

GeBP/GPL Transcription Factors Regulate a Subset of CPR5-Dependent Processes^{1[C][W]}

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The *CONSTITUTIVE EXPRESSOR OF PATHOGENESIS-RELATED GENES5 (CPR5)* gene of *Arabidopsis (Arabidopsis thaliana)* encodes a putative membrane protein of unknown biochemical function and displays highly pleiotropic functions, particularly in pathogen responses, cell proliferation, cell expansion, and cell death. Here, we demonstrate a link between *CPR5* and the *GLABRA1 ENHANCER BINDING PROTEIN (GeBP)* family of transcription factors. We investigated the primary role of the *GeBP/GeBP-like (GPL)* genes using transcriptomic analysis of the quadruple *gebp gpl1,2,3* mutant and one overexpressing line that displays several *cpr5*-like phenotypes including dwarfism, spontaneous necrotic lesions, and increased pathogen resistance. We found that *GeBP/GPLs* regulate a set of genes that represents a subset of the *CPR5* pathway. This subset includes genes involved in response to stress as well as cell wall metabolism. Analysis of the quintuple *gebp gpl1,2,3 cpr5* mutant indicates that *GeBP/GPLs* are involved in the control of cell expansion in a *CPR5*-dependent manner but not in the control of cell proliferation. In addition, to our knowledge, we provide the first evidence that the *CPR5* protein is localized in the nucleus of plant cells and that a truncated version of the protein with no transmembrane domain can trigger *cpr5*-like processes when fused to the VP16 constitutive transcriptional activation domain. Our results provide clues on how *CPR5* and *GeBP/GPLs* play opposite roles in the control of cell expansion and suggest that the *CPR5* protein is involved in transcription.

Pleiotropic genes participate in many seemingly unrelated traits and play a key role in fundamental

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aspects of life such as evolution, development, and aging. In *Arabidopsis (Arabidopsis thaliana)*, the *CONSTITUTIVE EXPRESSOR OF PATHOGENESIS-RELATED GENES5 (CPR5; CPR5/HYS1/OLD1)* gene is involved in highly pleiotropic developmental processes. Although initially identified based on their constitutive pathogen response phenotype, mutants of *CPR5* possess striking phenotypes including (1) enhanced constitutive expression of pathogen-related (PR) genes associated with increased pathogen resistance (Bowling et al., 1994; Boch et al., 1998); (2) defects in cell division, expansion, endoreduplication, and cell wall biogenesis associated with a reduced stature (Kirik et al., 2001; Brininstool et al., 2008); (3) spontaneous lesions mimicking cell death and accelerated leaf senescence (Jing et al., 2002, 2007; Yoshida et al., 2002); and (4) modified hormonal and metabolic responses in the salicylic acid, jasmonic acid, abscisic acid, ethylene, and sugar-sensing pathways (Clarke

et al., 2000; Aki et al., 2007; Jing and Dijkwel, 2008). Thus *CPR5* appears to play a broad role in plant growth and development.

Genetic analysis has revealed that *CPR5* independently controls many of these developmental processes, as blocking a particular signaling pathway does not affect the alterations in other signaling pathways in *cpr5* mutants (Bowling et al., 1997; Aki et al., 2007; Jing et al., 2008). This suggests that *CPR5* is a key factor that regulates the activity of distinct subpathways, modules, or subsets (Brininstool et al., 2008; Jing and Dijkwel, 2008). In the well-known pathogen-response pathway, *CPR5* appears to act just downstream of pathogen recognition and displays both *NON EXPRESSOR OF PR GENES1* (*NPR1*)-dependent and *NPR1*-independent disease resistance (Bowling et al., 1997) while it regulates PR gene expression in the *RPS2*-mediated pathway (Boch et al., 1998). One specific visible phenotype of *cpr5* mutants not found in the phenotype of any of the other constitutive pathogen response mutants is abnormal trichome cell development (Kirik et al., 2001; Brininstool et al., 2008). While most trichome cells on wild-type *Arabidopsis* leaves display multiple branches, *cpr5* leaves exhibit both reduced trichome branching and reduced trichome number. This phenotype is reminiscent of loss-of-function mutant phenotypes in genes involved in trichome initiation such as *GLABRA1* (*GL1*) or *GL3*. Genetic studies suggest that at least some of these trichome regulators are dependent on *CPR5* function (Brininstool et al., 2008). This is consistent with the recently described role of *GL1* in systemic acquired resistance in defense to both bacterial and fungal pathogens, a role that might be due to the regulation of cuticle development (Xia et al., 2010).

