CPR5 is involved in cell proliferation and cell death control and encodes a novel transmembrane protein

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Plants often respond to pathogens by sacrificing cells at the infection site. This type of programmed cell death is mimicked by the constitutive pathogene response5 (*cpr5*) mutant in *Arabidopsis* in the absence of pathogens, suggesting a role for *CPR5* in programmed cell death control [1]. The analysis of the cellular phenotypes of two T-DNA-tagged *cpr5* alleles revealed an additional role for *CPR5* in the regulation of endoreduplication and cell division. In *cpr5* mutant trichomes, endoreduplication cycles stop after two rounds instead of four, and trichome cells have fewer branches than normal. Eventually, *cpr5* trichomes die, the nucleus disintegrates, and the cell collapses. Similarly, leaf growth stops earlier than in wild-type, and, frequently, regions displaying spontaneous cell death are observed. The cloning of the *CPR5* gene revealed a novel putative transmembrane protein with a cytosolic domain containing a nuclear-targeting sequence. The dual role of *CPR5* in cell proliferation and cell death control suggests a regulatory link between these two processes.

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Results

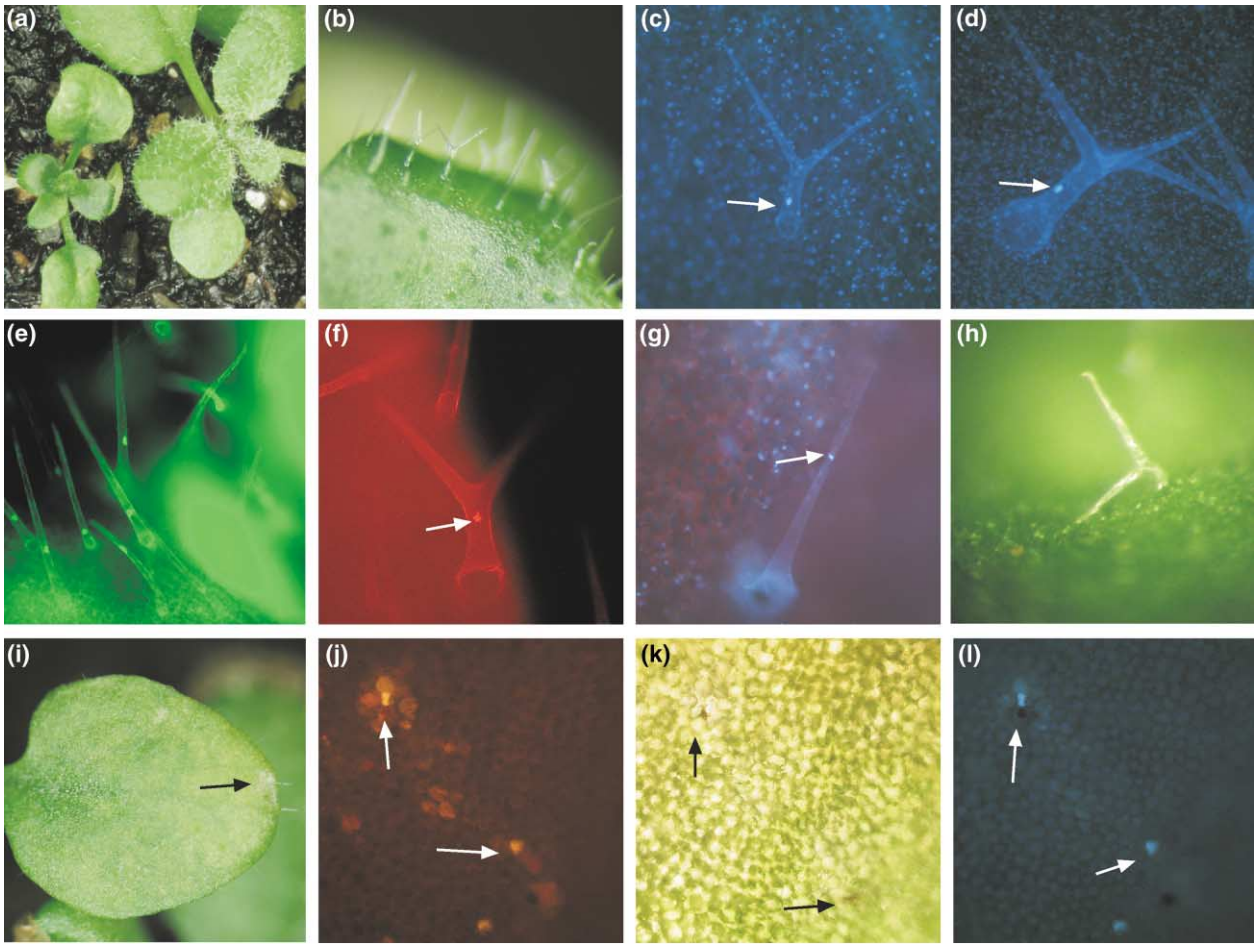
In animals and plants, genetically controlled mechanisms determine not only which cells should live, but also which cells should die. Thus, for homeostasis, cell division and cell death must be kept in balance. Cell death in plants occurs naturally during various developmental processes, including the differentiation of tracheary elements, root cap cells, aleuron cells, and during senescence [2, 3]. Cell death is also a common feature of plant disease resistance. At the site of pathogen entry, cell death lesions may occur in and around the infection site (called the hypersensitive response [HR]) and are thought to hinder the pathogen from spreading [4, 5]. This type of cell death is programmed, since many mutants have been identified that mimic the HR phenotype in the absence of a pathogen [1, 6–9] and are therefore potential candidates for genes involved in cell death control.

