Regulation of leaf senescence in Arabidopsis
Jing, Hai-Chun

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Chapter 4

*Arabidopsis* CPR5 is a senescence regulatory gene that exhibits early-life beneficial but late-life deleterious effects

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Abstract

Arabidopsis cpr5 mutants exhibit multiple phenotypes including enhanced pathogen resistance, abnormal trichome development, spontaneous lesion formation and accelerated leaf senescence, indicating that CPR5 is involved in multiple facets of the Arabidopsis life cycle. This study aimed to analyse the functions of CPR5 during development. Double mutants between cpr5 and hormonal mutants as well as transgenic plants with different CPR5 mRNA levels were constructed and studied. The cpr5 mutants exhibited enhanced responses to multiple signalling molecules and revealed complex interactions among salicylic acid, jasmonic acid and ethylene. The spontaneous lesion formation and early leaf senescence phenotypes of cpr5 mutants required discrete hormonal signals and could be uncoupled in different genetic backgrounds. Ectopic expression of CPR5 restored all the mutant phenotypes in plants up to the bolting stage. At late development, however, accelerated leaf senescence could be envisaged by the earlier occurrence of visible yellowing and increased levels of SAG12 mRNA. Thus, CPR5 has early-life beneficial effects by repressing cell death but late-life deleterious effects by promoting developmental senescence. As such, CPR5 appears to function as a typical senescence regulatory gene predicted by the evolutionary theories of senescence.

Keywords: Arabidopsis, leaf senescence, evolutionary senescence, hormones, CPR5/OLD1, cell death

Introduction

In animal and evolutionary biology, senescence is defined as a decline in age-specific fitness components due to internal physiological deterioration (Rose, 1991). Studies on evolutionary senescence aim to address why the mortality rates of individuals increase with advancing age and vary within populations and among species. Currently, two major theories of evolutionary senescence are widely acknowledged (Kirkwood and Austad, 2000). The Antagonistic Pleiotropy Theory points out that evolution acts to maximise the reproduction fitness and will allow the existence of genes that have beneficial effects for early-life survival and reproduction despite the fact that these genes may have deleterious late-life effects to promote senescence. The Mutation Accumulation Theory is based on the observation that the force of natural selection diminishes with age and predicts that mutations with deleterious late-life effects will allow the carrier to reproduce before dying and lead to senescence. These two theories implicate that two classes of genes are responsible for senescence: genes with beneficial early-life effects but deleterious late-life effects and late-acting mutations with purely deleterious effects (Kirkwood and Austad, 2000). In yeast and animal ageing paradigms, both types of gene action have been validated and genes involved in the insulin/IGF (insulin growth factor)-1 signalling, metabolic flux and resistance to oxidative stress, have been shown to be the important players for lifespan regulation (Sgro and Partridge, 1999; Guarente and Kenyon, 2000; Gem and Patridge, 2001; Kenyon, 2001; Arantes-Oliveira et al., 2002; Biesalski, 2002; Hughes et al., 2002; Tatar et al., 2003).

In plants, the term senescence is prevalently used in a physiological context to describe a genetically controlled developmental program that leads to the death of plant cells, tissues, organs, and whole plants. Leaves are a model system for plant senescence studies. There is a debate whether studies on leaf senescence can validate the evolutionary theories of
Senescence in plants (Thomas, 2002). Senescence in the evolutionary sense is based on studies on individuals at the population and species levels. One doubt is whether this definition can be ‘scaled down’ to individual leaves. However, it has been argued that leaves have clear lifespan and demographic features and hence can be viewed as cohorts in a population (Bleecker, 1998). Furthermore, leaf senescence is marked by the massive mobilization and recycling of the assimilated nutrients in the senescing leaf and hence considered to be essential for ensuring survivability of a species (Buchanan-Wollaston et al., 2003). Due to such a strong adaptive advantage, leaf senescence appears to violate the definition of evolutionary senescence that occurs at the absence of natural selection and is non-adaptive. This conflict might be reconciled by considering leaf senescence as a deleterious consequence of the selection for the traits that enable nutrient mobilization (Bleecker, 1998). In fact, at biochemical and molecular levels, leaf senescence resembles animal ageing in various aspects. It has been argued that in most cases, the strategies used by plants to regulate senescence are similar to those in animals (Gan, 2003; Lim et al., 2003). Leaf senescence is marked by changes in gene expression profiles. Many senescence-associated genes (SAGs) have been isolated and shown to include genes involved in protein and lipid degradation, transport, cellular stress- and defense-related responses, transcriptional regulation and signalling pathways (Buchanan-Wollaston, 1997; Nam, 1997; Quirino et al., 2000; Chen et al., 2002; Buchanan-Wollaston et al., 2003). In ageing yeast and animals, similar groups of genes displayed such senescence-associated changes in the expression profiles (Zou et al., 2000; Weindruch et al., 2001; Pletcher et al., 2002; Kyng et al., 2003). Thus, similar molecular and cellular processes may take place during leaf senescence and animal ageing.

