Journal of Integrative Plant Biology 2008, **50** (4): 475–483

PPF1 May Suppress Plant Senescence via Activating TFL1 in Transgenic *Arabidopsis* Plants

Da-Yong Wang[1,2*], Qing Li[3,4], Ke-Ming Cui[3,4] and Yu-Xian Zhu[3,4]

([1]*Department of Genetics and Developmental Biology, Southeast University*, Nanjing 210009, China;
[2]*Key Laboratory of Developmental Genes and Human Disease, Ministry of Education*, Nanjing 210009, China;
[3]*The National Laboratory of Protein Engineering and Plant Genetic Engineering*, Beijing 100871, China;
[4]*College of Life Sciences, Peking University*, Beijing 100871, China)

Abstract

Senescence, a sequence of biochemical and physiological events, constitutes the final stage of development in higher plants and is modulated by a variety of environmental factors and internal factors. PPF1 possesses an important biological function in plant development by controlling the Ca^{2+} storage capacity within chloroplasts. Here we show that the expression of PPF1 might play a pivotal role in negatively regulating plant senescence as revealed by the regulation of overexpression and suppression of PPF1 on plant development. Moreover, TFL1, a key regulator in the floral repression pathway, was screened out as one of the downstream targets for PPF1 in the senescence-signaling pathway. Investigation of the senescence-related phenotypes in *PPF1(–) tfl1-1* and *PPF1(+) tfl1-1* double mutants confirmed and further highlighted the relation of PPF1 with TFL1 in transgenic plants. The activation of TFL1 expression by PPF1 defines an important pathway possibly essential for the negative regulation of plant senescence in transgenic *Arabidopsis*.

Key words: *Arabidopsis thaliana;* plant senescence; PPF1; TFL1; target.

Wang DY, Li Q, Cui KM, Zhu YX (2008). PPF1 may suppress plant senescence via activating TFL1 in transgenic *Arabidopsis* plants. *J. Integr. Plant Biol.* **50**(4), 475–483.

Available online at www.jipb.net

Plant development ends with senescence, a process consisting of biochemical and physiological events of deterioration that ultimately leads to death (Woo et al. 2001). At present, both signal transduction pathway and genetic programming are thought to be involved in the control of cell senescence (Nooden et al. 1997). Ca^{2+} is one of the most important signal molecules to regulate cell senescence by exerting its toxic effects within cytosol (Li et al. 2004). Senescence is also considered as a programmed process and many of the effects, such as the chloroplast and mitochondria dysfunction and ordered proteolytic events are characteristics of this (Lam et al. 2001). Moreover, senescence is an active process programmed by genetic information since genetic variants with defects can affect the senescence program (Woo et al. 2001).

Whole plant senescence is an internally programmed degeneration occurring in different tissues and organs and leading to the death of a whole plant (Bleecker 1998). The whole plant senescence can be subdivided into several different forms such as leaf senescence and apical senescence. The regulation and mechanism of leaf senescence have been studied thoroughly and a series of related genes have been cloned and shown to be involved (Davis and Grierson 1989; Hensel et al. 1993; Buchanan-Wollaston 1997; Park et al. 1998; Woo et al. 2001; He and Gan 2002). Leaf senescence is an active process involving remobilization of nutrients from senescing leaves to other parts, under the control of genetic and physiological signals (He and Gan 2002). Compared with systematic studies on the leaf senescence, the regulation and mechanisms of whole plant senescence are largely unknown.

We noted that the apical senescence in the G2 pea mutant can be inhibited in short days, while plants undergo normal apical senescence in long days (Zhu and Davies 1997). PPF1 was cloned from the G2 pea mutant (Zhu et al. 1998) and it encodes an inward chloroplast calcium transporter (Wang et al. 2003). Overexpression of *PPF1* cDNA significantly prolongs

Received 24 Oct. 2006 Accepted 19 Dec. 2006
Supported by the Rockefeller Foundation of USA and the Southeast University Foundation for Excellent Young Scholars (4023001013).
*Author for correspondence.
Tel: +86 (0)25 8327 2504;
Fax: +86 (0)25 8332 4887;
E-mail: <dayongw@seu.edu.cn>.

doi: 10.1111/j.1744-7909.2008.00643.x

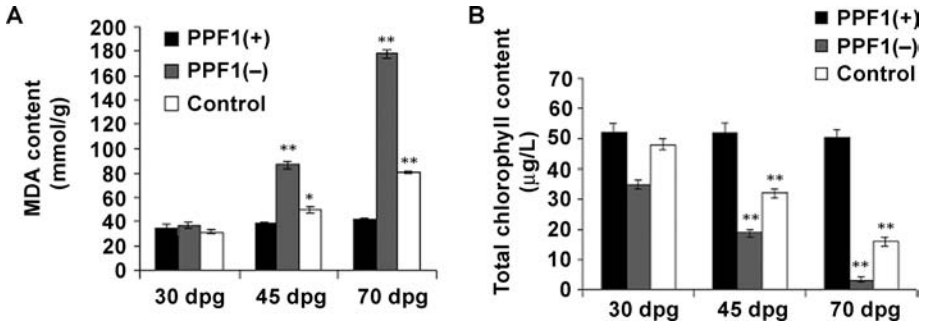

Figure 1. Changes of malondiadehyde (MDA) and chlorophyll levels in control and *PPF1* transgenic *Arabidopsis* plants.

(A) Changes of MDA content in plantlets of control and *PPF1* transgenic plants. Control, wild-type Columbia plants. dpg, days post germination. **(B)** Changes of chlorophyll in plantlets of control and *PPF1* transgenic plants. Results are presented as average values ± *SE* from three experiments. Bars represent ± *SD*. $^{*}P < 0.05$; $^{**}P < 0.01$.

the lifespan and the flowering time of transgenic *Arabidopsis thaliana* plants by changing the Ca^{2+} storage capacity within the chloroplast (Wang et al. 2003). Further results indicated that PPF1 regulates the apical senescence by varying calcium homeostasis to affect the timing of programmed cell death of G2 pea and *Arabidopsis* (Li et al. 2004).

