mechanism of leaf senescence. The most significant change of leaf senescence is chloroplast degradation, and 70% of leaf protein is concentrated in chloroplast, with the disintegration of chloroplast, a large number of proteins are degraded. For most plants, the critical period of seed and fruit formation is often accompanied by leaf senescence, which leads to the decline of photosynthesis and the reduction of yield (Quirino et al., 2000, Song et al., 2014). In addition, the regulation of leaf senescence time will also affect plant stress tolerance (Rivero et al., 2007, Zhao et al., 2016). In the growth and development of herbaceous plants, it is of great significance to study the role of leaf senescence and the leaf senescence regulatory network.

We found that CtbHLH41 was expressed in senescent safflower leaves and was also induced by SA; bhlh41 mutants showed accelerated SA-induced leaf senescence while plants overexpressing CtbHLH41 showed significantly delayed SA-induced leaf senescence compared with WT plants (figure 1) indicating that CtbHLH41 as a negative regulator SA-mediated leaf senescence. These SA-induced aging phenotypes are associated with changes in the expression of several aging-related genes, such as CtSAG12, CtSAG29, CtNYC1 and CtNYE1 (figure 1 and supplementary figure S2). As the second largest transcription factor family in plants, the bHLH family in Carthamus tinctorius is composed of 23 functionally diverse members (Yingqi et al., 2019). However, so far, there are few reports about their involvement in plant senescence. Therefore, our results provide new insights into the importance of bHLH protein in Carthamus tinctorius, especially in plant senescence response.

CtbHLH41 can regulate leaf senescence not only by regulating downstream genes, but also by interacting with other proteins. Here, we demonstrate the interaction between CtbHLH41 and CtCP1 (figure 2). Cysteine protease regulates SA-induced leaf senescence (Kovács et al., 2016, Poór et al., 2013, Havé et al., 2018, Gong et al., 2020). Recent studies have shown that cysteine proteases negatively regulate chloroplast development under SA treatment (Durian et al., 2020, Shi et al., 2021). It has been proved that OsPLCPs can negatively regulate the virulence of Xanthomonas oryzae pv in previous RNA-seq data (Niño et al., 2020). It has been found that leaf senescence is regulated by SAG12 directly activating the expression of NYC1 and NYE14 and inhibiting the expression of NPR1 (Morris et al., 2000). Here, our data shows that the promotion of leaf senescence is due to the overexpression of CtCP1 gene in plants, while inhibiting the expression of CtCP1 delays leaf senescence (figure 3). CtCP1 activated the expression of CtNYC1 and CtNYE1 in safflower leaves transformed by CtCP1-OE, which further proved that CtCP1 could positively regulate leaf senescence (supplement figure S4). We found that the transcriptional activation of CtNYC1 and CtNYE1 by CtbHLH41 was enhanced, which may be due to the acceleration of CtbHLH41-mediated leaf senescence by CtCP1 (Fig. S5). The transcriptional activity of CtbHLH41 to target genes may be affected by the interaction between CtCP1 and CtbHLH41, thus promoting leaf senescence. Our results show that CtCP1 plays an opposite role in leaf senescence promoted by CtbHLH41. All biological processes of plants are actively involved in proteolysis (van Wijk, 2015, Liao et al., 2019). Several studies have shown that cysteine proteases are involved in leaf senescence by hydrolyzing substrates (Carrión et al., 2013, Otegui et al., 2005). Our results show that the enhancement of leaf senescence induced by SA is due to the degradation of CtbHLH41 by CtCP1 (figure 5). CtCP1 can be released from CtbHLH41-mediated degradation and accelerate the degradation of CtbHLH41 in the presence of SA. Our results provide an in-depth understanding of the molecular mechanism of SA-induced leaf senescence mediated by cysteine protease. These results suggest that SA-induced leaf senescence can be regulated by CtbHLH41 regulating different target protein dynamics.

Despite the functional diversity, it was found that bHLH protein exerted its biological function (Jian-PingAn2019) by directly binding to G-box (CACGTG) in its target promoter. Interestingly, we reveal here that CtbHLH41 can directly target CtANS1 through G-box in its promoter and subsequently regulate SA-mediated leaf senescence (figure 6). Due to the comparative expression patterns of CtbHLH41 and CtANS1 in CtbHLH41 mutants and overexpressed plants, as well as the enhanced LUC signal in transient

expression assay (Fig. 6), we infer that CtbHLH41 plays a role as a positive regulator of CtANS1. Genetic analysis further showed that CtbHLH41 acted on the upstream of CtANS1 and participated in SA-mediated leaf senescence in a CtANS1-dependent manner (Fig. 6). Previously, bHLH has also been shown to directly activate several senescence-related genes and chlorophyll degradation regulatory genes (NYE1), but inhibit chloroplast activity maintenance genes (GLK2) during leaf senescence (Song et al., 2014, Qi et al., 2015). Therefore, CtbHLH41 can be used as both an activator and an inhibitor to fine-tune leaf senescence. We find that CtbHLH41 interacts with CtCP1 and CtANS1, and the same region of CtbHLH41 interacts with CtCP1 and CtANS1, and CtCP1 and CtANS1 interact with full-length CtbHLH41, but it is not clear whether these two interactions are related (figure 2). CtCP1 and CtANS1 may interact competitively with CtbHLH41 to antagonize the regulation of leaf senescence, but this hypothesis needs to be further verified.

Finally, the role of CtbHLH41 in leaf senescence is summarized by a working model (figure 7). On the one hand, in the presence of SA, leaf senescence is due to CtCP1 interacting with CtbHLH41 and hydrolyzing CtbHLH41 to degrade CtbHLH41. On the other hand, CtbHLH41 interacts with CtANS1 to delay leaf senescence by reducing the transcriptional activity of CtbHLH41 to senescence-related genes CtSAG12 and CtSAG29 and chlorophyll degradation genes CtNYC1 and CtNYE1. In this study, the role of CtbHLH41 in the regulatory network of leaf senescence was characterized by direct interaction with CtCP1 and CtANS1.

Materials And Methods

Plant materials and growth conditions

The safflower (Carthamustinctorius) variety of "Jihong 1" was used as material in our study. The seeds were sown in the experimental field of Jilin Agricultural University in Changchun, China in late March 2018. Then the leaves of Carthamus tinctorius at different developmental stages were picked, put in liquid nitrogen and stored in the ultra-low temperature refrigerator at-80 ℃. The Columbia ecotype of Arabidopsis thaliana was used. The seeds were disinfected with 20% bleach for 15 minutes, then sowed at 4°C on half-intensity Murashige and Skoog (MS) medium for 3 days. The plants transferred to the soil 7 days after germination and grew in a greenhouse at 22°C under 16 hours of light / 8 hours of dark photoperiod. Tobacco grows in a greenhouse at 25°C under 16 hours of light / 8 hours of dark photoperiod. The mutants of Arabidopsis thaliana: bhlh41(SALK_085759C), cp1(SALK_065256C) and ans1(SALK_120680C) were purchased from AraShare (http://www.arashare.cn/). Taq DNA polymerase is purchased from Takara Biotechnology Co. Ltd (Japan), SA is purchased from Sigma Co. Ltd (USA), and other chemicals are purchased from Shanghai Sangon Biotechnology Co., Ltd. (China).