In Arabidopsis, several senescence regulatory genes have been identified through mutational analyses (Gan, 2003; Lim et al., 2003). The cpr5/hys1 alleles were isolated in a screen for mutants with altered dark-induced whole plant senescence (Yoshida et al., 2002a). They were also recovered in screens for mutants with constitutive expresser of pathogenesis related (PR) genes (Bowling et al., 1997) or abnormal trichome development (Kirik et al., 2001) suggesting that leaf senescence may share common regulatory components with pathogen resistance and cell proliferation. Besides the aforementioned phenotypes, cpr5 mutants showed an elevated salicylic acid (SA) level, enhanced sugar sensitivity, reduced plant size, and spontaneous lesion formation (Bowling et al., 1997; Yoshida et al., 2002a). CPR5 encodes a plant-specific protein that has two distinct signatures: a nuclear localization signal and at least four transmembrane domains (Kirik et al., 2001; Yoshida et al., 2002a). The stunted stature of cpr5 mutants indicates that CPR5 acts to ensure normal growth and development at early life. The abnormal early development of cpr5 mutants may relate to the elevated SA and the enhanced sugar sensitivity since both SA and sugar are able to regulate cell growth in Arabidopsis (Vanacker et al., 2001; Rolland et al., 2002). In addition, the prominent senescence-associated and lesion mimic cell death processes may contribute to the compromised growth and development.

The accelerated leaf senescence in cpr5 mutants might be a consequence of constitutive activation of pathogen resistance responses. Plant disease resistance responses-and senescence have several intrinsic associations. Both processes are genetically controlled and require the active participation of the cell for its own diminishing (Pennell and Lamb, 1997; Dangl and Jones, 2001; Lam et al., 2001; Lim et al., 2003). SAGs were found to express in cells near the sites of pathogen challenge (Pontier et al., 2000), whereas PR genes were shown to be up-regulated during leaf senescence as well (Buchanan-Wollaston, 1997; Quirino et al., 1999;
Buchanan-Wollaston et al., 2003). A genetic hierarchy elucidating the role of CPR5 in mediating a broad spectrum of disease resistance has been constructed, in which CPR5 was placed upstream of salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) signalling pathways (Bowling et al., 1997; Clarke et al., 2000; Jirage et al., 2001). Thus, it is possible that the accelerated senescence is caused by the alteration in these hormonal signals, which have been demonstrated to be important senescence modulators (Grbic and Bleecker, 1995; Morris et al., 2000; He et al., 2001).

In an effort to isolate Arabidopsis mutants that exhibit altered ethylene-induced leaf senescence, we obtained old1 mutants, which caught our special attention for two distinct alterations: accelerated age-regulated senescence and enhanced ethylene responses (Jing et al., 2002). old1 was found to be allelic to cpr5, and hence in the cpr5-induced phenotypes, two more items were added. More importantly, old1 mutants differed from previous reported cpr5 mutants in that they showed predominantly senescence but no macroscopic lesions. We took advantage of this genetic variation and the available hormonal mutants to dissect the diverse functions of CPR5 and to explore the possible roles of the affected signalling pathways in cpr5-induced phenotypes. Transgenic plants with increased CPR5 mRNA levels were also constructed to examine the effects of ectopic expression of CPR5 on leaf senescence. We showed that CPR5 mediated multiple signals to control seedling growth and to repress cell death in adult plants. However, CPR5 can promote leaf senescence at late development. These results are consistent with the notion that CPR5 may be a senescence regulatory gene as predicted by the Antagonistic Pleiotropy Theory of Evolutionary Senescence.

### Results

#### Identification of Ler-0 alleles of cpr5

The old1 mutants were isolated in a screen for leaf senescence mutants that showed accelerated visible yellowing upon exposure to 10µl/l ethylene for 3 days; the details of this screen were reported previously (Jing et al., 2002). Map-based cloning showed that old1 alleles were allelic to cpr5 and hence were renamed as cpr5-1l, cpr5-12 and cpr5-13, respectively (Table 1). These Ler-0 cpr5 mutants showed phenotypes different from cpr5 or hys1 mutants derived from Col-0 (see below). We took the advantage of the available cpr5 alleles to examine the roles of CPR5 in various aspects of Arabidopsis development, especially its role in senescence regulation.

### Table 1. cpr5 alleles identified or studied in this paper

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Accession</th>
<th>Nucleotide change</th>
<th>AA change</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpr5-1l</td>
<td>Ler-0</td>
<td>GGT to AGT</td>
<td>459G to A</td>
</tr>
<tr>
<td>cpr5-12</td>
<td>Ler-0</td>
<td>TGG to TGA</td>
<td>391W to stop</td>
</tr>
<tr>
<td>cpr5-13</td>
<td>Ler-0</td>
<td>GGG to GAG</td>
<td>126G to D</td>
</tr>
<tr>
<td>cpr5-1</td>
<td>Col-0</td>
<td>GGT to GAT</td>
<td>420G to D</td>
</tr>
<tr>
<td>cpr5-2</td>
<td>Col-0</td>
<td>TGG to TAG</td>
<td>473W to stop</td>
</tr>
<tr>
<td>hys1-1</td>
<td>Col-0</td>
<td>TGG to TGA</td>
<td>477W to stop</td>
</tr>
</tbody>
</table>
Enhanced responses of \textit{cpr5} mutant to ET, sugar, JA and ABA