The function of PPF1 in regulating lifespan suggests the possible association of senescence inhibition with flowering-time delay, as well as changes in apical meristem state or architecture (Wang et al. 2003; Li et al. 2004). In *Arabidopsis*, TFL1 might have similar effects on senescence regulation to PPF1, because the *tfl1-1* mutation causes early flowering and early whole plant senescence, and limits the development of the normally indeterminate inflorescence by promoting the formation of a terminal floral meristem (Shannon and Meeks-Wagner 1991; Bradley et al. 1997; Ruiz-Garcia et al. 1997; Kobayashi et al. 1999). In the present study, we investigate the effects of PPF1 expression on the plant senescence and the regulation relation of PPF1 with TFL1 in controlling this. Our results suggest that PPF1 might negatively regulate plant senescence by at least partially activating or stimulating TFL1 expression. This work provides a direct clue for a relationship between flowering regulation and whole plant senescence.

Results

Phenotype analysis of plant senescence in transgenic *Arabidopsis* plants that over- or under-express PPF1 cDNA

In a previous study, we showed that PPF1 expression is possibly related to the regulation of apical senescence in G2 pea mutants (Zhu et al. 1998). Moreover, we showed that expression of PPF1 takes part in the regulation of the lifespan of higher plants by controlling flowering time in *Arabidopsis* (Wang et al. 2003). Control plants underwent apical terminal-differentiation at 80 days post-germination (dpg), PPF1(−) plants ceased to grow and became senescent early at 50 dpg, and PPF1(+) plants maintained vigorous apical growth even at late 80 dpg (Wang et al. 2003). To examine the possible roles of PPF1 in regulating plant senescence, we first determined whether PPF1 overexpression or anti-sense inhibition resulted in altered phenotypes of plant senescence in *Arabidopsis*. Malondiadehyde (MDA) content and chlorophyll content were used to monitor plant senescence to reflect the accumulation of oxide-free radicals and chlorophyll content, respectively. Consistent with previous studies (Wang et al. 2003; Li et al. 2004), MDA content increased and chlorophyll content decreased gradually during development from 30 dpg to 70 dpg in control plants (Figure 1A, B). In PPF1-overexpressing plants, no pronounced differences of MDA and chlorophyll content were recorded during development from 30 dpg to 70 dpg, and at 30 dpg they were similar to those in control plants at the same developmental stage (Figure 1A, B). In PPF1(−) transgenic plants, MDA content increased sharply from 30 dpg to 70 dpg ($P < 0.01$) and at 70 dpg was more than fivefold that at 30 dpg (Figure 1A). Compared with the changes of chlorophyll content in control plants, the chlorophyll content decreased in these plants during development from 30 dpg to 70 dpg (Figure 1B) and the chlorophyll content at 75 dpg was nearly undetectable (data not shown). These results indicate that PPF1 may function in the inhibition of senescence during higher plant development.

To determine the possible mechanisms mediated by PPF1 to inhibit plant senescence, we further examined the expression pattern of *AtTERT* in control and transgenic plants, since accumulation of oxide-free radicals could result in the shortening of telomere length (von Zglinicki et al. 1995). *AtTERT* encodes telomerase reverse transcriptase and is directly involved in the telomere length regulation. The expression

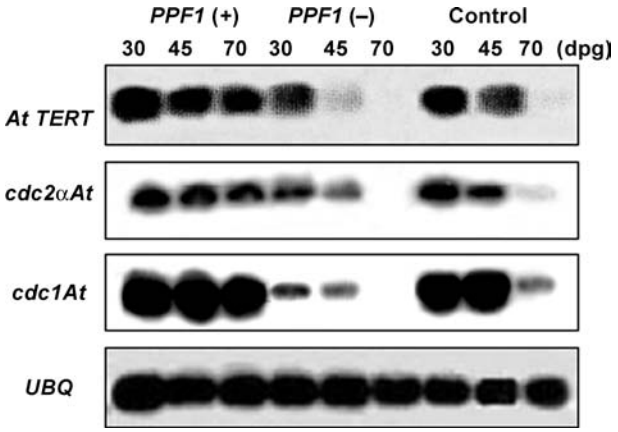

Figure 2. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *AtTERT*, *cdcAt* and *cyclAt* in control and *PPF1* transgenic *Arabidopsis* plants.

Blots show the expression of *AtTERT*, *cdcAt* and *cyclAt* during development in control and transgenic plants. *UBQ* was used to show the equal amount of loading. Semi-quantitative RT-PCR was carried out with *AtTERT*-specific primers, *cdcAt*-specific primers, *cyclAt*-specific primers and *UBQ*-specific primers, respectively.

of *AtTERT* diminished slowly during development in control plants and the *AtTERT* expression was nearly undetectable at 70 dpg when plants underwent senescence (Figure 2). In PPF1-overexpressing plants, *AtTERT* maintained a very high expression even at 70 dpg, like at 30 dpg (Figure 2). However, *AtTERT* expression was inhibited slightly already at 30 dpg in *PPF1(−)* plants compared with that in control plants at 30 dpg, and diminished markedly even early at 45 dpg (Figure 2). Therefore, PPF1 expression might inhibit senescence partially by maintaining high *AtTERT* expression in higher plants.

Our previous work revealed that the expression of PPF1 may inhibit programmed cell death in the apical meristem of flowering plants by keeping a low cytoplasmic calcium content (Li et al. 2004). Thus, PPF1 might play an important role in preventing the occurrence of terminal differentiation in plants. To examine this possibility further, we also determined whether overexpression of PPF1 altered the expression patterns of genes related to the regulation of cell division. Expression of *cdc2aAt* reflects the potential for cell division, and *cyclAt* is especially expressed in dividing plant cells (Shaul et al. 1996). As shown in Figure 2, *cdc2aAt* and *cyclAt* exhibited a similar expression pattern to *AtTERT,* decreasing gradually during development in control plants. Furthermore, expression of *cdc2aAt* and *cyclAt* remained sustained at a very high level during development even at 70 dpg in *PPF1(+)* transgenic plants (Figure 2). Interestingly, neither the expression of *AtTERT* nor the expression of *cdc2aAt* and *cyclAt* was maintained in *PPF1(+)* plants compared with that in control plants. Moreover, inhibition of PPF1 resulted in the obvious decrease of *cdc2aAt* and *cyclAt* expression (Figure 2). Therefore, PPF1 could prevent the plant senescence by maintaining the activities of genes related to cell division, and by inhibiting the DNA fragmentation in *PPF1(+)* transgenic plants.