Plasmid vector construction and plant genetic transformation

The full-length coding sequences of CtCP1 and CtANS1 were cloned into pCAMBIA3301-Flag vector, and the full-length coding sequence CtbHLH41 was cloned into pCAMBIA1302-GFP vector. Finally, the fragments of CtbHLH41, CtCP1 and CtANS1 sequences were cloned into pCAMBIA3301 vector to obtain antisense inhibitory vectors (CtbHLH41-Anti, CtCP1-Anti and CtANS1-Anti). The instantaneous transformation of safflower leaves by CtbHLH41-GFP was obtained according to the previous study and with slight improvement (An et al., 2018). In order to generate proCtCP1GUS and proCtANS1:GUS constructors, the promoter fragments of CtCP1 and CtANS1 were inserted into pCAMBIA3301-GUS vector and then transformed into Arabidopsis thaliana. Transgenic Arabidopsis seedlings were obtained based on previously described methods (An et al., 2020). The instantaneous transformed safflower leaves (An et al., 2020) were obtained according to the methods described by others and slightly improved. All the primers used for gene cloning in the experiment can be seen in the supplementary table S1.

RNA extraction and gene expression analysis

10 mM solution was prepared by dissolving SA in 90μl ethanol and adding water. The SA solution was diluted to 100um with distilled water and sprayed on the plants. Spray water on the plants as a control. The total RNA in safflower, safflower, Arabidopsis and treated seedlings was extracted using the RNAplantPlus kit (Coolaber) and according to the manufacturer's instructions. The first strand cDNA is synthesized using the PrimeScript strand RT kit (TaKaRa) and according to the manufacturer's instructions. Use UltraSYBR Mixture (Takara) to qRT-PCR on the Mx3000P instrument (Multiplex Quantitative PCR System). The primers used in the experiment can be seen in Supplementary Table S1. Each experiment performed three biological replicates and three technical replicates.

Analysis of senescence induced by SA

The fifth and sixth rosette leaves of 4-week-old plants were placed in a petri dish containing distilled water with or without 100μM SA, and then the petri dish was placed in low light (20μmol m-2s-1 photosynthetic photon flux density) at 22°C for 7 days. Three-week-old plants growing in the soil were sprayed with or without 100μM SA and placed under 22°C under low light (20μmol m-2s-1 photosynthetic photon flux density) for 7 days. According to (Schoefs et al., 2001), 80% acetone was used to extract chlorophyll from isolated leaves. Cell mortality was detected by trypan blue staining; leaves of specified genotypes treated with or without 100 μM SA were soaked in 0.05% trypan blue solution, kept at 80°C for 2 minutes, and then cleared with chloral hydrate.

Yeast two-hybrid screening and confirmation

The recombinant plasmid pGBKT7-CtBHLH41 was constructed by cloning the full-length CtbHLH41 coding sequence into the bait vector pGBKT7, and then transforming it into the yeast strain Y2H Gold (Clontech, USA). The safflower cDNA library and the amplified CtCP1 or CtANS1 full-length coding sequence are fused with the prey vector pGADT7. In order to confirm the protein-protein interaction, we performed a yeast two-hybrid screen, and performed it according to the hybridization protocol described in the Clontech Matchmaker TM Gold Yeast Two-Hybrid manufacturer. See attached table S1 for Y2H screening primers.

Pull-down and BiFC assays

The recombinant plasmids CtCP1-pET32a and CtbHLH41-pGEX-KG were generated by cloning the full-length coding sequences of CtCP1 and CtbHLH41 into pET32a and pGEX-KG vectors. The recombinant plasmids were then transformed into E. coli BL21 (DE3), and the fusion proteins CtCP1-His and CtbHLH41-GST were induced and expressed by 0.8mM isopropyl β-Dmurmurl 1-galactoside (IPTG). Finally, the eluted proteins were detected by Western blotting using anti-GST and anti-His antibodies (Abmart, Shanghai, China). CtCP1-cYFP and CtbHLH41-nYFP were generated by cloning the coding sequences of CtCP1 and CtbHLH41 into 35S:pSPYCE-cYFP and 35S:pSPYNE-nYFP vectors. The recombinant plasmid was then transformed into Agrobacterium tumefaciens EHA105 and then injected into tobacco leaves. Finally, the YFP fluorescence signal was detected by confocal laser scanning microscope (Leica).

Transient expression assay

For transient expression analysis, CtCP1 and CtANS1 promoters were inserted into pGreenII0800-LUC vector. The recombinant plasmid pGreen62-SK-CtbHLH41 was obtained by cloning the full-length CtbHLH41 into pGreen62-SK vector. The recombinant plasmid was transformed into tobacco leaf by Agrobacterium tumefaciens, and the final LUC/REN activity ratio was obtained by double luciferase reporter gene detection system (Promega)(An et al., 2018).

GUS analysis

We prepared X-gluc buffers (1mM X-Gluc, 0.5mM ferricyanide, 0.5mM ferricyanide, 0.1mM EDTA and 0.1% Triton X-100), and then incubated transgenic Arabidopsis thaliana at 37°C for 14 hours. The final GUS activity was detected by fluorescence spectrophotometer.

Y1H assay

The natural promoters (about 2.0kb) of CtANS1 and CtCP1 were cloned into pHIS2 vector. The ORF of CtbHLH41 was amplified and cloned into pGADT7 vector. Each pHIS2 bait vector containing target gene and pGADT7-CtbHLH41 prey vector were co-transformed into competent yeast strain Y187. The transformants were selected on SD/-Trp/-Leu medium and treated with 30mM 3-amino-1 3-AT (SD/-Trp/-Leu/-His + 30mM 3-AT). The combination of pGADT7-CtbHLH41 and pHIS2 was used as negative control plasmid.

Quantification of chlorophyll content and Fv/Fm ratio

We obtained the total chlorophyll concentration by extracting total pigment from plant leaves with 95% ethanol and incubating it overnight, and then using ultraviolet / visible spectrophotometer (SOPTOPUV2800S, Shanghai, China) to measure the absorbance at 649 and 665 nm. Finally, using a closed chlorophyll fluorescence imaging system (Photon system instrument, Brno, Czech Republic) and analyzing the safflower leaves according to the manufacturer's instructions, calculate the Fv/Fm ratio, as mentioned earlier (Sakuraba et al., 2020).

Protease specificity

In order to determine the specificity of protease, azo casein, albumin, gelatin, collagen, l.leucine.p.nitroaniline and CtbHLH41-His fusion protein were used as substrates to measure enzyme activity. For azo casein, 90 μ l substrate 1% and 10 μ l enzyme solution were incubated at the optimum temperature and pH value for 30 minutes. The reaction was then terminated by adding cold TCA 15%. The solution was centrifuged at 4000 × g for 15 minutes. The absorbance of the supernatant was measured at 440 nm. When using l.leucine.p.nitroaniline for detection, 30 μl substrate (10 mM), 100 μ l protease, 120 μ l distilled water and 250 μ l Tris-HCL (100 mM) were incubated for 30 minutes at the optimum temperature and pH value. In order to stop the reaction, 100 μ l 30% (w / w) acetic acid was used. The sample was centrifuged at 4000 × g for 10 minutes. The absorbance of the supernatant was measured at 410 nm. Albumin, collagen, gelatin and CtbHLH41-His fusion proteins were also determined in the same way as azo casein.