Two prominent phenotypes of \textit{cpr5} mutants were the enhanced responses to sugar and ET (Jing et al., 2002; Yoshida et al., 2002a). Several \textit{cpr5} mutants are in the Col-0 background while the \textit{cpr5-ll} mutants have the Ler background. The \textit{cpr5-ll} mutant was crossed to Col-0 and several F2 plants, homozygous for the \textit{cpr5-ll} allele, were selected and designated as \textit{cpr5-ll/C}. These mutants have a Col-0 and Ler mixed background and allow for a better comparison between the \textit{cpr5} alleles from the different genetic backgrounds. Original exams showed that the individual \textit{cpr5-ll/C} alleles performed similar in a variety of assays. Therefore, one line was selected for further study. Figure 1 shows the observed variation among \textit{cpr5} alleles in their enhanced responses to ET and sugar. In the ET triple response assay, \textit{cpr5-ll} and \textit{cpr5-ll/C} seedlings exhibited the strongest hypersensitivity and the seedlings were ring-shaped. While the \textit{cpr5-2} mutants displayed modest shortening in the hypocotyl length,
no obvious differences between *hys1*-1 mutants and the wild types were observed (Figure 1B). Similar allelic differences were observed in the sugar sensitivity assay (Figure 1C). Interestingly, the allelic variations appear to correlate with the positions of the mutations (Table 1), the closer the defect is to the C-terminal end of the protein, the less pronounced the response. To further examine the relationship between these two signalling pathways in *cpr5* mutants, double mutants between *cpr5*-1 and *ctr1*-1, *ein2* and *abi4* were constructed and their ET and sugar sensitivity examined (Figure 1D-E). These hormonal mutants were isolated from the Col-0 background, thus the responses of the double mutants were compared with *cpr5*-1/C. The results showed that *ctr1*-1 and *ein2* mutations exaggerated or blocked the enhanced ET response of *cpr5*-1, respectively. However, the *ein2* mutation did not affect the sugar hypersensitivity. On the other hand, *abi4* alleviated the exaggerated sugar response of *cpr5*, but not its ET hypersensitivity. These results suggested that the enhanced ET and sugar sensitivities are independent downstream events after the *cpr5* mutation.

The *cpr5* mutations also resulted in hypersensitivity to JA and ABA (Figures 1F-H). The enhanced JA response of *cpr5*-11 was restored by *jar1*, and ABA sensitivity by *abi4*, suggesting that *CPR5* acts upstream of *JAR1* and *ABI4*.

Taken together, *cpr5* mutants exhibit enhanced responses to ET, sugar, JA, and ABA, indicating that in seedlings *CPR5* may function by controlling multiple signalling pathways.

**Elevated JA levels in *cpr5* mutants**

The altered hormone responses may be a result of changed hormone levels. Therefore, the levels of several hormones were measured in the *cpr5* mutants and wild types. Figure 2 shows the endogenous levels of SA and JA in rosette leaves 3 and 4 of 21-d-old soil-grown *cpr5*-11 mutants and various double mutants. Consistent with the analysis of other *cpr5* mutants (Clarke et al., 1997; Clarke et al., 2000; Clarke et al., 2001; Jirage et al., 2001), the *cpr5* mutants showed a similar elevated endogenous SA level (data only shown for *cpr5*-1/C mutant). The SA level was substantially reduced by the bacterial *nahG* gene or increased by the *npr1* mutation (Figure 2A). Interestingly, *ein2* did not affect the SA level in *cpr5*-11. This effect of *ein2* was different from previously reported in another *cpr5* mutant in which *ein2* was shown to increase the SA level 3 fold (Clarke et al., 2000). However, *ctr1*-1 reduced the *cpr5*-11 SA level, suggesting that constitutively activating ET signalling had a negative effect on SA production. Similarly, *jar1* was found to repress the SA production in *cpr5*-11 as envisaged by the lower level of SA in *cpr5*-11/*jar1*.

The JA level was also increased in the *cpr5* single mutants (Figure 2B, data only shown for *cpr5*-11/C), which may be the cause for the observed hypersensitivity to JA. In *cpr5*-11/*nahG* and *cpr5*-11/*npr1* plants, the JA level was ~5 and ~2 fold as high as in *cpr5*-11 mutants, respectively. *ein2* increased JA level in *cpr5*-11 mutants, but *ctr1*-1 did not affect the JA level. *jar1* had a slightly but significantly higher JA level than the wild types and it substantially increased the JA level in *cpr5*-11. We also measured the ET production in etiolated *cpr5*-11 mutants and its various double mutants and the results showed that *cpr5*-11 had a wild-type ET production level, and that SA and JA seemed not to affect ET production (data not shown).

Thus, *cpr5* mutants possessed not only elevated SA levels, but also high JA levels. *ctr1* and *jar1* reduced the high level of SA in *cpr5*-11, while *ein2*, *nahG* and *jar1* increased JA levels in *cpr5*-11.
The senescence syndrome in cpr5 mutants and double mutants

The effects of hormones in cpr5-induced senescence were characterised using a genetic approach. We examined the senescence syndrome using a combination of visible, physiological and molecular markers. A distinction was made between whole leaf yellowing and the occurrence of chlorotic lesions. Representative plants were photographed after 30 days of growth in air and are shown in Figure 3. Leaf yellowing was quantified by measuring average chlorophyll contents in cotyledons and the first and second true leaf pair separately (Figure 4). The senescence syndrome was further examined by determining the expression profiles of several senescence and cell death marker genes as shown in Figure 5. We included a reliable developmental senescence marker \textit{SAG12}, a general cell death marker \textit{SAG13}, and the pathogenesis-related gene \textit{PR-1} (Lohman et al., 1994; Bowling et al., 1997; Weaver et al., 1998; Miller et al., 1999; Noh and Amasino, 1999; Brodersen et al., 2002).

The wild type plants and the single hormonal mutants did not show signs of senescence under our growth conditions, whereas the cpr5 mutants showed clear visible yellowing (Figure 3, data shown for the cpr5 mutants). As opposed to cpr5-11, in which the visible yellowing appeared uniformly, cpr5-11/C and cpr5-2 also showed a mixture of yellow patches and randomly spreading chlorotic lesions. Moreover, in rosette leaves 3 and 4 the chlorophyll content of cpr5-11 was lower than those of the other two mutants (Figure 4). These results indicate that the genetic background affects the pattern of visible yellowing caused by cpr5 alleles. Despite the differences in the visible yellowing, cpr5-11, cpr5-11/C and cpr5-2 exhibited
very similar SAG expression and all the senescence marker genes SAG12, SAG13, SAG14, and SAG21 showed higher expression levels than those observed in wild type. PR-1 mRNA level was also higher in cpr5 mutants (Figure 5).