The *CPR5* protein is well conserved throughout the plant kingdom and is predicted to be a type IIIa membrane protein (Kirik et al., 2001). A well-defined bipartite nuclear localization signal (NLS) in the non-membrane region predicts nuclear targeting. Although its intracellular localization and mode of action are unknown, several authors have proposed that *CPR5* might be anchored in a membrane, such as that of the endoplasmic reticulum or the inner nuclear envelope, and might function upon proteolytic cleavage as a transcription (co)factor (Kirik et al., 2001; Brininstool et al., 2008).

We have previously identified a family of transcription factors whose founding member, *GL1 ENHANCER BINDING PROTEIN* (*GeBP*), is a putative regulator of *GL1*. The four *GeBP/GeBP-like* (*GPL*) genes encode unconventional Leu-zipper transcription factors (Curaba et al., 2003; Chevalier et al., 2008) and redundant roles of *GeBP/GPLs* in the indirect regulation of some cytokinin response genes have been shown (Chevalier et al., 2008). However, *GeBP/GPLs* primary role remains unknown. We show here that the *GeBP/GPL* family fulfills a subset of *CPR5* functions. In this study we performed a transcriptomic profiling of the quadruple *gebp gpl1,2,3* mutant and

one overexpressing line and found that *GeBP/GPLs* control genes involved in defense responses and cell wall metabolism that overlap with a subset of *CPR5*-regulated genes. Our genetic analysis demonstrates that *GeBP/GPL* genes play a repressive role in cell expansion by counteracting the positive role of *CPR5* in this process. In contrast to *CPR5*, *GeBP/GPLs* do not regulate cell proliferation or endoreduplication. Finally, we show that *CPR5* is a nuclear protein and that its putative nucleosolic domain alone is sufficient for transcriptional regulatory responses. These results demonstrate that *GeBP/GPL* genes have a role in *CPR5*-dependent processes, such as stress and cell expansion, and suggest that *CPR5* may directly participate in transcription through a proteolytic activation mechanism.

RESULTS

GeBP/GPL Downstream Genes Represent a Subset of *CPR5*-Regulated Genes

We have previously shown that *GeBP/GPL* genes play a redundant role in the indirect regulation of the cytokinin negative feedback loop (Chevalier et al., 2008). However, their primary function was unknown. To assess the function of *GeBP/GPL* genes in *Arabidopsis* development, we constructed the quadruple *gebp gpl1,2,3* mutant and performed a transcriptomic analysis, which we compared to that of wild-type *Arabidopsis*. A transgenic line overexpressing a version of *GPL2* with constitutive transcriptional activation activity (*VP16:GPL2* line; Chevalier et al., 2008) was also included in the analysis. Three-week-old rosettes were used to prepare RNAs, a developmental stage at which all *GeBP/GPL* genes are expressed. At this stage, the *gebp gpl1,2,3* mutant showed no visible phenotype but the *VP16:GPL2* line showed retarded growth, early senescence, and necrotic lesion phenotypes (see below). The CATMA v2 arrays, which represent 24,576 gene sequence tags, were used to hybridize three independent biological replicates for each genotype and data were treated as previously described (Gagnot et al., 2008). In the quadruple *gebp gpl1,2,3* mutant, the transcripts of 88 genes were reproducibly misregulated (37 up- and 51 down-regulated genes), while in the *VP16:GPL2* line, the transcripts of 332 genes were affected (251 up- and 81 down-regulated genes; Supplemental Tables S1 and S2). The datasets were mined with the software tools Genevestigator (Calikowski et al., 2003), Mapman (Thimm et al., 2004; Rotter et al., 2007), and EasyGO/AgriGO (Ashburner et al., 2000) to search for specific correlations. These analyses indicated an enrichment of genes involved in response to stress (10.39-fold; hypergeometric test: P value = 0.0) and cell wall metabolism (26.5-fold; P value < $5.6 \cdot 10^{-9}$) pathways (Fig. 1A) and revealed, more specifically, that transcriptomic alterations in both *gebp gpl1,2,3* and