Protein degradation and hydrolysis analysis

For the determination of protein degradation in vitro, the degradation buffer (25mM Tris-HCl, pH7.5, 10mM NaCl, 10 mM MgCl2, 4mM, 4mM phenylmethylsulfonyl fluoride, 5mM DTT, and 10mM ATP) was used. The CtbHLH41-His fusion protein was incubated with various plant proteins at 23°C and evaluated with anti-His antibody (Abmart) by Western blotting. For in vivo hydrolysis detection, the abundance of CtbHLH41-GFP protein in transiently transformed safflower leaves (CtbHLH41-GFP and CtbHLH41-GFP/CtCP1-OE) was evaluated by Western blotting using anti-GFP antibodies.

Statistical analyses

Each experiment in this study carried out at least three biological replicates and three technical replicates. The data was processed using Origin 2019b software for t-test, and asterisks indicate significant differences (* P <0.05).

Declarations

Availability of data and materials

Vectors and Arabidopsis seeds can be requested from the corresponding authors. The vectors pEASY-T1 are also available via Miaoling (https:// miaolingbio.com).

Authors' contributions

L.X and Y.N designed the project; H Y. performed the experiments and analyze the data; J.Z, and Y.X carried out formal analysis; H Y. performed statistical analysis; H Y. conducted software analysis, H.Y wrote the original draft.

Conflicts of Interest:

The authors declare that they have no conflict of interest.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree with submission of this version.

Competing interests

The authors declare that they have no competing interests.

References

An JP, Wang XF, Zhang XW*, et al.*, 2020. An apple MYB transcription factor regulates cold tolerance and anthocyanin accumulation and undergoes MIEL1-mediated degradation. *Plant Biotechnol J* **18**, 337-53.

An JP, Yao JF, Xu RR, You CX, Wang XF, Hao YJ, 2018. Apple bZIP transcription factor MdbZIP44 regulates abscisic acid-promoted anthocyanin accumulation. *Plant Cell Environ* **41**, 2678-92.

An JP, Zhang XW, Bi SQ, You CX, Wang XF, Hao YJ, 2019. MdbHLH93, an apple activator regulating leaf senescence, is regulated by ABA and MdBT2 in antagonistic ways. *New Phytol* **222**, 735-51.

Atchley WR, Terhalle W, Dress A, 1999. Positional dependence, cliques, and predictive motifs in the bHLH protein domain. *J Mol Evol* **48**, 501-16.

Carrión CA, Costa ML, Martínez DE, Mohr C, Humbeck K, Guiamet JJ, 2013. In vivo inhibition of cysteine proteases provides evidence for the involvement of 'senescence-associated vacuoles' in chloroplast protein degradation during dark-induced senescence of tobacco leaves. *J Exp Bot* **64**, 4967-80.

Deng J, Zhu F, Liu J*, et al.*, 2019. Transcription Factor bHLH2 Represses CYSTEINE PROTEASE77 to Negatively Regulate Nodule Senescence. *Plant Physiol* **181**, 1683-703.

Durian G, Jeschke V, Rahikainen M*, et al.*, 2020. PROTEIN PHOSPHATASE 2A-B'γ Controls Botrytis cinerea Resistance and Developmental Leaf Senescence. *Plant Physiol* **182**, 1161-81.

Girondé A, Etienne P, Trouverie J*, et al.*, 2015. The contrasting N management of two oilseed rape genotypes reveals the mechanisms of proteolysis associated with leaf N remobilization and the respective contributions of leaves and stems to N storage and remobilization during seed filling. *BMC Plant Biol* **15**, 59.

Gong W, Li B, Zhang B, Chen W, 2020. ATG4 Mediated Psm ES4326/AvrRpt2-Induced Autophagy Dependent on Salicylic Acid in Arabidopsis Thaliana. *Int J Mol Sci* **21**.

Havé M, Balliau T, Cottyn-Boitte B*, et al.*, 2018. Increases in activity of proteasome and papain-like cysteine protease in Arabidopsis autophagy mutants: back-up compensatory effect or cell-death promoting effect? *J Exp Bot* **69**, 1369-85.

Kovács J, Poór P, Szepesi Á, Tari I, 2016. Salicylic acid induced cysteine protease activity during programmed cell death in tomato plants. *Acta Biol Hung* **67**, 148-58.

Liao Z, Yu H, Duan J*, et al.*, 2019. SLR1 inhibits MOC1 degradation to coordinate tiller number and plant height in rice. *Nat Commun* **10**, 2738.

Lim PO, Kim HJ, Nam HG, 2007. Leaf senescence. *Annu Rev Plant Biol* **58**, 115-36.

Liu Y, Li M, Li T*, et al.*, 2020. Airborne fungus-induced biosynthesis of anthocyanins in Arabidopsis thaliana via jasmonic acid and salicylic acid signaling. *Plant Sci* **300**, 110635.

Martínez-García JF, Huq E, Quail PH, 2000. Direct targeting of light signals to a promoter element-bound transcription factor. *Science* **288**, 859-63.

Massari ME, Murre C, 2000. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol Cell Biol* **20**, 429-40.

Miller JD, Arteca RN, Pell EJ, 1999. Senescence-associated gene expression during ozone-induced leaf senescence in Arabidopsis. *Plant Physiol* **120**, 1015-24.

Morris K, Mackerness SA, Page T*, et al.*, 2000. Salicylic acid has a role in regulating gene expression during leaf senescence. *Plant J* **23**, 677-85.

Niño MC, Kang KK, Cho YG, 2020. Genome-wide transcriptional response of papain-like cysteine protease-mediated resistance against Xanthomonas oryzae pv. oryzae in rice. *Plant Cell Rep* **39**, 457-72.

Otegui MS, Noh YS, Martínez DE*, et al.*, 2005. Senescence-associated vacuoles with intense proteolytic activity develop in leaves of Arabidopsis and soybean. *Plant J* **41**, 831-44.

Poór P, Kovács J, Szopkó D, Tari I, 2013. Ethylene signaling in salt stress- and salicylic acid-induced programmed cell death in tomato suspension cells. *Protoplasma* **250**, 273-84.

Qi T, Wang J, Huang H*, et al.*, 2015. Regulation of Jasmonate-Induced Leaf Senescence by Antagonism between bHLH Subgroup IIIe and IIId Factors in Arabidopsis. *Plant Cell* **27**, 1634-49.

Quirino BF, Noh YS, Himelblau E, Amasino RM, 2000. Molecular aspects of leaf senescence. *Trends Plant Sci* **5**, 278-82.

Rivero RM, Kojima M, Gepstein A*, et al.*, 2007. Delayed leaf senescence induces extreme drought tolerance in a flowering plant. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 19631-6.

Robinson WD, Carson I, Ying S, Ellis K, Plaxton WC, 2012. Eliminating the purple acid phosphatase AtPAP26 in Arabidopsis thaliana delays leaf senescence and impairs phosphorus remobilization. *New Phytol* **196**, 1024-9.

Sakuraba Y, Kim D, Han SH*, et al.*, 2020. Multilayered Regulation of Membrane-Bound ONAC054 Is Essential for Abscisic Acid-Induced Leaf Senescence in Rice. *Plant Cell* **32**, 630-49.