Our work with one of these mutants, the *cpr5* mutant, revealed an unexpected link to the growth regulation in leaves and the regulation of endoreduplication in leaf trichomes. In a screen of T-DNA-mutagenized plants [10] for trichome mutants, we found two recessive mutants exhibiting reduced trichome branching, the *ctz8* mutant and the *5758-1* mutant (Figure 1b). Both mutants exhibited the same phenotypes and resulted from a T-DNA insertion in the *CPR5* gene (accession number AY033229). We therefore renamed the *ctz8* mutant and the *5758-1* mutant *cpr5-T1* and *cpr5-T2*, respectively.

In the *cpr5-T1* mutant, the number of trichome branches is drastically reduced (58% unbranched, 38% two branches, 3% three branches, n = 661) compared to the corresponding wild-type WS (0% unbranched, 10% two branches, 90% three branches, n = 1029) (Figure 1b). Because trichome cells appeared to be reduced in size, we determined whether the ploidy level is also reduced (Figure 1c,d). The comparison of the relative fluorescence of DAPI-stained nuclei in *cpr5-T1* trichomes with that of wild-type (32C) and the *glabra3* (16C) mutant revealed that the DNA content in *cpr5-T1* trichomes corresponds to approximately 8C, suggesting that endoreduplication cycles stop after the second cycle is completed (Figure 2a). We also found that the cell size and the nuclear size of epidermal pavement cells was greatly reduced (Figure 2c), such that, in 3-week-old plants, cell size and nuclear size of 98% of all cells in *cpr5* mutants corresponded to about 50% of the smallest cells in wild-type. This suggests that endoreduplication levels are also reduced in epidermal cells.

As *cpr5* mutant plants are much smaller than wild-type plants (Figure 1a), we tested whether cell divisions are generally affected. We compared the number of pavement cells on rosette leaves of 3-week-old plants along the