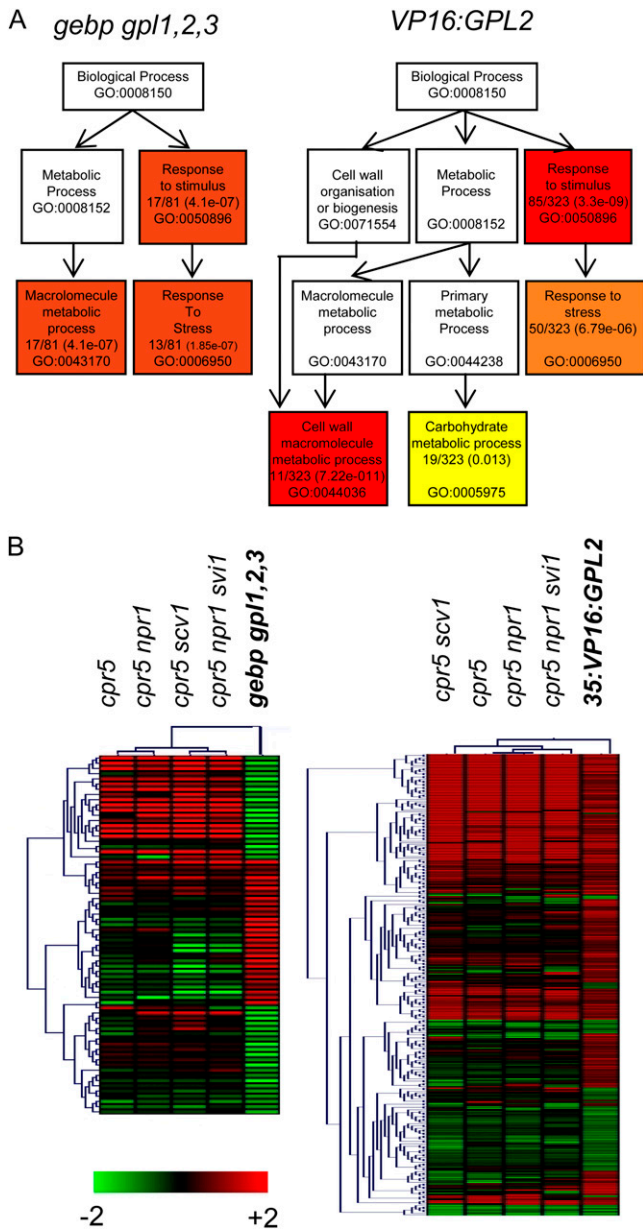


Figure 1. Gene ontology of *gebp gpl1,2,3* and *VP16:GPL2* transcriptomic data and transcriptomic similarities with the *cpr5* mutant series. A, Gene ontology of *gebp gpl1,2,3* (left) and *VP16:GPL2* (right) transcriptomic data. Distribution of gene sets among functional biological pathways using singular enrichment analysis of AgriGO are shown. Colors are as in AgriGO, and only the most significant pathways are shown. The ratio of genes involved in each pathway and *P* values are indicated within boxes together with the gene ontology accession number. The highest probabilities are for the stimuli/stress response (*gebp gpl1,2,3* and *VP16:GPL2*) and cell wall process (*VP16:GPL2*) pathways. Several entries were not associated to GO terms. Hence 81 genes instead of 88 were used in this analysis for the *gebp gpl1,2,3* data and 323 genes instead of 332 for the *VP16:GPL2* data. B, Hierarchical clusterings of genes misregulated in the *gebp gpl1,2,3* (left) or *VP16:GPL2* (right) and *cpr5* mutant series. Graphics were generated using Genevestigator and MultiExperiment viewer software.