Schippers JH, Schmidt R, Wagstaff C, Jing HC, 2015. Living to Die and Dying to Live: The Survival Strategy behind Leaf Senescence. *Plant Physiol* **169**, 914-30.

Schoefs B, Darko E, Rodermel S, 2001. Photosynthetic pigments, photosynthesis and plastid ultrastructure in RbcS antisense DNA mutants of tobacco (Nicotiana tabacum). *Z Naturforsch C J Biosci* **56**, 1067-74.

Shi B, Zhao X, Li M*, et al.*, 2021. Wheat Thioredoxin (TaTrxh1) Associates With RD19-Like Cysteine Protease TaCP1 to Defend Against Stripe Rust Fungus Through Modulation of Programmed Cell Death. *Mol Plant Microbe Interact* **34**, 426-38.

Song Y, Yang C, Gao S, Zhang W, Li L, Kuai B, 2014. Age-triggered and dark-induced leaf senescence require the bHLH transcription factors PIF3, 4, and 5. *Mol Plant* **7**, 1776-87.

Van Wijk KJ, 2015. Protein maturation and proteolysis in plant plastids, mitochondria, and peroxisomes. *Annu Rev Plant Biol* **66**, 75-111.

Woo HR, Kim HJ, Lim PO, Nam HG, 2019. Leaf Senescence: Systems and Dynamics Aspects. *Annu Rev Plant Biol* **70**, 347-76.

Xiao HJ, Yin YX, Chai WG, Gong ZH, 2014. Silencing of the CaCP gene delays salt- and osmotic-induced leaf senescence in Capsicum annuum L. *Int J Mol Sci* **15**, 8316-34.

Yan S, Ning K, Wang Z*, et al.*, 2020. CsIVP functions in vasculature development and downy mildew resistance in cucumber. *PLoS Biol* **18**, e3000671.

Yingqi H, Ahmad N, Yuanyuan T*, et al.*, 2019. Genome-Wide Identification, Expression Analysis, and Subcellular Localization of Carthamus tinctorius bHLH Transcription Factors. *Int J Mol Sci* **20**.

Zhang Y, Liu Z, Chen Y, He JX, Bi Y, 2015. PHYTOCHROME-INTERACTING FACTOR 5 (PIF5) positively regulates dark-induced senescence and chlorophyll degradation in Arabidopsis. *Plant Sci* **237**, 57-68.

Zhao Y, Chan Z, Gao J*, et al.*, 2016. ABA receptor PYL9 promotes drought resistance and leaf senescence. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 1949-54.

Figures

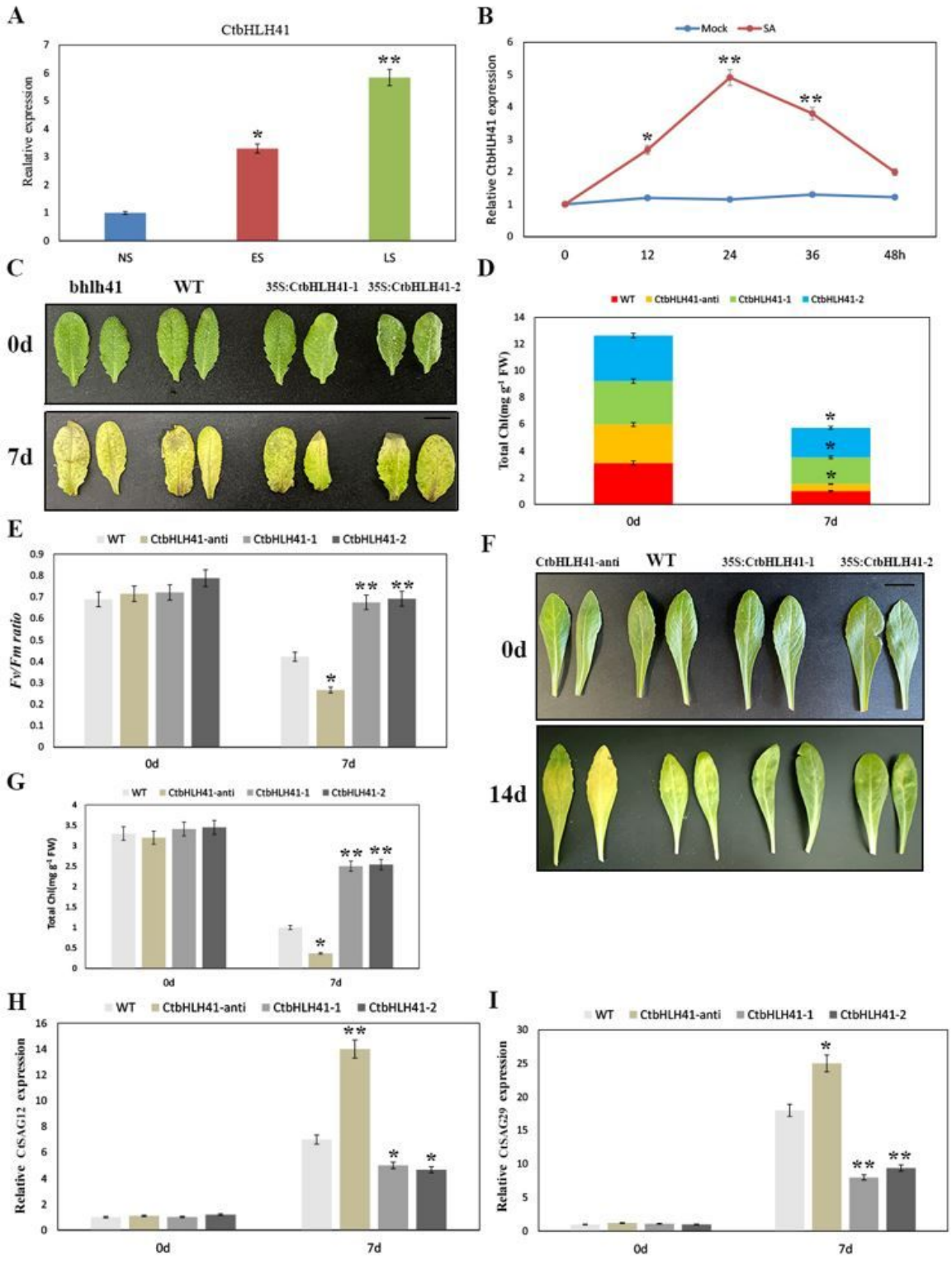

Figure 1

CtbHLH41 negatively regulates SA-induced leaf senescence. (A) CtbHLH41 transcription level in late senile (LS), premature senile (ES) and non-senile (NS) leaves. (B) qRT-PCR analysis of CtbHLH41 transcription level in 4-week-old WT safflower leaves after 100um SA treatment. (C) Senescence phenotype of 4-week-old Arabidopsis leaves of designated genotypes treated with or without 100um SA for 7 days. Scale = 1 cm. (D) chlorophyll content in specified genotypes treated with or without 100um SA

for 7 days. (E) Fv/Fm in specified genotypes treated with or without 100um SA for 7 days. (F) Leaf senescence phenotype of specified genotypes of Carthamus tinctorius treated with or without 100um SA for 14 days. Scale = 1cm. (G) chlorophyll content in designated genotypes treated with or without 100um SA for 14 days. qRT-PCR analysis of SAG12 and SAG29 expression in leaves of specified genotypes of Carthamus tinctorius treated with or without 100um SA for 14 days. The asterisk indicates the significant difference determined by t-test (* P < 0.05, P < 0.01).