Figure 1



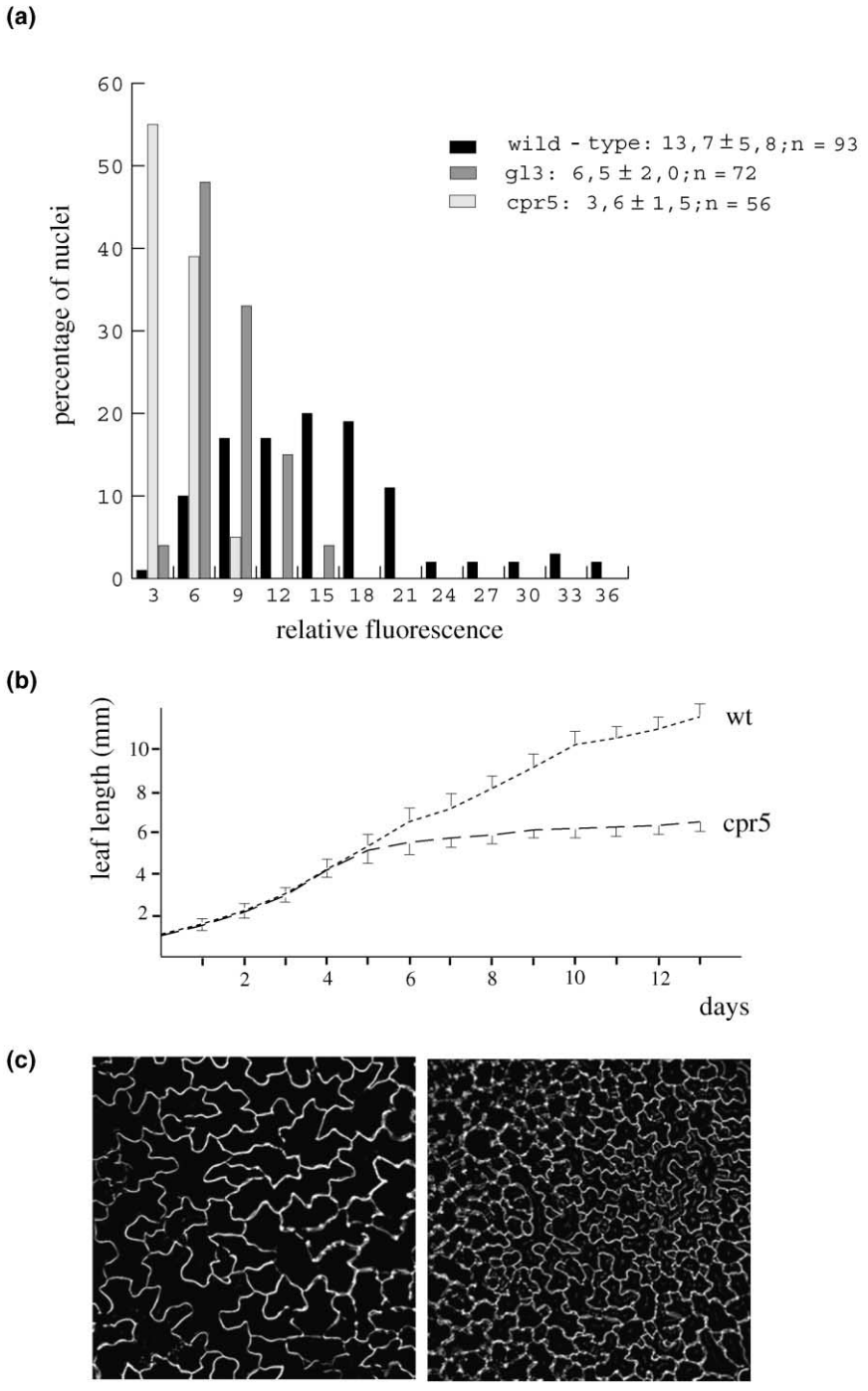
Trichome and lesion phenotype in *cpr5* mutants. **(a)** A mature wild-type (right) and *cpr5* mutant plant (left). **(b)** *cpr5* mutant trichomes on rosette leaves. **(c)** DAPI-stained *cpr5* mutant trichome; the nucleus is marked with an arrow. **(d)** DAPI-stained mature wild-type trichome cell; the nucleus is marked with an arrow. **(e)** Mature FDA-stained *cpr5* mutant trichomes; note that the cytoplasmic region around the nucleus and the thin lining of the cytoplasm is stained, indicating that the cell is alive. **(f)** Propidium iodide-stained *cpr5* mutant cell; staining indicates that membranes are not intact anymore and that the cell is dead. Note that the nucleus still has a normal morphology (arrow). **(g)** DAPI-stained *cpr5* mutant trichome cell, the nucleus is drastically reduced in size and is condensed (arrow). **(h)** Collapsed trichome cell. **(i)** Lesions on rosette leaves of *cpr5* mutants (arrow). **(j)** Propidium iodide-stained leaf tissue; note that single cells have started to die (arrow). **(k)** Light micrograph of the same leaf area as in (j); note the single brownish dead cells (arrow). **(l)** Same leaf area as in (j) and (k), using the UV filter set. Note that autofluorescent cells are different from the brown cells shown in (k) (arrow).