VP16:GPL2 plants largely overlapped with one specific set of publicly available microarray data (Fig. 1B). This data set is from a series of mutants that all share a mutation in the *CPR5* gene (NASCARRAYS-355 performed by Dr. Yang). Among the 409 genes misregulated both in *gebp gpl1,2,3* and *VP16:GPL2*, 20% were included in *cpr5* transcriptomic data (*P* value < 1.0 10⁻¹⁹), indicating that a substantial fraction of *GeBP/GPL*-dependent genes are found in the *CPR5* pathway. Transcriptomic alterations induced by multiple abiotic stresses (Zeller et al., 2009) showed no overlap with *gpl1,2,3* and a limited overlap with *VP16:GPL2* data sets, suggesting that the *GeBP/GPL* pathway is closer to *CPR5* than to a more general stress-related pathway (Supplemental Fig. S1). Genes that were down-regulated in the *gebp gpl1,2,3* mutant were often up-regulated in *cpr5* plants (Fig. 1B; Supplemental Fig. S1). This negative correlation was also true for genes that were up-regulated in the *gebp gpl1,2,3* mutant, suggesting that *GeBP/GPL* and *CPR5* genes are involved, directly or indirectly, in opposite regulation of the same downstream genes.

The overlap between transcriptomic alterations in *gebp gpl1,2,3* and *cpr5* mutants suggests that *GeBP/GPL* transcription factors control a subset of *CPR5*-regulated genes.

GeBP/GPL Genes Are Involved in Several, But Not All, *CPR5*-Like Pathways

In addition to their substantial overlapping transcriptomic profiles, strong phenotypic similarities were observed between *cpr5-2* and *VP16:GPL2* plants. As shown in Figure 2A, both types of plant displayed a reduced stature relative to the wild type, together with spontaneous necrotic lesions on cotyledons and leaves. A similar phenotype was observed in transgenic lines that overexpress a stable form of *GeBP* fused to the *VP16* domain (Supplemental Fig. S2). Interestingly, while *cpr5* mutant plants made smaller trichomes (Kirik et al., 2001), trichomes of *VP16:GPL2* plants were indistinguishable from those of the wild type (Fig. 2A). Trichomes of wild-type *Arabidopsis* have an average DNA content of 32C due to endoreduplication, a cell cycle bypass that permits replication of the DNA without mitosis (Sugimoto-Shirasu and Roberts, 2003; John and Qi, 2008). We measured the DNA levels in the trichomes of wild-type, *cpr5-2*, and *VP16:GPL2* plants (Supplemental Fig. S3). Relative to the wild type, trichomes of *cpr5* had a lower DNA level as previously reported (Kirik et al., 2001), while trichomes of *VP16:GPL2* plants reached a DNA level similar to the wild type. This indicates that, in contrast to *CPR5*, *GeBP/GPL* genes are not involved in the control of endoreduplication, at least in trichomes.

A common consequence of defense responses to infection or stimuli is the hypersensitive response (HR), a local and rapid cell death that helps halt the spread of pathogens. Necrotic lesions in *VP16:GPL2* plants were similar to those observed in the *cpr5*