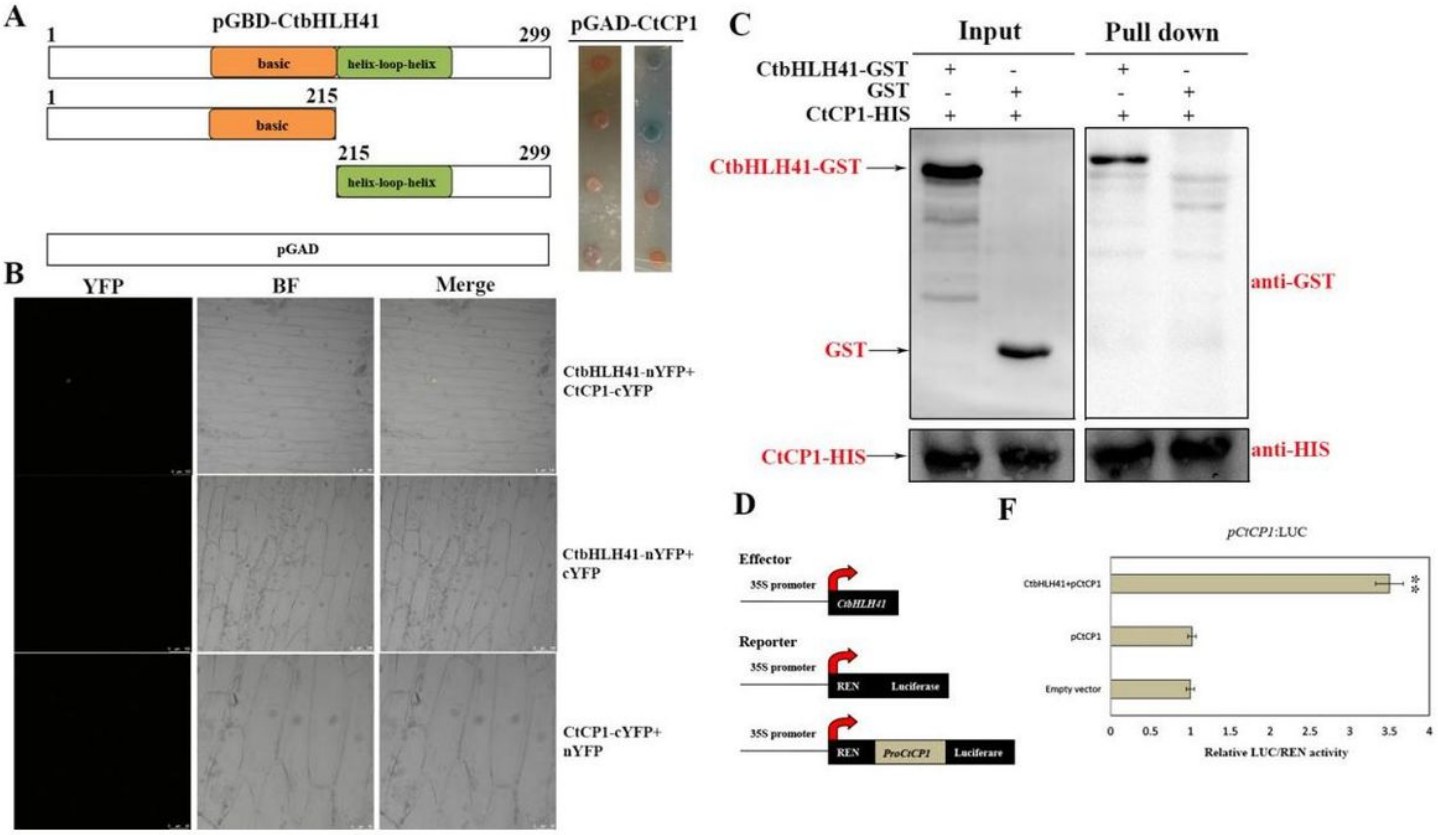

Figure 2

CtbHLH41 physically interacts with CtCP1. (A) yeast two-hybrid (Y2H) assay showed the interaction between CtbHLH41 and CtCP1. The full-length CtbHLH41 and truncated CtbHLH41 sequences were cloned into pGBKT7-BD vector. The full-length CtCP1 was cloned into pGADT7-AD vector. Empty pGAD vector was used as negative control. (B) BiFC determination. CtCP1-cYFP and CtbHLH41-nYFP constructs were transiently expressed in tobacco leaves, and the fluorescence signals were observed by fluorescence microscope. Scale, 50μm. (C) CtbHLH41 and CtCP1 pull-down assay in vitro. CtCP1-His protein was incubated with CtbHLH41-GST and GST. Anti-GST and anti-His antibodies were used to detect proteins pulled down with GST beads. (D) schematic diagram of reporter and effector constructors. (F) luciferase assay showed that the transient transformation of CtbHLH41 into tobacco activated the expression of CtCP1. Empty vector (SK + LUC) was used as negative control. The LUC/REN ratio represents the ability of CtbHLH41 to activate CtCP1 expression. The asterisk indicates that there is a significant difference between * P < 0.05 and P < 0.01.