The ET signalling mutations ein2 and ctr1 had opposite effects on the senescence syndrome in rosette leaves. In cpr5-1/ein2 only prominent chlorotic lesions were observed, whereas in cpr5-1/ctr1-1, the whole leaf blades were uniformly yellow and no macroscopic lesions were observed (Figure 3). Trypan blue staining and subsequent observation of microscopic lesions revealed that the cpr5 mutants showed microscopic lesions, comparable to those that were observed in 45-day-old senescing Ler-0 and Col-0 leaves (data not shown). In cpr5-1/ein2 the observed microscopic lesions corresponded to the macroscopic lesions, whereas in cpr5-1/ctr1-1 no microscopic lesions were observed. Compared with cpr5-1/C, the rosette leaves 3 and 4 of cpr5-1/ein2 showed less pronounced yellowing and higher chlorophyll content, whereas cpr5-1/ctr1-1 showed more visible yellowing and lower chlorophyll content. Gene expression was similarly differentially affected by the two ET signalling mutations. The SAG12 mRNA level of cpr5-1/ein2 was not detectable, whereas in cpr5-1/ctr1-1, it was increased to a higher level than that of cpr5-1/C. In contrast, the SAG13 mRNA level in cpr5-1/ein2 was comparable to that of cpr5-1/C, whereas it was not detected in cpr5-1/ctr1. The SAG14 and PR-1 mRNA levels in cpr5-1 were not affected by ein2, but were reduced by ctr1-1. Compared to cpr5-1/C, the SAG21 mRNA level was reduced in cpr5-1/ein2, but was similar in cpr5-1/ctr1-1.
The bacterial *nahG* gene and the *npr1* mutation were shown to reduce the senescence syndrome in *Arabidopsis* (Morris et al., 2000). The senescence syndrome in *cpr5-1*npr1 was weaker than in *cpr5-1/C*. For instance, the yellowing in *cpr5-1*npr1 did not cover the whole leaf blades and the chlorophyll contents in the examined rosette leaves were higher than those in the *cpr5-1/C* leaves (Figures 3, 4). In *cpr5-1*nahG plants the yellowing was more
lesion-like, as compared to cpr5-l1/C, but there was no difference in chlorophyll content. Introducing nahG or npr1 into cpr5-l1 could reduce the mRNA levels of SAG12 and PR-1 but did not affect those of SAG13, SAG14 and SAG21 (data not shown). JA, ABA and sugar were shown to regulate SAG expression (He et al., 2002; Rolland et al., 2002). However, we did not observe obvious differences among cpr5-l1/C, cpr5-l1jar1 and cpr5-l1abi4.

Thus, enhancing ET signalling hastened the senescence syndrome but inhibited chlorotic lesion formation in cpr5 mutants, whereas impaired ET signalling led to reduced senescence symptoms and increased lesion formation. The nahG transgene and npr1 mutation could suppress some aspects of the senescence syndrome in cpr5 mutants.

Accelerated senescence in CPR5 overexpression lines

Transgenic plants that express varied amounts of CPR5 transcripts were constructed to examine the effects of CPR5 expression on leaf senescence. The CPR5 mRNA levels in leaves from 21-d-old soil-grown plants were measured with a light cycler and the results showed that lines C5-7, C4-3 and N5-6 had higher than wild type CPR5 mRNA levels, line C8-6 and cpr5-l1 mutants had lower levels (Figure 6A). Figure 7 shows the phenotypes of the silenced line C8-6, which displayed all the cpr5-mutant phenotypes such as the enhanced hormonal sensitivity, reduced leaf and plant size and abnormal trichome development. These altered phenotypes were not observed in the overexpression lines.

We examined senescence phenotypes in the transgenic lines. Figures 6B and 6C show the results of the detachment-induced senescence experiment. As could be expected, cpr5-l1 mutants and line C8-6 contained less chlorophyll in comparison with the wild type. The chlorophyll contents in the overexpression lines C5-7, C4-3 and N5-6 were lower than that of the wild type, but were higher than those of cpr5-l1 mutants and C8-6 plants. Similar results
Figure 7. Characterisation of hormonal sensitivity and growth of CPR5 transgenic lines, cpr5 mutants and wild type.