TFL1 might be one of the triggered targets for the function of *PPF1* in regulating plant senescence in transgenic *Arabidopsis* plants

As described above, PPF1 is only involved in the maintenance of *AtTERT*, *cdc2aAt* and *cyclAt* expression. Then, which gene(s) would serve as the possibly triggered target(s) in *PPF1* transgenic *Arabidopsis* plants? Our previous work indicated that PPF1 can significantly prolong lifespan and postpone flowering time (Wang et al. 2003), suggesting a possible correlation between flowering time and plant senescence. In *Arabidopsis*, the photoperiodic promotion pathway is only responsible for floral induction in long days, and the vernalization promotion pathway induces flower initiation after an exposure to low temperatures for several weeks (Levy and Dean 1998; Amouradov et al. 2002). The antagonistic floral repression pathways monitor the internal developmental status and target to *LFY* and floral meristem gene expression (Levy and Dean 1998; Amouradov et al. 2002). To determine the possible flowering-regulation gene(s) targeted by PPF1 in the control of plant senescence, we examined expression patterns of a series of flowering regulation genes. No differences were found between the expression patterns of *CO*, *GI* and *FLC* in *PPF1(+)* plants at 55 dpg and those in control plants (data not shown). However, *PPF1(+)* transgenic lines showed an obvious increase of *TFL1* expression, and the *TFL1* expression was markedly reduced in *PPF1(−)* than that in control plants (Figure 3A), suggesting that the TFL1 might just be one of the downstream target(s) for PPF1 in plant senescence regulation.

Again, we selected transgenic lines of *PPF1(−)* and *PPF1(−)a* at 45 dpg to further address this interaction. The expression patterns of ALBINO3 in control and transgenic plants confirmed the differences of *PPF1(−)* and *PPF1(−)a* transgenic plants (Figure 3B). As the endogenous ALBINO3 was suppressed more strongly in *PPF1(−)a* plants compared with that in *PPF1(−)* plants, both *PPF1(−)* and *PPF1(−)a* transgenic lines showed an obvious decrease of *TFL1* expression and the *TFL1* expression was reduced more in *PPF1(−)a* than that in *PPF1(−)* plants (Figure 3B). Although, expressing the *PPF1* cDNA driven by the CaMV 35S promoter in antisense orientation had no effects on the expression of *CO*, *GI* and *FLC* (data not shown). At the same time, the flowering occurred earlier in *PPF1(−)a* plants with an average of only five rosette leaves at bolting compared with control plants with an average of 22 rosette leaves at bolting (Figure 3E). Moreover, MDA content increased and the chlorophyll content decreased more

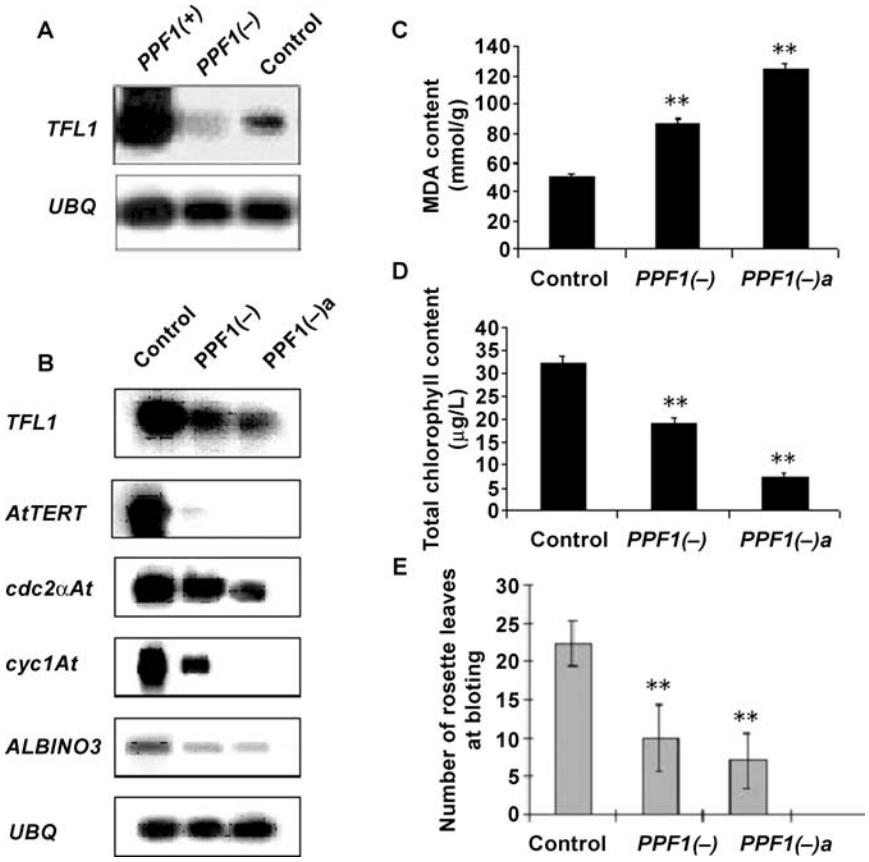

Figure 3. Expression patterns of genes affecting plant senescence and flowering in *PPF1(–)* transgenic plants.

(A) Expression patterns of *TFL1* in control and *PPF1* transgenic plants, collected at 55 d post germination (dpg). *UBQ* was used to show equal amount of loading.

(B) Expression patterns of *TFL1*, *AtTERT*, *cdcAt, cyclAt* and *ALBINO3* in control and *PPF1(–)* transgenic plants, collected at 45 dpg. *UBQ* was used to show equal amount of loading.