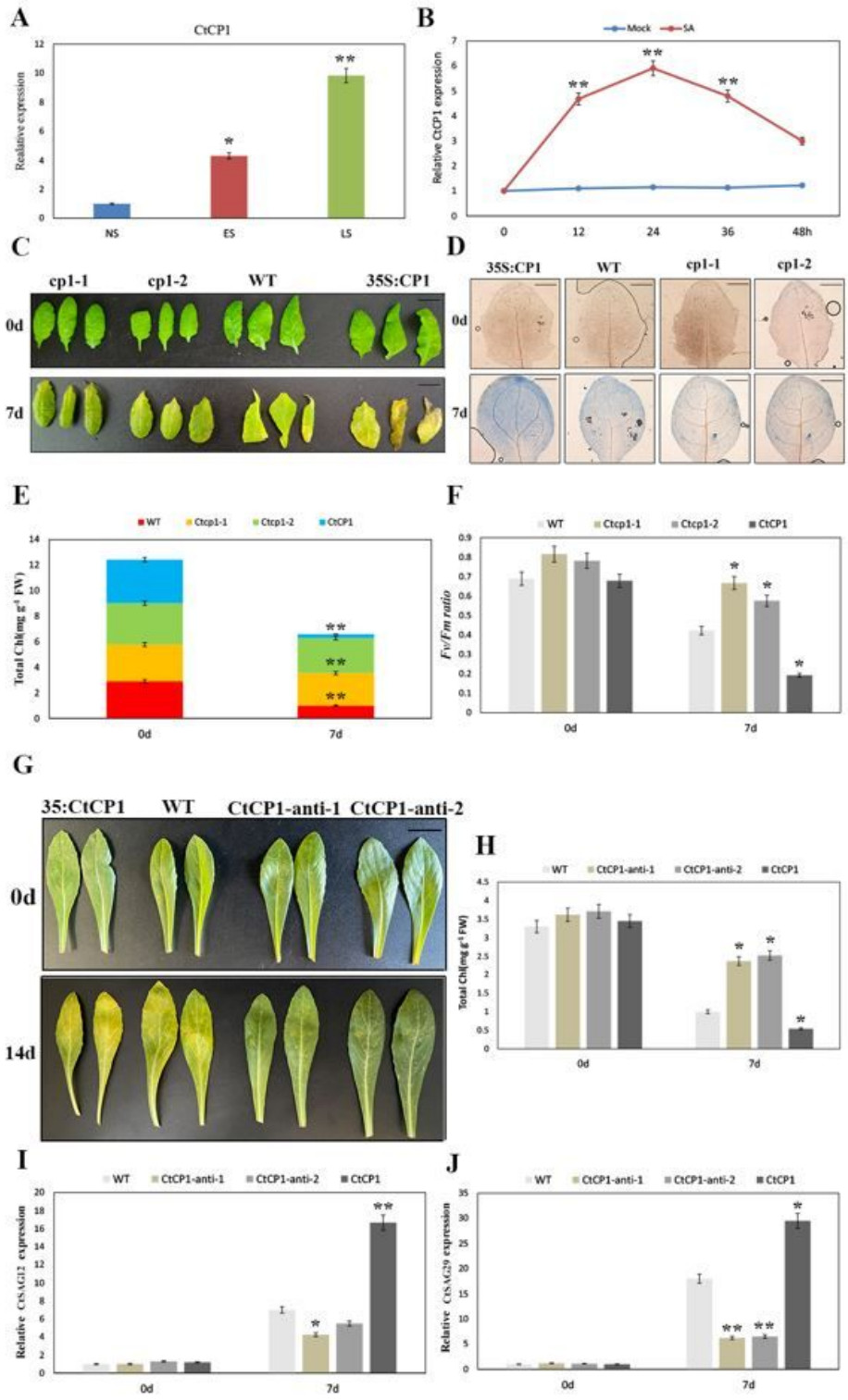

Figure 3

CtCP1 positively regulates SA-induced senescence of safflower leaves. (A) CtCP1 transcription level in late senile (LS), premature senile (ES) and non-senile (NS) leaves. (B) qRT-PCR analysis of CtCP1 transcription level in 4-week-old WT safflower leaves after 100um SA treatment. (C) Senescence phenotype of 4-week-old Arabidopsis leaves of designated genotypes treated with or without 100um SA for 7 days. Scale = 1 cm. (D) Trypan blue staining of the indicated genotypes treated with or without SA

for 7d . Scale bar =2 mm. (E) chlorophyll content in specified genotypes treated with or without 100um SA for 7 days. (F) Fv/Fm in specified genotypes treated with or without 100um SA for 7 days. (G) Leaf senescence phenotype of specified genotypes of Carthamus tinctorius treated with or without 100um SA for 14 days. Scale = 1cm. (HJ) chlorophyll content in designated genotypes treated with or without 100um SA for 14 days. qRT-PCR analysis of SAG12 and SAG29 expression in leaves of specified genotypes of Carthamus tinctorius treated with or without 100um SA for 14 days. The asterisk indicates the significant difference determined by t-test (* P <0.05, P <0.01).

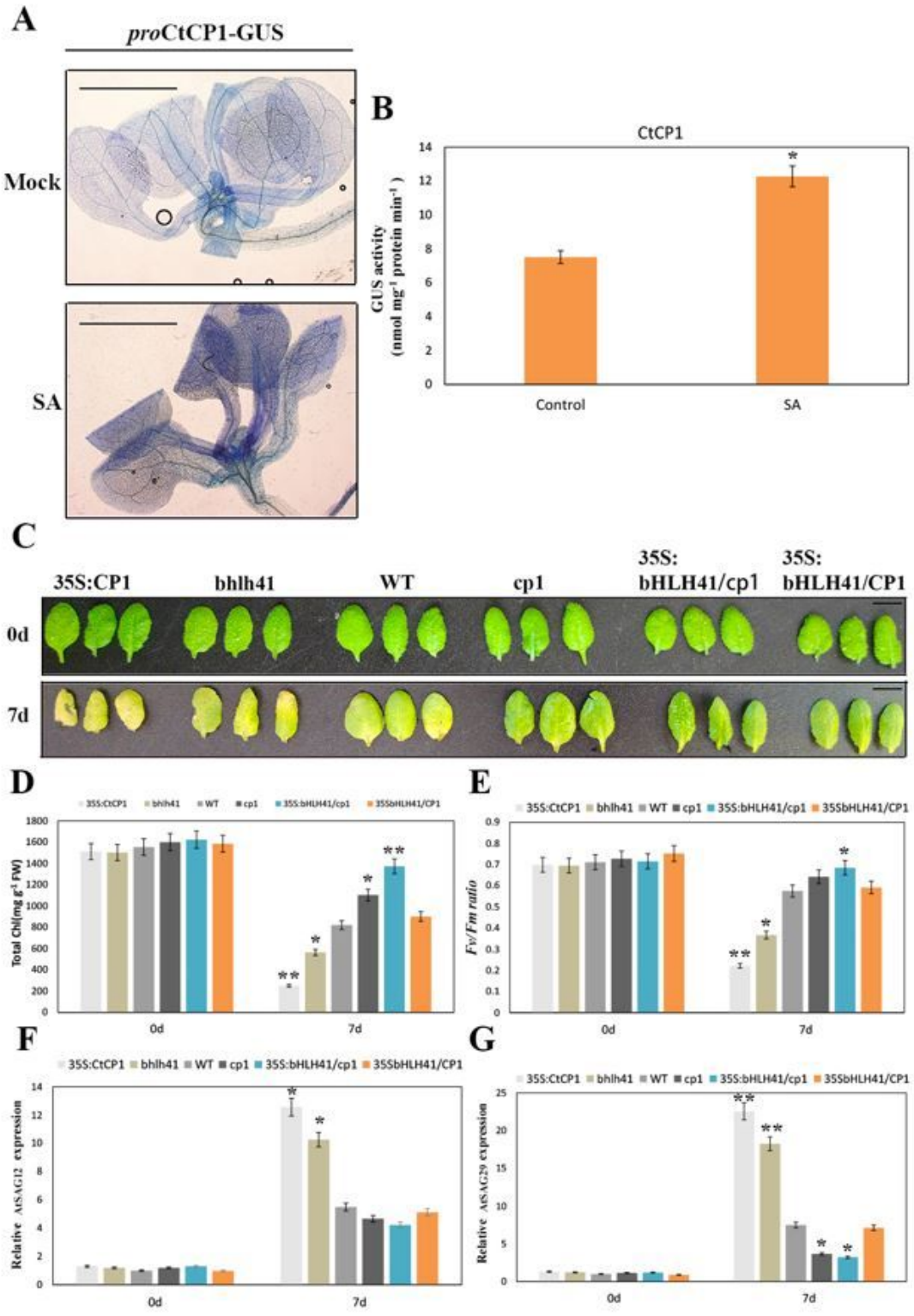

Figure 4

CtbHLH41 negatively regulates SA-induced leaf senescence in a CtCP1-dependent manner. (A) GUS staining (B) GUS in proCtCP1::GUS transgenic Arabidopsis thaliana was treated with (SA) or without (simulated) 100um SA for 12h. (C) Aging phenotype of specified genotypes treated with or without 100um SA for 7 days. Scale=1 cm. (D-E) Chlorophyll content and Fv/Fm in specified genotypes treated with or without SA for 7 days. (F-G) qRT-PCR was used to analyze the transcription levels of SAG12 and SAG29 in the specified genotypes with or without SA treatment. The transcription level of SAG12 and SAG29 in untreated WT leaves was arbitrarily set to 1. The asterisk indicates the significant difference in t-test (* $P < 0.05$, $P < 0.01$).