length and the width axis. Along the length axis, *cpr5* mutant leaves had approximately 70% fewer cells than wild-type (wild-type: 471 ± 65; *cpr5*: 149 ± 35, n = 20). Along the width axis, the cell number was reduced by about 60% (wild-type: 269 ± 23; *cpr5*: 109 ± 35, n = 20). In order to determine whether these phenotypes are caused by growth retardation or by a premature growth arrest, we compared leaf elongation between *cpr5* mutants and wild-type (Figure 2b). Initial growth rates were indistinguishable. After 5 days, *cpr5* mutant leaves stopped growing, while wild-type leaves continued to grow.

During the course of experiments, we noticed that *cpr5-T1* mutant trichomes on mature leaves eventually died and collapsed (Figure 1h). FDA, which labels only living cells, was found to stain mature *cpr5-T1* mutant trichomes (Figure 1e), indicating that these trichomes had completed their normal differentiation program before cell death occurred. On 4-week-old plants, trichomes began to die, as indicated by propidium iodide staining (Figure 1f). Initially, the nucleus appeared to be normal. However, eventually trichomes were found containing extremely small and condensed nuclei, indicating that the nucleus disintegrated (Figure 1g). On 6- to 7-week-old plants, all trichome cells were collapsed, leaving the cell wall remnants behind (Figure 1h).

Cell death was also observed in other cell types. On 6-

Figure 2



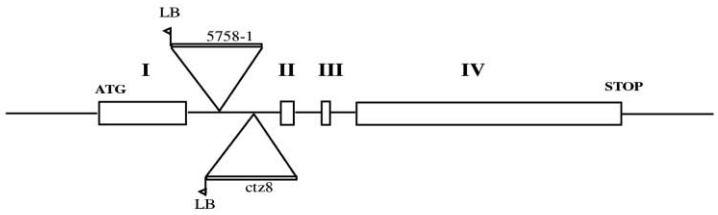
Cell proliferation in *cpr5* mutants. **(a)** The relative fluorescence of trichome nuclei is blotted against the percentage of nuclei that were grouped into classes of three fluorescence units. The distribution of nuclei in *cpr5-T1*, *gl3*, and wild-type is shown in the diagram, and the mean values and standard deviations are shown at the side. **(b)** Leaf length measurements of leaf number 5 at daily intervals. The standard deviation is shown for wild-type toward the top and for the *cpr5* mutant toward the bottom. **(c)** Comparison of wild-type (left) and *cpr5* mutant (right) pavement cells at the same magnification.

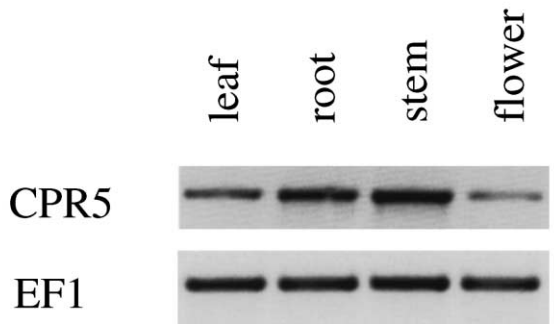
to 7-week-old plants, single cells (Figure 1j) or small leaf areas (Figure 1i) were found to form lesions containing brownish cells (Figure 1k). In propidium iodide-stained leaves, single cells or cell groups were stained, indicating that cell death had occurred. Frequently, cells displaying a high autofluorescence were found (Figure 1l), which is indicative of the production of high amounts of phenolic compounds and often correlates with cell death.

The *cpr5-T1* and *cpr5-T2* mutants were isolated from screens of two different *Arabidopsis* T-DNA insertion mutant populations [10, 11]. The genomic DNA sequences flanking the T-DNA insertions mapped to the P1 clone MXK3 on chromosome V. According to the annotated sequence information, both T-DNA insertions are located in the first intron of the *CPR5* gene (Figure 3a) and most likely result in a complete loss of gene function. In order to

Figure 3

Structure and expression analysis of the *CPR5* gene. **(a)** Structure of the *CPR5* gene. Exons are shown in boxes. The positions of the T-DNA inserts in the first intron are marked. **(b)** Expression of the *CPR5* gene as revealed by semiquantitative RT-PCR, with the translation elongation factor *EF1* used as a control.





prove the correct identity of the gene, we used a 6340-bp BamHI fragment containing 3.407 bp 5′ and 500 bp 3′ of the gene for rescue. A total of 16 transgenic lines were recovered, which all showed complete rescue of the *cpr5-T1* mutant phenotype.