(C) Comparison of malondiadehyde (MDA) content changes in *PPF1(–)* transgenic and control plants, collected at 45 dpg.

(D) Comparison of chlorophyll content changes in *PPF1(–)* transgenic and control plants, collected at 45 dpg.

(E) Comparison of rosette leaf number at bolting in *PPF1(–)* transgenic and control plants. Bars represent ± *SD*. $^{*}P < 0.05$; $^{**}P < 0.01$.

sharply in *PPF1(–)a* plants than those in controls or *PPF1(–)* plants ($P < 0.01$) (Figure 3C,D); the expression of *AtTERT*, *cdc2aAt* and *cyclAt* in *PPF1(–)a* plants was much lower than those in control or *PPF1(–)* plants (Figure 3B). In *PPF1(–)a* plants, the expression of *AtTERT* and *cyclAt* were nearly undetectable (Figure 3B). Therefore, the regulation of PPF1 on plant senescence in transgenic plants might just be mediated by the activation of TFL1 expression.

The importance of TFL1 activation for PPF1 function in regulating plant senescence

To examine the importance of TFL1 expression for PPF1 function and whether *tfl-1* is the only activated target for *PPF1*, we further constructed double mutants of *PPF1(–) tfl1-1* and *PPF1(+) tfl1-1*. The double mutants were screened at F3 progeny according to the phenotypes of *tfl1-1* and the transgenic plants, and the expression patterns of PPF1 in double mutants and single mutants (Figure 4A).

Two lines of evidence are provided here to indicate the important role of TFL1 activation for PPF1 function to regulate plant senescence using the *PPF1(–) tfl1-1* double mutant. First, the MDA content at 60 dpg of *PPF1(–) tfl1-1* double mutants was very high like in the *tfl1-1* mutant, and not like that in the *PPF1(–)* plants (Figure 4B). Second, the chlorophyll content of the *PPF1(–) tfl1-1* double mutant was also similar to that of *tfl1-1* plants and was not like that of the *PPF1(–)* plants (Figure 4C). These data suggest that the activation of TFL1 could explain most of the PPF1 function in plant senescence regulation in transgenic plants.

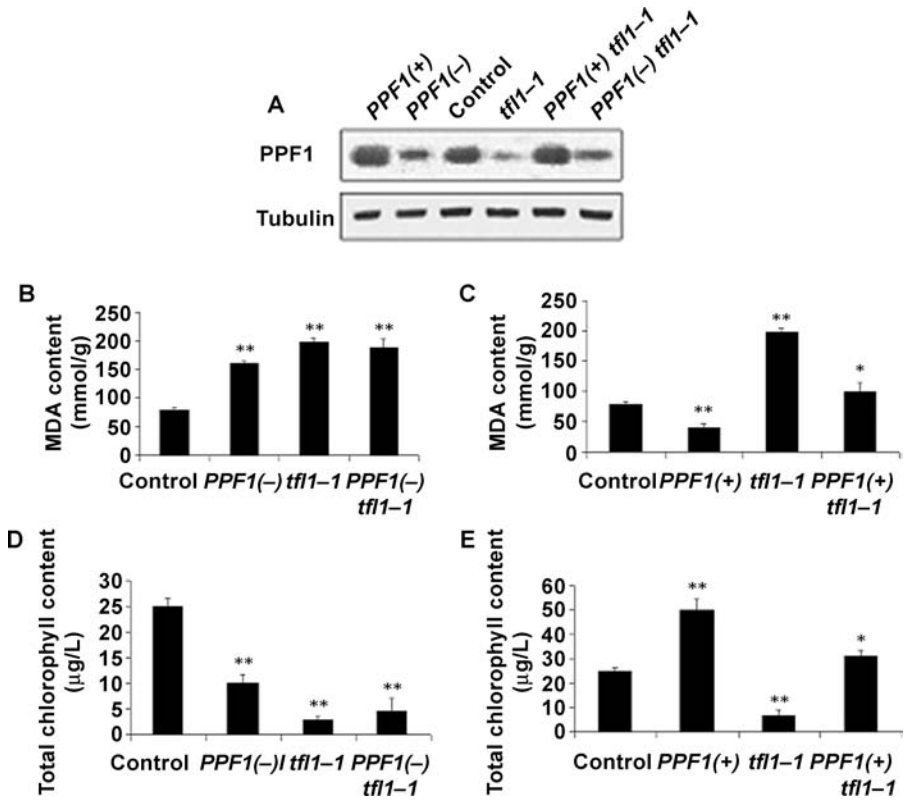

Figure 4. Altered phenotypes of plant senescence in *PPF1(−) tfl1-1* and *PPF1(+) tfl1-1* double mutants.

(A) Western blot analysis of the PPF1 expression in *PPF1(−) tfl1-1* and *PPF1(+) tfl1-1* double mutants. Equivalent amounts of total protein were determined by blotting with antibody against tubulin. Samples were collected at 60 d post germination (dpg).

(B) Comparison of malondiadehyde (MDA) content changes in control, *PPF1(−)* transgenic line, *tfl1-1* and *PPF1(−) tfl1-1* double mutant plants.

(C) Comparison of MDA content changes in control, *PPF1(+)* transgenic line, *tfl1-1* and *PPF1(+) tfl1-1* double mutant plants.

(D) Comparison of chlorophyll content changes in control, *PPF1(−)* transgenic line, *tfl1-1* and *PPF1(−) tfl1-1* double mutant plants.

(E) Comparison of chlorophyll content changes in control, *PPF1(+)* transgenic line, *tfl1-1* and *PPF1(+) tfl1-1* double mutant plants. Bars represent ± *SD*. $^{*}P < 0.05$; $^{**}P < 0.01$.