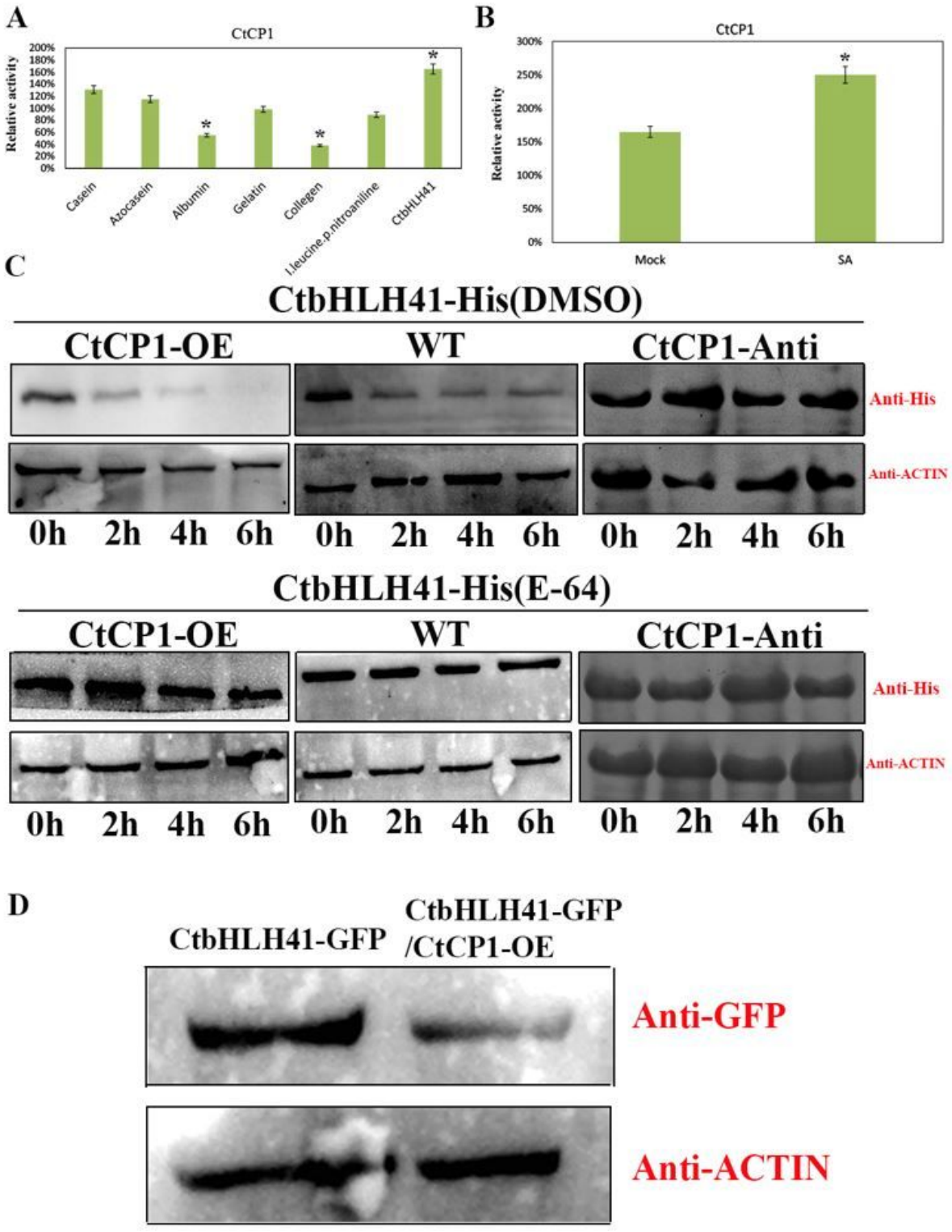

Figure 5

CtCP1 promotes the hydrolysis of CtbHLH41 protein. (A) CtCP1 substrate specificity. (B) the relative activity of the enzyme with or without SA treatment. (C) acellular CtbHLH41-His degradation test. The CtbHLH41-His fusion protein was incubated with total proteins extracted from wild type (WT), CtCP1-O1 and CtCP1-Anti transgenic leaves for a specified period of time. (D) using anti-GFP antibody to evaluate

the abundance of CtbHLH41-GFP protein in transgenic safflower leaves (CtbHLH41-GFP and CtbHLH41-GFP/CtCP1-OE) by Western blotting.