(A-F) The first 8 seedlings are Ler-0, cpr5-l1, Col-0, cpr5-1, C5-7, C8-6, C4-3, and N5-6, and the rest are ctrl-1, ein2 and abi4-1 (A), ctrl-1 and ein2 (B), abi4-1 (C), jar1-1 and abi4-1 (D), jar1-1 (E), and abi4-1 (F). Seedlings were grown on plates containing the indicated components, in darkness for 5 days (A-C) or in light for 7 days (D-F). (G) Representative mature leaves from the indicated lines. White bars represent 0.5cm.
were obtained when the detached leaves were incubated with JA or ABA (data not shown). Thus, the leaves of the transgenic lines exhibited accelerated drops in chlorophyll content upon detachment. We further characterised the developmental senescence. Similar to cpr5 mutants, the C8-6 plants displayed visible yellowing at young plant stages and at the time of bolting yellow cotyledons and at least two yellow rosette leaves were observed (data not shown). The CPR5-overexpressing lines were indistinguishable from the wild type up to the bolting stage (approximately 40 days after germination). At ~40 days after germination visible yellowing was observed both in the wild type and the transgenic lines. In the overexpression lines, however, visible yellowing proceeded faster and occurred in younger leaves. Figures 6D and 6E show that at 50 days after germination similar numbers of yellow leaves were observed in cpr5-1 mutants and lines C8-6 and C5-7, and that these numbers were significantly higher than those observed in wild type plants. Lines C4-3 and N5-6 also showed significantly more yellowing than the wild type. Thus, visible yellowing was accelerated in the transgenic plants. The correlation between CPR5 expression and the advanced senescence syndrome was further studied using molecular markers (Figure 6F). Northern blotting detection showed that at this developmental stage, the CPR5 mRNA level was the highest in C5-7, slightly lower in N5-6 and comparable with the wild type in C4-3. This was a surprising finding, since CPR5 mRNA levels were very high in 21 day-old C4-3 plants. Nevertheless, there was a good correlation between the CPR5 and SAG12 mRNA levels. Very low CPR5 levels (cpr5-1 and C8-6) as well as higher than wild type CPR5 levels (C5-7 and N5-6), correlated with increased SAG12 mRNA levels. The increased visible yellowing and SAG12 mRNA levels in C4-3 plants may be the result of the higher CPR5 expression levels during earlier development. PR-1 mRNA levels, furthermore, correlated well with the visible yellowing.

Taken together, plants with reduced CPR5 mRNA levels had phenotypes similar to cpr5 mutants, whereas CPR5 overexpression only caused early leaf senescence during later stages of plant development.

Discussion

CPR5 differentially mediates multiple signalling pathways

The cpr5 mutants have many phenotypes and were recovered in several mutant screens, indicative of its involvement in various aspects of the plant life cycle. On the basis of the results presented here and those obtained from several groups, we propose that CPR5 functions to repress a common signalling integration complex as shown in Figure 8. We examined the responses of cpr5 seedlings to various signalling molecules at different developmental stages. Seedlings carrying cpr5 alleles were found to exhibit enhanced ET and sugar responses (Jing et al., 2002; Yoshida et al., 2002a) and were hypersensitive to JA and ABA. These signalling molecules all contribute to seedling growth and development in various ways (Creelman and Mullet, 1997; Johnson and Ecker, 1998; Rolland et al., 2002). It has been documented that cross-talks exist among ET, sugar and ABA signalling pathways (reviewed by Rolland et al., 2002; Cheng et al., 2003; Leon and Sheen, 2003). For instance, ein2 mutants are insensitive to ET but hypersensitive to ABA and sugar, whereas ctr1 mutants exhibit constitutive ET response but are ABA and sugar insensitive (Guzman and Ecker, 1990; Zhou et al., 1998; Beaudoin et al., 2000). These results suggest that ET signalling acts antagonistically with sugar and/or ABA signalling. However, we did not observe similar
interactions in cpr5 seedlings, under our experimental conditions. Epistatic analyses indicated that compromising sugar signalling by introducing abi4, did not affect ET hypersensitivity, and vice versa. Furthermore, we observed a mutation-position specific variation in the sensitivity to ET and sugar among different alleles, which was not observed in JA or ABA hypersensitivity. Thus, the enhanced hormonal responses appeared to be partially parallel and independent events in cpr5 seedlings.

In adult cpr5 plants, we observed complex interactions in the control of the endogenous levels of ET, SA and JA among the cpr5 mutants, as outlined in Figure 8. Previous studies have shown that a high level of SA suppresses the accumulation of JA (Peña-Cortés et al., 1993; Doherty et al., 1988) and that the JA levels increase after pathogen infection only in the absence of SA accumulation (Spoel et al., 2003). It has also been shown that cpr5 mutations result in elevated SA levels (Bowling et al., 1997). Here we found that in the cpr5 mutant, the JA level was also increased, suggesting that both biosynthetic pathways can be activated simultaneously. Nevertheless, nahG or npr1 strongly increased the JA levels in cpr5-l1, confirming that SA has an antagonistic effect on JA production. On the other hand, JA signalling might also act antagonistically to control the SA level since jar1 decreased the SA level in cpr5. ET signalling appeared to influence the SA and JA levels in cpr5. Interestingly, this effect was unidirectional, with only constitutive ET signalling repressing SA and JA since we did not observe differences in the ET production among cpr5 alleles and various double mutants.

Two prominent phenotypes of adult cpr5 mutants are the abnormal trichome development and reduced plant size. Both phenotypes were not affected in all of the examined double mutants, suggesting that SA, ET, ABA and JA signalling components are not involved in these abnormalities.

Taken together, we conclude that CPR5 differentially mediates multiple hormonal signals depending on the plant developmental stage.

**CPR5 controls different cell death processes**

CPR5 is a repressor of cell death and two different processes were readily visible in cpr5 plants: senescence-associated and lesion-mimic. The senescence-like cell death, however, could be a result of massive lesion formation. Similarly, the lesion formation could be the result of localized senescence. Making use of double mutants between cpr5 and hormonal pathway mutants in combination with molecular markers, we provided several lines of evidence that cpr5 controls the two cell death processes independently.