In addition, we also noticed that *in situ* cell death detection by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay showed that the patterns of distribution, amount and strength of reaction signals in the apical indeterminate inflorescence meristem from *PPF1(−) tfl1-1* double mutants at 60 dpg were nearly the same as in the apical inflorescence meristem from *PPF1(−)* plants. Stronger reaction signals for DNA fragmentation accumulated in the apical terminal floral meristem of the *tfl1-1* single mutant (Figure 5B). Moreover, the expression patterns of *AtTERT*, *cdc2aAt* and *cyclAt* in *PPF1(−) tfl1-1* double mutants were also similar to those in *PPF1(−)* plants, but the *AtTERT*, *cdc2aAt* and *cyclAt* expressions were very low or nearly undetectable in the *tfl1-1* single mutant at 45 dpg (Figure 5A). These results suggest the mechanism regulating the cell death and cell division might be different from that in the plant senescence control.

TFL1 is not the only activated target for PPF1 in regulating plant senescence

Although we have shown that PPF1 upstream targets TFL1 to function in the control of plant senescence, we cannot exclude the possibility that PPF1 activates other downstream targets during development. Thus, a series of phenotypes of the *PPF1(+) tfl1-1* double mutant were further analyzed. First, the MDA content in the *PPF1(+) tfl1-1* double mutant at 60 dpg was more than twofold of that in *PPF1(+)* plants and much less than that in the *tfl1-1* mutant alone (Figure 4C). Second, the chlorophyll content of *PPF1(+) tfl1-1* double mutants was much lower than that of *PPF1(+)* plants, but it did not reach the low chlorophyll content of the *tfl1-1* mutant (Figure 4E). Third, the expression of *AtTERT*, *cdc2aAt* and *cyclAt* was suppressed to different degrees in the *PPF1(+) tfl1-1* double mutant compared

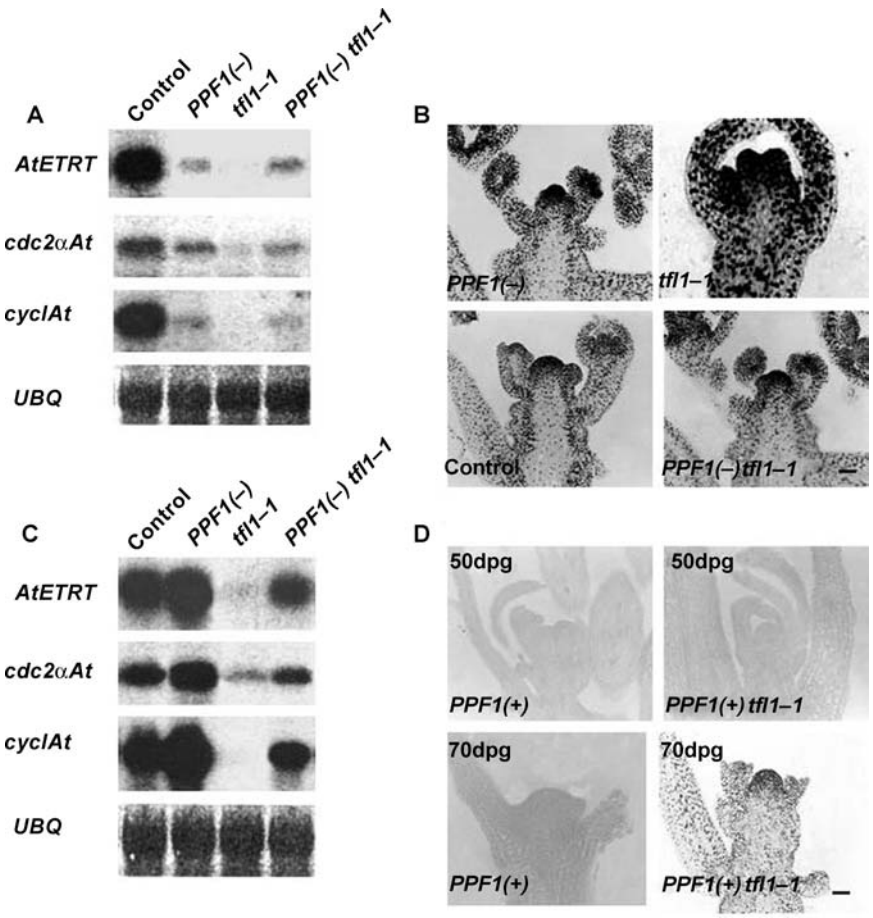

Figure 5. Altered plant terminal differentiation in *PPF1(−) tfl1-1* and *PPF1(+) tfl1-1* double mutants.

(A) Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *AtTERT*, *cdcAt* and *cyclAt* in *PPF1(−) tfl1-1* double mutant and *PPF1(−)*, *tfl1-1* single mutants. *UBQ* was used to show equal amount of loading. Samples were collected at 45 d post germination (dpg) from control, *tfl1-1*, *PPF1(−)* and double mutant plants.

(B) *In situ* cell death detection by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using apical meristem prepared from *PPF1(−) tfl1-1* double mutant and *PPF1(−)*, *tfl1-1* single mutants. Bars, 100 μm.

(C) Quantitative RT-PCR analysis of *AtTERT*, *cdcAt* and *cyclAt* in *PPF1(+) tfl1-1* double mutant and *PPF1(+)*, *tfl1-1* single mutants. *UBQ* was used to control the equal amount for each loading line. Samples were collected at 60 dpg from control, *tfl1-1*, *PPF1(+)* and double mutant plants.

(D) *In situ* cell death detection by TUNEL assay using apical meristem prepared from *PPF1(+) tfl1-1* double mutant and *PPF1(−)* transgenic plants. Samples were collected at 50 dpg and 70 dpg respectively. Bars, 100 μm.

with that in *PPF1(+)* plants at 60 dpg, whereas the expression of these three genes in the *tfl1-1* single mutant was very low or undetectable at the same developmental stage (Figure 5C). Finally, we compared the occurrence of DNA fragmentation in apical inflorescences between *PPF1(+) tfl1-1* double mutants and *PPF1(+)* plants. No pronounced differences were found at 50 dpg, but a large number of reaction signals occurred at 70 dpg in the apical meristem of *PPF1(+) tfl1-1* double mutants compared with no reaction signals in the apical meristem of *PPF1(+)* plants (Figure 5D). These results suggest that TFL1 is a pivotal downstream target for the function of PPF1 in regulating plant senescence. Nevertheless, TFL1 might not be the only target for PPF1, and PPF1 might still have the potential ability to activate other downstream pathway(s) to control plant senescence.