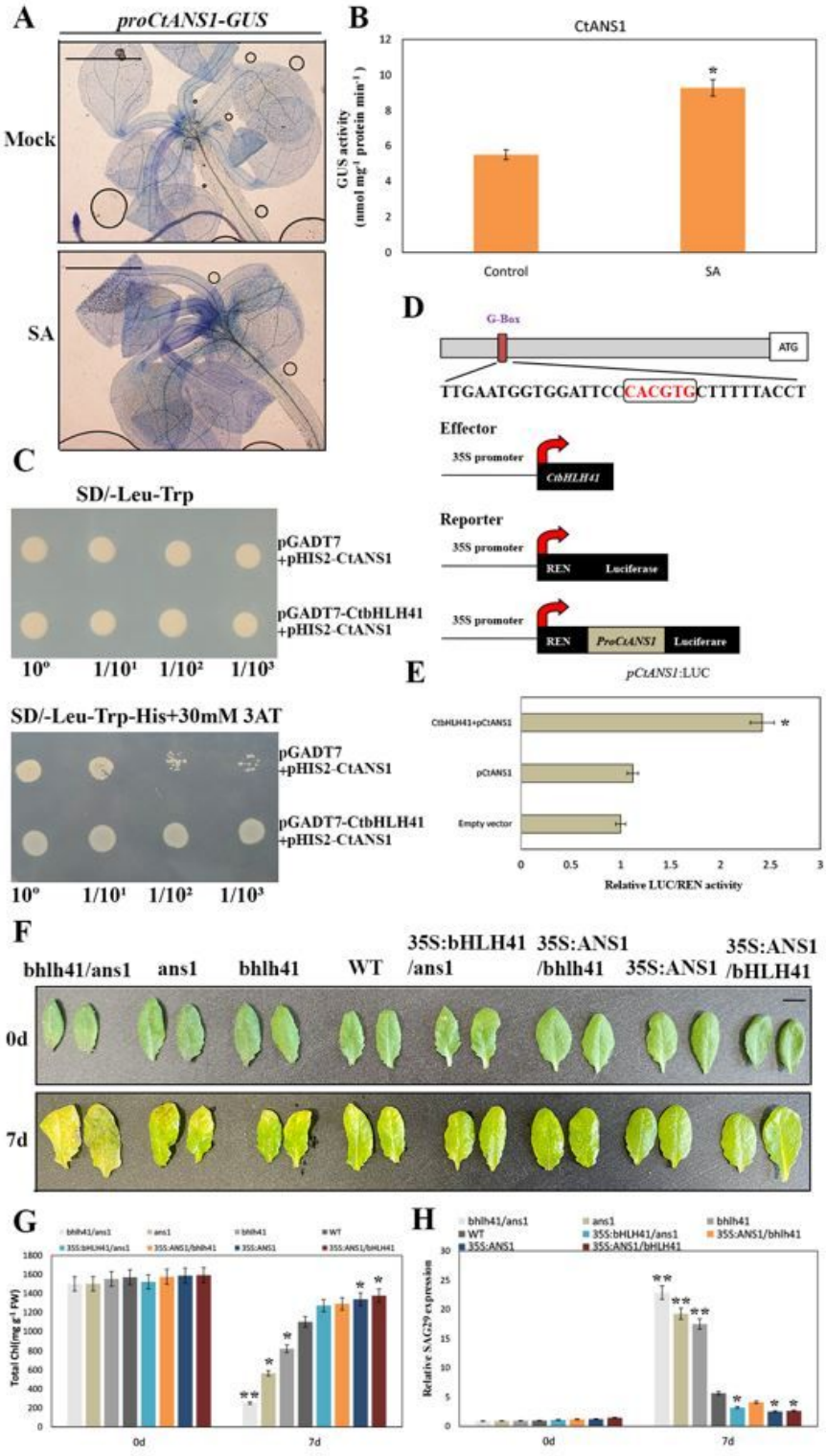

Figure 6

CtbHLH41 delays SA-mediated leaf senescence in a CtANS1-dependent manner. (A) GUS staining (B) GUS in proCtANS1:GUS transgenic Arabidopsis thaliana was treated with (SA) or without (simulated) 100um SA for 12h. (C) The interaction between CtbHLH41 and candidate target gene CtANS1 in Y187

yeast cells. Based on the growth status of transformed Y187 yeast cells on SD-TL and SD-TLH + 30 mM 3-AT medium, the binding ability of CtbHLH41 to target promoter sequence was identified. The combination of pGADT7-CtbHLH41 and pHIS2 is used as a negative control. (D) schematic diagram of G-box cis-elements and reporter and effector constructors in the promoter of CtANS1 for the LUC assay. (E) luciferase assay showed that the transient transformation of CtbHLH41 into tobacco activated the expression of CtANS1. Empty vector (SK + LUC) was used as negative control. The LUC/REN ratio represents the ability of CtbHLH41 to activate CtANS1 expression. (F) Aging phenotype of specified genotypes treated with or without 100um SA for 7 days. Scale=1 cm. (G) Chlorophyll content in specified genotypes treated with or without SA for 7 days. (H) qRT-PCR was used to analyze the transcription levels of SAG29 in the specified genotypes with or without SA treatment. The transcription level of SAG29 in untreated WT leaves was arbitrarily set to 1. The asterisk indicates the significant difference in t-test (* $P < 0.05$, $P < 0.01$).

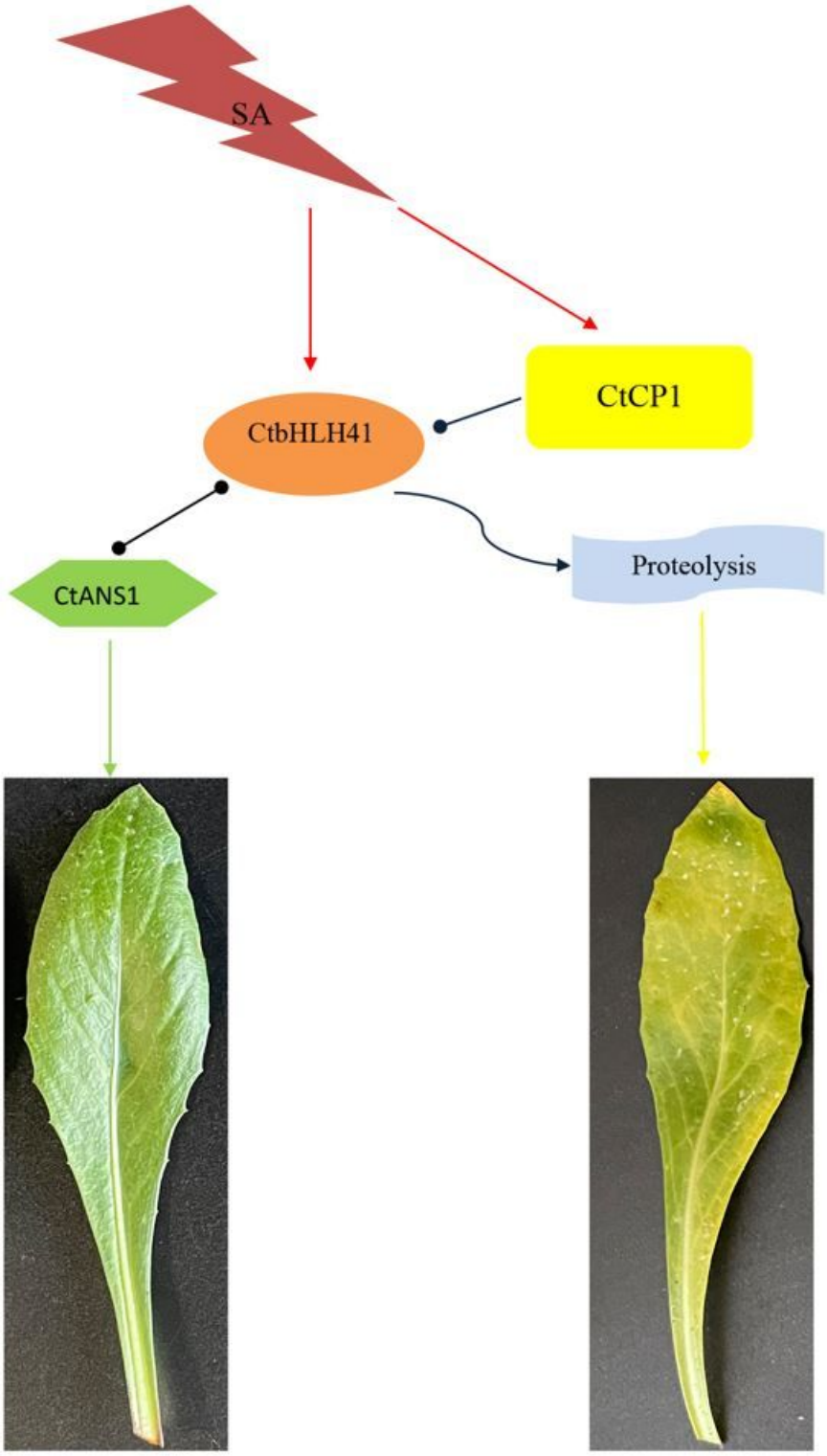

Figure 7

Model of interaction between CtCP1-CtbHLH41-CtANS1 in SA-mediated safflower leaf senescence. CtCP1 and CtbHLH41 were both up-regulated during the senescence of safflower leaves and induced by SA. The SA pathway usually promotes leaf senescence of safflower. CtCP1 interacts with CtbHLH41 to hydrolyze its protein and positively regulate SA-mediated safflower leaf senescence. CtANS1 negatively regulates

SA-mediated safflower leaf senescence, and CtbHLH41 directly binds to the promoter of CtANS1 to inhibit their expression during SA-mediated safflower leaf senescence.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- CtbHLHCP.rar