Sequence analysis revealed that the *CPR5* gene encodes a novel putative transmembrane protein containing five putative transmembrane helices at the C terminus. *CPR5* is predicted to be a Type IIIa membrane protein, with the N terminus being cytoplasmatic (PSORT). In addition, a bipartite NLS is found at the N terminus at position 40–56 (PSORT).

The pleiotropic phenotype of *cpr5* mutants affecting virtually all organs suggests that *CPR5* is ubiquitously expressed. Using RT-PCR, we found *CPR5* expression in all tissues analyzed. Expression levels were similar in roots, leaves, stems, and flowers (Figure 3c).

Discussion

The finding that the *CPR5* gene is involved in several processes including cell death, cell cycle, and pathogen response raises the question of what the primary cellular function of CPR5 is. Initially, the *cpr5* mutant was identified as an important component in the plant pathogen response pathway [1]. *cpr5* belongs to a class of mutants that show several plant pathogen response reactions in the absence of a pathogen attack [1, 6–9]. In *Arabidopsis*, most of these spontaneous lesion mutants express features characteristic for systemic-acquired resistance (SAR) and are more resistant to pathogens. SAR is frequently associated with the HR and leads to an enhanced immunity in secondary plant tissues by systemic signaling [12]. Features characteristic of SAR that are also expressed in the spontaneous lesion mutants include an enhancement of salicylic acid (SA) levels and the expression of PR genes (pathogenesis-related genes). In some of these mutants' lesion formation, PR gene expression and resistance can be suppressed by overexpressing the bacterial salicylate hydroxylase gene, which leads to a reduction of endogenous SA levels [9, 13]. This indicates that, in these mutants, SA mediates all other responses. In other mutants, including *cpr5*, the reduction of SA compromises the expression of PR genes and the pathogen resistance, but not lesion formation [1, 14]. Therefore, the constitutive pathogen response phenotypes are considered secondary [1]. Whether *CPR5* controls SA accumulation and cell death by different pathways or whether SA accumulation in *cpr5* mutants is a consequence of cell death is unclear.

Our finding that *cpr5* mutants are severely affected in the ploidy levels of trichomes and exhibit a marked reduction in cell number indicates that *CPR5* is also involved in the control of cell proliferation. Our finding that FDA stains mature *crp5* mutant trichome cells indicates that the endoreduplication defects precede the initiation of cell death. Thus, as judged by the relative timing of events, the primary defect of *cpr5* mutants is a defect in the proper control of cell proliferation. It is, however, unlikely that

this cell cycle defect as such leads to the observed cell death, since trichome mutants such as the *gl3* mutant that display a reduced ploidy level without showing a cell death phenotype [15] are known. Similarily, a large fraction of pavement cells also remains diploid in the wild-type without undergoing cell death [16].

The sequence analysis of the CPR5 amino acid sequence reveals several domains, leading to contradictory predictions on the intracellular localization of CPR5. On the one hand, CPR5 is predicted to be a Type IIIa membrane protein with five transmembrane helices at the C terminus and a cytoplasmatic N terminus. On the other side, a well-conserved bipartite NLS sequence in the cytoplasmatic region predicts a nuclear localization. An exciting possibility that would explain this apparent contradiction is that CPR5 might function similarly to membrane-bound transcription factors that are kept in a dormant state in the cytosol by membrane anchors and are released by proteolytic cleavage, enabling the transcription factors to enter the nucleus [17, 18].

While the potential biochemical function of the CPR5 protein remains highly speculative, the prediction that it represents a membrane protein suggests that CPR5 is involved in signal transduction, controlling cell proliferation and cell death.

Supplementary material

Details of methods and biological material, plasmids, and oligonucleotides used in this study can be found with the electronic version of this article at http://images.cellpress.com/supmat/supmatin.htm.

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