Discussion

Our previous work indicated that PPF1 is involved in the regulation of lifespan and flowering time by controlling calcium storage capacity within chloroplasts (Wang et al. 2003), and that this

gene plays an important part in inhibiting programmed cell death in the apical meristem of higher plants (Li et al. 2004). Therefore, we hypothesized that PPF1 affects plant senescence as an important component of Ca^{2+} signaling. Here, we have shown that PPF1 negatively regulates plant senescence in transgenic *Arabidopsis*. Compared with the senescence phenotypes in control plants, the chlorophyll content and the expression of *AtTERT*, *cdc2aAt* and *cyclAt* were significantly suppressed; the MDA content and the DNA fragmentation in the apical meristem were obviously enhanced in *PPF1(−)* transgenic plants (Li et al. 2004; Figures 1 and 2). Moreover, overexpression of PPF1 in transgenic plants resulted in a decrease in the MDA content and in the occurrence of the DNA fragmentation in the apical meristem, and was sufficient for the maintenance of the chlorophyll content and the gene activities of *AtTERT*, *cdc2aAt* and *cyclAt* (Li et al. 2004; Figures 1 and 2). Therefore, together with the previous finding that the expression of PPF1 inhibits apical terminal differentiation by keeping a low cytoplasmic calcium content in plant cells (Wang et al. 2003; Li et al. 2004), this prompts us to conclude that PPF1 might be a pivotal and specific component in the senescence signaling processes during higher plant development.

Based on the important roles of PPF1 in plant senescence, we further elucidated the mechanism involving PPF1 in senescence-regulation by determining the downstream target(s) activated by PPF1. We first used the *PPF1(−)* and *PPF1(−)a* transgenic lines with different degrees of suppression of endogenous ALBINO3 expression to screen for possible targets. We found that expressing the *PPF1* cDNA driven by the CaMV 35S promoter in antisense orientation caused a decrease in expression of *AtTERT*, *cdc2aAt*, *cyclAt* and *TFL1* (Figure 3), but had no effects on the expression of *CO*, *GI* and *FLC*. We further analyzed the expression pattern of the four proteins encoded by *AtTERT*, *cdc2aAt*, *cyclAt* and *TFL1* in *PPF1(+)* transgenic lines and noted that overexpression of PPF1 only enhanced *TFL1* expression (Figure 2). TFL1 mediates the floral repression pathway in the flowering network pathways (Levy and Dean 1998; Amouradov et al. 2002), and PPF1 plays a similar role to TFL1 in the flowering signaling pathway. Therefore, our results suggest that TFL1 may be one of the targets activated by PPF1 to negatively regulate plant senescence. Another interesting result is that PPF1 expression was essential for the maintenance of activities of *AtTERT*, *cdc2aAt* and *cyclAt,* but couldn't enhance the expression of these three genes (Figures 2 and 3). This finding suggests that expression of *AtTERT*, *cdc2aAt* and *cyclAt* for normal development of plant cells has specific limits, or that excess activities of these proteins might cause defects in plant development or be toxic to plant cell growth and differentiation.

To confirm our assumption that TFL1 might be the downstream target for PPF1 to regulate plant senescence, we analyzed the relation of TFL1 with PPF1 using the double mutant *PPF1(−) tfl1-1*. Senescence phenotypes of this double mutant suggested that TFL1 may be in the PPF1-mediated senescence signaling pathway, and that TFL1 is the downstream target of PPF1 in transgenic plants. Thus, we established an interaction of the flowering pathway with the senescence regulation, which is integrated by the putative calcium transporter PPF1 expression. Nevertheless, TFL1 is not the only downstream target activated by PPF1. PPF1 might be involved in a more complex regulation mechanism, as revealed by investigation of the phenotypes of *PPF1(+) tfl1-1* double mutant. In the *PPF1(−) tfl1-1* double mutant, the *tfl1-1* mutation resulted in a decrease in chlorophyll content and gene activities of *AtTERT*, *cdc2aAt* and *cyclAt*, and an increase in MDA content and the possibility of DNA fragmentation in the apical meristem, compared with *PPF1(+)* plants (Figures 4 and 5). However, the *tfl1-1* mutation doesn't cause the complete loss of the PPF1 function in negatively regulating plant senescence, although it does explain much about PPF1 function in the control of plant senescence. As a result, PPF1 must still activate at least another downstream target in the senescence signaling pathway.

It is known that Ca^{2+} serves as a second messenger in many signal transduction pathways and a number of intermediate components may play a role in this signaling process from calcium to gene expression (reviewed by Bush 1995; Sanders et al. 1999). In the present study, we have identified PPF1, a putative calcium transporter localized within chloroplast membrane, as a critical component in plant senescence regulation. We have further provided evidence that PPF1 may play a pivotal role in regulating plant senescence by activating its downstream target TFL1, which provides a novel pathway essential for the negative control of the plant senescence process. These findings may contribute to our understanding of important mechanisms underlying calcium functions in senescence signaling pathways.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (cv. Columbia) plants were grown at 23 °C during the light period and 21 °C during the dark period in fully automated growth chambers (Conviron, Winnipeg, Canada). Plants were maintained at 9-h (short-day) photoperiods with cool-white fluorescence lamps supplemented by incandescent lamps. Transgenic *Arabidopsis* plants overexpressing the *PPF1* gene in sense (*PPF1*(+)) and anti-sense orientation (*PPF1*(−)) were obtained as described (Wang et al. 2003). The *PPF*1(+) transgenic plants had an average of 45 rosette leaves at bolting. The *PPF*1(−) transgenic plants had an average of eight rosette leaves at bolting. The *PPF1(−)a* transgenic plants (another name is *PPF1(−)*24) had an average of five rosette leaves at bolting.