First, at the macroscopic level, the Ler-0 cpr5 mutants showed predominantly accelerated senescence and lacked chlorotic lesions, whereas lesions were evident in cpr5 mutants in Col-0 or Col-0 and Ler-0 mixed background. Thus, the genetic background changes the appearance of the two cell death processes. More importantly, they involved different signalling pathways: the senescence phenotype of cpr5-l1/C mutants depended on the ethylene signalling pathway, while the lesion mimic phenotype did not. Introducing ein2 into cpr5-l1 reduced the senescence symptoms and caused a reduction of the expression of the natural senescence marker SAG12 (Lohman et al., 1994; Weaver et al., 1998; Miller et al., 1999; Noh and Amasino, 1999), to undetectable levels. In contrast, neither chlorotic lesion formation nor the mRNA levels of the general cell death markers SAG13 and PR-1 (Uknes et al., 1992; Brodersen et al., 2002) were affected. In a complementary experiment, ctrl-1 was introduced into cpr5-l1 mutants. In cpr5-l1/ctr1-1 there was a concomitant increase in SAG12 expression.
and senescence symptoms in younger leaves. In the same plants, SAG13 and PR-1 mRNA levels were reduced and lesions were absent. Thus, in cpr5 mutants, ET signalling promoted leaf senescence but inhibited lesion formation. Such functions of ET in cpr5-induced cell death differ from its role in pathogen resistance-associated cell death where ET was shown to be a stimulator of lesion expansion after pathogen attack (Bent et al., 1992; Lund et al., 1998; Pilloff et al., 2002). Other hormone signalling pathways had a varied effect on senescence symptoms and lesion formation. Reducing SA levels or blocking SA signalling reduced SAG12 and PR-1 mRNA levels, but had no effect on lesion formation in cpr5 mutants (Bowling et al., 1997), suggesting that CPR5 might regulate additional signals to control lesion formation (Figure 8). Remarkably, our results showed that inhibiting JA responses by jar1 affected neither lesion formation nor senescence, even though the role of JA in both senescence and defence has been well documented (Penninckx et al., 1998; Pieterse et al., 1998; He et al., 2002). Thus, the precocious cell death in cpr5 mutants may not involve JA signalling (Figure 8). Finally, in CPR5-overexpressing plants, early leaf senescence was uncoupled from lesion formation and a correlation was found between CPR5 and SAG12 expression levels. PR-1 mRNA levels, however, did not correlate with CPR5 mRNA levels in all transgenic lines. Taken together, these observations indicated that distinct cell death processes occurred in cpr5 mutants as outlined in Figure 8.

The discussion presented above argues that mutations in CPR5 resulted in accelerated cell death including early leaf senescence and hence indicates that CPR5 functions to repress senescence. However, CPR5 overexpression lines exhibited enhanced leaf senescence both upon detachment and in planta, as envisaged by the earlier appearance of visible yellowing, faster drop of chlorophyll content, and a correlation of the SAG12 mRNA levels with the CPR5 mRNA levels. The promoting effect of CPR5 on senescence was obtained by ectopic expression, which might not reflect the real function of CPR5 in the wild type. Nonetheless, accelerated leaf senescence was the only unique phenotype in CPR5 overexpression lines.

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**Figure 8.** A tentative model depicting the functions of CPR5 in regulating cell death via mediating multiple signalling pathways. CPR5 is proposed to function through an integration complex and employ ET, SA and an undefined signalling pathway (dotted line) to regulate leaf senescence and lesion formation. The promotive effect of CPR5 on senescence is shown as a green T-bar. The interactions among ET, SA and JA are also shown. Arrows indicate promotion and T-bars stand for inhibition. See discussion for details.
Furthermore, the expression pattern of CPR5 was examined in transgenic plants harbouring CPR5 promoter:GUS reporter constructs and the results indicated that CPR5 levels increased in mature leaves (unpublished results), which is consistent with the reports that a roughly 2-fold increase in CPR5 transcript was found in senescing leaves (Zimmermann et al., 2004; https://www.genevestigator.ethz.ch.). Finally, ectopic expression was employed to demonstrate the regulatory function of another senescence regulatory gene SAG101 (He and Gan, 2002). Thus, it seems likely that the accelerated leaf senescence obtained from CPR5 overexpression indeed reflects the promotion effect of CPR5 in wild type plants.

Together, analyses on cpr5 mutants show that CPR5 is required to repress cell death, while overexpression analyses show that CPR5 can promote senescence-associated cell death. A plausible explanation for these seemingly contradicting results is that CPR5 may display changed functions during development. Indeed, the senescence syndrome in cpr5 mutants or transgenic plants with low CPR5 mRNA levels was distinctively different from the one in CPR5 overexpression lines. In the former ones, senescence started early in adult plants, whereas in CPR5 overexpression lines it started late in life after bolting. Thus, at adulthood, CPR5 was required to repress leaf senescence and another cell death process, whereas at late development it could promote senescence (Figure 8).

**Evolution of senescence regulating genes in plants**
Besides leaf senescence, CPR5 is also involved in seedling growth, trichome development, and pathogen resistance, indicative of its pleiotropic nature. A common feature of several senescence regulatory genes is that they are involved in plant growth and development as well. ORE9 encodes an F-box protein and is part of the ubiquitination protein degradation machinery (Woo et al., 2000). ore9/max2 alleles were also recovered in screen for mutants with altered shoot lateral branching (Stirnberg et al., 2002). Furthermore, SAG101 was shown to be involved in lipid metabolism (He and Gan, 2002), and ORE4 encodes the plastid ribosomal small subunit protein 17 (PRPS17) that is important for protein synthesis (Woo et al., 2002). This fits very well to the prediction of the antagonistic pleiotropy theory of senescence (Kirkwood and Austad, 2000). Moreover, these observations agree with the view that similar molecular strategies appear to have evolved for senescence regulation in both plant and animal kingdoms (Gan, 2003; Lim et al., 2003).