Plant senescence measurements

Chlorophyll content was measured as described (Moran and Porath 1980), and calculated as described (Inskeep and Bloom 1985) in plantlets collected into 2 mL of *N*,*N*-dimethylformamide. MDA content assay was carried out as described (Health and Packer 1968; Takahama and Nishimura 1976) from the plantlets collected into ddH_2O. Three repeats were averaged from each apical inflorescence sampled.

Semi-quantitative RT-PCR assay

The basic method was carried out as described (Kardalsky et al. 1999). Total RNA was extracted from plantlets of control and transgenic plants for cDNA synthesis using a Qiagen RNeasy Kit (Hilden, Germany). cDNA synthesis and reverse transcription-polymerase chain reaction (RT-PCR) was then carried out according to the protocol supplied by the cDNA Synthesis Kit (GIBCO BRL Co., Carlsbad, CA, USA). One mircogram of RNA was used as a template in each reaction. Gene-specific primers were designed for *AtTERT* (AtTERTa, 5′-ATGCCGCGTAAACCTAGACAT-3′; and AtTERTb, 5′-TGAGTGGTCCCAAGC AAACT-3′), for *cdc2aAt* (cdc2aAta, 5′-GTGGTTTATAA GGCTCGTGAC-3′; and cdc2aAtb, 5′-TACCTCGTGTGTAAATGTTCTG-3′), for *cyclAt* (cyclAta, 5′-ATGGCTGACAAAGAGAACTG-3′, and cyclAtb, 5′-ACTCTGATTCTCAAATATCTTC-3′), for *ALBINO3* (ALBINO3a, 5′-CCGATGCTATGGAATCGGTT-3′; and ALBINO3b, 5′-TCGTCAGGCTGAGCAATAGA-3′), and for *TFL1* (TFL1a, 5′-CGGGATCCATGGGGAGAGTGGTAGGAGAT-3′; and TFL1b, 5′-CCGGTA CCGATTCAACTCATCTTTGGCAG-3′). *UBQ* was used to determine the equal loading for each sample and primers specific for UBQ were used in control reactions (UBQa, 5′-GGTGCTAAGAAGAGGAAGAAT-3′; UBQb, 5′-CTCCTTCTTTCTGGTAAACGT-3′). Amplification of all DNA fragments for RT-PCR or probes was carried out for 30 cycles in a Perkin-Elmer 480 thermal cycler (Waltham, MA, USA) using a 55 °C annealing temperature (60 °C for *ALBINO3*, 58 °C for *TFL1* and 63 °C for *AtTERT*) and a 1-min extension. Aliquots of individual PCR products were resolved by agarose gel electrophoresis and were made visible with ethidium bromide under UV light. The gels were transferred onto a Nytran membrane with a Turboblotter (Schleicher and Schuell, Dassel, Germany), followed by membrane baking at 80 °C for 2 h and auto-crosslink in a UV Stratalinker. The amplified probe fragments (~50 ng) were labeled with (α-^{32}P) dCTP using a Prime-it II Kit (Stratagene, Santa Clara, CA, USA). Membranes were probed with labelled DNA fragments in 0.3 mol/L sodium phosphate buffer, pH 7.2, containing 7% (w/v) sodium dodecyl sulfate (SDS), 1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 8.0, and 2% (w/v) bovine serum albumin (BSA) at 65 °C for 16–24 h. Filters were washed at 65 °C twice with 2× standard saline citrate (SSC) and 0.1% (w/v) SDS for 30 min, once with 1× SSC and 0.1% (w/v) SDS for 20 min, and once with 0.5× SSC and 0.1% (w/v) SDS for 10 min. The membranes were exposed to Kodak XAR film at −80 °C for autoradiography.

Immunodetection

Plantlets of various *Arabidopsis* plants were harvested at noon. Samples were immediately frozen, ground and homogenized in extraction buffer containing 50 mmol/L HEPES-KOH (pH 7.5), 5 mmol/L EDTA, 0.1% BSA, 1 mmol/L phenylmethanesulfonyl fluoride (PMSF), 2 mmol/L dithiothreitol (DTT), 1% (w/v) polyvinyl polypyrrolidone (PVP), and 0.25 M sucrose. Cell debris was removed by centrifugation at 10 000 *g* for 15 min. Standard methods were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein gel blot analysis, and detection. Immunoblotting of the protein samples transferred to nitrocellulose membrane from a 12% SDS-PAGE gel was carried out using 1:3 000 dilutions of the primary anti-PPFC antibody. Pre-immune serum was used as the negative control for antibody specificity. Equivalent amounts of total proteins of different samples, as determined by blotting with antibody against tubulin, were loaded in each of the lanes. The secondary antibody was an anti-rabbit immunoglobulin G (IgG) alkaline phosphatase conjugate (Promega, Madison, WI, USA), and the detection reagent was 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium alkaline phophatase substrate (Promega, Madison, WI, USA).

Strains constructions

To construct the *PPF1(+) tfl1-1* double mutant, we fertilized *PPF1(+)* plants with pollen from *tfl1-1* plants and collected seed from individual F1 plants confirmed by the *tfl1-1* mutant phenotypes. We further screened for the plants with *tfl1-1* phenotypes in a F_2 progeny. From the F_3 progeny in a single F2 plant, the double mutants of *PPF1(+) tfl1-1* were isolated, and the double mutants were identified by the *PPF1(+)* phenotype and Western blot with the antibody against PPF1.

***In situ* cell death detection by TUNEL assay**

The apical inflorescences of various lines of *Arabidopsis* were fixed in formalin-acetic acid (FAA) fixation solution, dehydrated in a series of graded ethanol and embedded in paraffin. Samples in sections of 10 μm thickness were digested with 10 μg/mL proteinase K at 37 °C for 30 min. The TUNEL assay was carried out according to the manufacturer's (Boehringer Mannheim, Mannheim, Germany) instructions. In brief, a 50-μL TUNEL (TdT-mediated dUTP nick end labeling) reaction mixture, including 5 μL terminal deoxynucleotidyl transferase and 45 μL nucleotide mixture in reaction buffer were added to each sample and paraffin-covered slides were incubated at 37 °C for 60 min in a humidified chamber. Intensity of the labeling was analyzed

by using an Olympus microscope after washing the slides twice with phosphate buffer. Each sample received 50 μL Converter-AP (Roche Molecular Biochemicals, Mannheim, Germany) and was incubated at 37 °C for 30 min. After another rinse with phosphate buffer, to each slide was added 50 μL substrate solution (3.5 μL 55-bromo-4-chloro-3-indolyl-phosphate [BCIP] and 4.5 μL 4-nitroblue tetrazolium chloride [NBT]) and incubated at room temperature in darkness for 0.5–2.0 h. The results were analyzed using an Olympus microscope under light field optics. Negative controls were carried out with no addition of TdT and positive controls were carried out with an addition of DNase I (final concentration of 0.5 μg/μL) to the reaction mixture.

Statistical analysis

All data in the present study were expressed as mean ± *SD* and analyzed by SPSS 13.0 software (Chicago, IL, USA). Paired-sample *t* test were carried out between control and transgenic or mutant plants. A probability level of 0.05 was considered statistically significant.

References

Amouradov A, Cremer K, Coupland G (2002). Control of flowering time: interacting pathways as a basis for diversity. *Plant Cell* **14**, S111–S130.

Bleecker AB (1998). The evolutionary basis of leaf senescence: method to the madness? *Curr. Opin. Plant Biol.* **1**, 73–78.

Bradley D, Ratcliffe O, Vincent C, Carpenter R, Coen E (1997). Inflorescence commitment and architecture in *Arabidopsis*. *Science* **275**, 80–83.

Buchanan-Wollaston V (1997). The molecular biology of leaf senescence. *J. Exp. Biol.* **307,** 181–199.

Bush DS (1995). Calcium regulation in plant cells and its role in signaling. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 95–122.

Davis KM, Grierson D (1989). Identification of cDNAs clones for tomato (*Lycopersicon esculentum* Mill.) mRNAs that accumulate during fruit ripening and leaf senescence in response to ethylene. *Planta* **179**, 73–80.

He Y, Gan S (2002). A gene encoding acyl hydrolase is involved in leaf senescence in *Arabidopsis*. *Plant Cell* **14**, 805–815.

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

Hensel LL, Grbic V, Banmgarten DA, Bleecker AB (1993). Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in *Arabidopsis*. *Plant Cell* **5**, 553–564.

Inskeep WP, Bloom PR (1985). Extinction coefficients of chlorophyll a and b in *N, N*-dimethyl formamide and 80% acetone. *Plant Physiol.* **77**, 483–485.

Kardalsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT et al. (1999). Activation tagging of the floral inducer FT. *Science* **286**, 1962–1965.

Kobayashi Y, Kaya H, Goto K, Iwabuchi M, Araki T (1999). A pair of related genes with antagonistic roles in mediating flowering signals. *Science* **286**, 1960–1962.

Lam E, Kato N, Lawton M (2001). Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* **411**, 848–853.

Levy YY, Dean C (1998). The transition to flowering. *Plant Cell* **10,** 1973–1998.

Li J, Wang DY, Li Q, Xu YJ, Cui KM, Zhu YX (2004). PPF1 inhibits programmed cell death in apical meristem of both G2 pea and transgenic *Arabidopsis* plants possibly by delaying cytosolic Ca^{2+} elevation. *Cell Calcium.* **35**, 71–77.

Moran R, Porath D (1980). Chlorophyll determination in intact tissues using *N, N*-dimethyl formamide. *Plant Physiol.* **65**, 478–479.

Nooden LD, Guiamet JJ, Joh I (1997). Senescence mechanism. *Physiol. Plant* **101**, 746–753.

Park JH, Oh SA, Kim YH, Woo HR, Nam HG (1998). Differential expression of senescence-associated mRNAs during leaf senescence induced by different senescence-inducing factors in *Arabidopsis*. *Plant Mol. Biol.* **37**, 445–454.

Ruiz-Garcia L, Madueno F, Wilkinson M, Haughn G, Salinas J, Martinez-Zapater JM (1997). Different roles of flowering-time genes in the activation of floral initiation genes in *Arabidopsis*. *Plant Cell* **9**, 1921–34.

Sanders D, Brownlee C, Harper JF (1999). Communicating with calcium. *Plant Cell* **11**, 691–706.

Shannon S, Meeks-Wagner DR (1991). A mutation in the *Arabidopsis* TFL1 gene affects inflorescence meristem development. *Plant Cell* **3**, 877–892.

Shaul O, Montagu MV, Inze D (1996). Cell cycle control in *Arabidopsis*. *Ann. Bot.* **78**, 283–288

Takahama U, Nishimura M (1976). Effects of electron donor and acceptors, electron transfer mediators, and superoxide dismutase on lipid peroxidation in illuminated chloroplast fragments. *Plant Cell Physiol.* **17**, 111–118.

von Zglinicki T, Saretzki G, Docke W, Lotze C (1995). Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: A model for senescence? *Exp. Cell Res.* **220**, 186–193.

Wang DY, Xu YJ, Li Q, Hao XM, Cui KM, Sun FZ et al. (2003). Transgenic expression of a putative calcium transporter affects the time of *Arabidopsis* flowering. *Plant J.* **33**, 285–292.

Woo HR, Chung KM, Park JH, Oh SA, Ahn T, Hong SH et al. (2001). ORE9, an T-box protein that regulates leaf senescence an *Arabidopsis*. *Plant Cell* **13**, 1779–1790.

Zhu YX, Davies PJ (1997). The control of apical bud growth and senescence by auxin and gibberellin in genetic lines of peas. *Plant Physiol.* **113**, 631–637.

Zhu YX, Zhang YF, Luo JC, Davies PJ, Ho DT (1998). PPF-1, a post-floral-specific gene expressed in short-day-grown G2 pea, may be important for its never-senescing phenotype. *Gene* **208**, 1–6.

(Handling editor: Jian-Ru Zuo)