This notion might be particularly true for CPR5. The results obtained in this study showed that CPR5 differentially exerts its functions during Arabidopsis growth and development. CPR5 exhibits early-life beneficial effects by ensuring normal seedling growth and repressing cell death in adult plants. Interestingly, CPR5 also shows high expression in embryos (https://www.genevestigator.ethz.ch/). However, at late developmental stage, a functional CPR5 promotes “normal” senescence and hence is deleterious. Such a separation of the functions of CPR5 throughout development mimics the action of the insulin/IGF-1 signalling pathway and p53 in animal and human cells. The insulin/IGF-1 signalling pathway has pleiotropic functions and is shown to control lifespan, reproduction and stress resistance in many organisms (Guarente and Kenyon, 2000; Gems and Partridge, 2001; Kenyon, 2001; Tatar et al., 2003). A recent study in nematode worms indicates that DAF-2, an insulin/IGF-1-like receptor, employs independent mechanisms to regulate lifespan and reproduction, and that the insulin/IGF-1-like pathway influences development and reproduction at early life, but acts exclusively
during adulthood to control adult lifespan (Dillin et al., 2002). p53 is a genome guardian, the deficiency of p53 proteins leads to cancer and tumor development due to increased cellular damages, suggesting that p53 has clear early–life beneficial effects (Levine, 1997; Sharpless and DePinho, 2002). Nonetheless, a p53 mutant mouse line (p53<sup>−/−</sup>), in which the stability and activities of the wild-type p53 protein were augmented in the presence of a mutant allele, developed fewer tumors than wild-type (p53<sup>+/+</sup>) homozygotes, but exhibited faster ageing (Tyner et al., 2002). Clearly, maintaining a higher p53 level at late life is deleterious.

Thus, CPR5 seems to function as a typical senescence regulatory gene as predicted by the evolutionary theory of senescence. However, at the DNA and protein levels, CPR5 shares no similarities with any genes in the insulin/IGF-1 signalling pathway, or p53, in agreement with the notion that although plants may use similar strategies to control senescence, the particular molecular mechanisms can be different (Jing et al., 2003). Further molecular genetic and biochemical studies that unravel how CPR5 works in a plant cell will allow a better comparison of senescence regulatory mechanisms across kingdoms.

**Experimental procedures**

**Plant material and growth conditions**

*Arabidopsis thaliana* accessions Ler-0 and Col-0 were the wild types. The mutant alleles and transgenic plants used were *old1-1* (renamed as *cpr5-I1* in this paper) (Jing et al., 2002), *cpr5-I1* (Bowling et al., 1997), *cpr5-2* (Boch et al., 1998), *hys1-1* (Yoshida et al., 2002a), *npr1-1* (Cao et al., 1994), *ein2-1* (Guzman and Ecker, 1990), *ctr1-1* (Kieber et al., 1993), *jar1-1* (Staswick et al., 1992), *abi4-1* (Finkelstein et al., 1998), and *Arabidopsis* plants expressing the bacterial *nahG* gene (Bowling et al., 1994). Depending on the specific phenotypes and identities of the particular mutations, a variety of screening methods employing hormonal responses and PCR-based marker assays were used to isolate double mutants.

Plants were grown in an organic-rich soil (TULIP PROFI No.4, BOGRO B.V., Hardenberg, The Netherlands) or in Murashige and Skoog (MS) medium containing 0.8% agar under the conditions described by Jing et al. (2002).

**Map-based cloning, complementation test and construction of transgenic lines**

*cpr5-I1* was originally placed ~3 centiMorgans south of the single nucleotide polymorphism (SNP) marker SGCSNP84 at the bottom of chromosome 5. To perform fine mapping, 2000 F<sub>2</sub> *cpr5-I1* seedlings were selected from a mapping population generate by crossing *cpr5-I1* to Col-0. DNA was isolated using the SHORTY quick preparation method (http://www.biotech.wisc.edu/Arabidopsis). By comparing the genomes of Col-0 (TAIR database) and Cereon Ler-0 (Monsanto SNPs and Ler) (http://www.arabidopsis.org; Jander et al., 2002), potential SNPs were selected. Primers were designed, using the WebSNAPER programme, that specifically amplified Col-0 DNA fragments and used for PCR (Drenkard et al., 2000; http://ausubellab.mgh.harvard.edu/resources). The mutation was mapped onto a 15 kb region spanning three open reading frames including CPR5. Sequence analyses revealed a single nucleotide change inside CPR5. The other two Ler-0 *cpr5* alleles were subsequently sequenced (Table1). *Agrobacterium*-mediated transformation was performed to further confirm the identity of *old1* as a *cpr5* allele.

For constructing CPR5 transgenic plants, full-length CPR5 cDNA was amplified using primers designed either with or without an in-frame fusion of the HA epitope tag (YPYDVPDYA)
and cloned behind a modified 35S CaMV promoter in the plant transformation vector pBI1.4T. All constructs were verified by sequencing and subsequently electroporated into Agrobacterium tumefaciens strain GV3101. The resulting bacteria were used to transform wild-type Col-0, mutant cpr5-1 (lacking NptII) and cpr5-2 plants (Clough and Bent, 1998). Transformants were selected on MS media containing 50 µg/mL kanamycin (Murashige and Skoog, 1962) and complementation of cpr5 was determined by restored wild-type trichome development and lack of macroscopic lesions. Lines homozygous for single insertion events were used in further experiments.

**Hormonal sensitivity assay**
For ET sensitivity, seedlings were grown on MS media containing 1µM ACC (1-aminocyclopropane-1-carboxylic acid) in the dark for 5 days, and the triple response was observed (Guzman and Ecker, 1990). Sugar sensitivity was determined by growth for 5 days in darkness on MS medium containing 1% sucrose. The hypocotyl lengths of the seedlings were subsequently compared (Dijkwel et al., 1997). The effect of JA on the inhibition of root elongation of light-grown seedlings was examined as described (Staswick et al., 1992). Briefly, seeds were germinated in light in vertical plates containing MS medium, 0.5% sucrose and 20µM MeJA, and the root elongation of 7-d light-grown seedlings was examined. A low concentration of sucrose (0.5%) was used to minimize the inhibiting effect of sugar. For ABA sensitivity, seeds were germinated in light on vertical plates containing MS medium, 0.5% sucrose and 0.3µM ABA. The growth of 7-d light-grown seedlings was examined.

**Chlorophyll content measurement and gene expression analysis**
For chlorophyll content and Northern blotting, rosette leaf samples were collected from 30-day-old soil-grown Arabidopsis plants and were prepared and analyzed as described by Jing et al. (2002).

For light cycler measurements of CPR5 mRNA levels, approximately 150 mg of tissue was harvested from 3-week-old soil-grown wild-type Col-0, mutant cpr5-1, and wild-type plants overexpressing CPR5 cDNA (C5-7, C4-3 and C8-6) or HA-tagged CPR5 cDNA (N5-6). Subsequently, total RNA was extracted as described by Cao et al. (1994). Ten µg of RNA was treated with DNase I according to manufacturer’s instructions (Ambion Inc., Austin, Texas). One µg of RNA was incubated with Superscript II reverse transcriptase and oligo dT in a 20µl reaction to synthesize cDNA (Invitrogen, Carlsbad, California). For the quantitative PCR half reactions (10µl each), 2µl of the cDNA product was used as template with the CPR5 specific primers, whereas 2µl of a 20-fold dilution was used for reactions with Ubiquitin5 (UBQ5, At3g62250) specific primers. The final primer concentration in all reactions was 0.5µM. Quantitative PCR was carried out using the SYBR green PCR kit (QIAGen, Valencia, California) and a Roche Lightcycler real-time PCR machine according to the manufacturer’s instructions (Roche, Mannheim, Germany). The relative number of CPR5 specific transcripts was determined in 3 replicate experiments by normalization to UBQ transcript levels.

**HPLC-MS (High Pressure Liquid Chromatograph-Mass Spectrometry) analyses of salicylic acid and jasmonic acid**
Rosette leaves number 3 and 4 without any signs of visible yellowing were taken from 21-day-old soil-grown plants and used to measure the SA and JA concentrations according to a procedure derived from Wilbert et al. (1998). Briefly, ~200mg leaf tissues were ground in liquid
nitrogen into fine powder and extracted with 500µl acidified MeOH (methanol with 0.1%
concentrated HCl) overnight at 4°C. After centrifugation, the supernatant was collected,
diluted to 35% with water, and centrifuged before injecting 100µl into HPLC coupled on-line
with a mass spectrometer for quantification. The injection was done with a Perkin-Elmer series
200 autosampler. Before and after injection the injector and the needle were flushed twice with
0.1% NH₄OH in 50% MeOH to remove the residual SA or JA. MeOH (gradient grade), formic
acid (p.a, 98-100%) and ammonia solution (p.a., 25%) were purchased from Merck, Darmstadt.

For HPLC, both JA and SA were negatively charged by post-column adding 1% NH₄OH
solution in MeOH with a flow of 100µl/min, delivered by a Kratos spectroflow 400-pump. JA
and SA were separated under acidic conditions by running a gradient of aqueous 0.1% formic
acid and 0.1% formic acid in MeOH over a 2.1mm-column (Alltech Alltima C18 5µ). The gradient
was delivered by 2 Perkin-Elmer series 200 LC-pumps at a flow rate of 200µl/min, started with
30% MeOH for 1min, raised to 95% in 5min and retained for 5min, then dropped to 30% in
2min. A 6-min interval was used for equilibration.

For mass spectrometry, the free acids JA and SA were analysed in the negative ion-mode by
measuring a small range in a Q1-profile-scan-mode combined with “up-front” collision to see
the M-44-ion of SA (loss of CO₂) and to avoid association of the formate-ion to JA (M+45).
The M-44-ion of JA was not observed. The MS system consisted of an API3000 mass
spectrometer (Applied Biosystems/MDS-SCIEX, Toronto, Canada) and a triple quadrupole
mass spectrometer equipped with a Turbo Ion-spray interface. The 200µl/min HPLC-flow,
combined with a 100µl/min, post-column flow, were introduced through the ion-spray interface
with the temperature of the heater set to 450°C. The state file was as follows: NEB(Zero-air)=14,
CUR=14, IS=−4500, TEM=450, OR=−50, RNG=−200, Q0=11, IQ1=11, St=15, RO1=11,
IQ2=-20, RO2=100, St3=120, RO3-102, DF=300, CEM=2500. For SA, the range of 136-139amu
and 92-95amu with a step-size of 0.100amu and a dwell-time of 15ms was analyzed. The molecular
weight of SA is 138. The M-1-ion is m/z 137 and the M-1-44 is m/z 93. For JA, the range of 208-
211amu with the same step-size and dwell-time was analyzed. The molecular weight of JA is
210. The M-1-ion is m/z 209. We avoided SIM to double-check the isotope-patterns of the free
acids. Due to the interference of many unknown products, slightly shifting of the retention
time for the same ions was observed. The area under the ion-signals was calculated with
MacQuan 1.7 (PE SCIEX). To confirm the authenticity, SA and JA standards were added into
the plant extracts as controls.

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