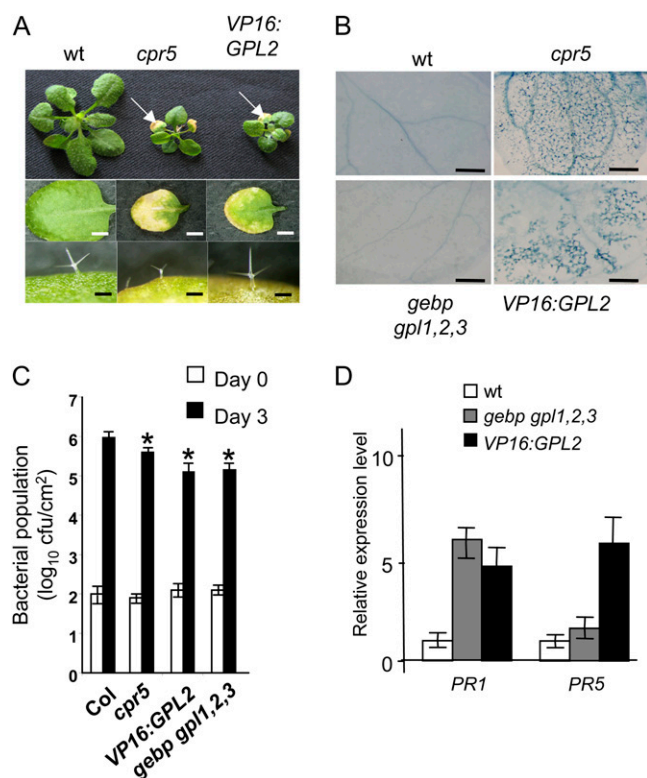


Figure 2. Phenotypic similarities between *VP16:GPL2* and *cpr5-2* mutants. **A**, Top row, rosettes of 3-week-old wild-type, *cpr5-2*, and *VP16:GPL2* plants grown in soil. White arrows indicate early senescing leaves. Middle row, individual third leaves of 4-week-old plants. Control lines expressing the VP16 domain alone showed no visible phenotypes as previously described (Chevalier et al., 2008). Scale bars: 1 mm. Bottom row, sizes of trichomes. Scale bars: 100 μ m. **B**, Trypan blue staining of wild-type, *cpr5-2*, *gebp gpl1,2,3*, and *VP16:GPL2* leaves. Scale bars: 500 μ m. **C**, Bacterial populations in wild-type, *cpr5-2*, *VP16:GPL2*, and *gebp gpl1,2,3* plants. Inoculations with *Pst DC3000* strain were performed on leaves without lesions with a bacterial suspension at 2×10^5 cfu mL⁻¹. Bacterial populations were measured at 0 (white bars) and 3 d (dark bars) postinoculation. Mean bacterial densities are shown (three to five replicates with corresponding sds) for one representative experiment from two or three independent experiments. Asterisks denote significantly different values from bacterial number in the wild type according to the Student's *t* test ($P \leq 0.05$). **D**, Transcript levels of *PR1* and *PR5* genes in *gebp gpl1,2,3* and *VP16:GPL2* relative to the wild type. Real-time RT-PCR was performed on 3-week-old rosettes. [See online article for color version of this figure.]

mutant, mimicking HR in the absence of pathogen. To determine whether the ectopic immune response suggested by the gene expression profiles correlated with HR-like cell death, we first stained leaves of *VP16:GPL2*, *cpr5*, and quadruple *gebp gpl1,2,3* plants with Trypan blue, an indicator of dead cells. Leaves of *VP16:GPL2* and *cpr5* showed staining, indicating spontaneous cell death, while the quadruple *gebp gpl1,2,3* mutant was indistinguishable from the wild type (Fig. 2B).

To confirm unambiguously a role for *GeBP/GPL* genes in immune responses, we assayed the response

of the *gebp gpl1,2,3* mutant and the *VP16:GPL2* line to the pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000. Plant resistance was evaluated by the measurement of in planta bacterial growth 3 d after leaf infiltration. As shown in Figure 2C, in planta pathogen growth was significantly decreased in *VP16:GPL2* as well as *cpr5* relative to wild-type plants. The *gebp gpl1,2,3* mutant also showed a similar phenotype (Fig. 2C). This suggests that pathogen response pathways were activated in both *gebp gpl1,2,3* and *VP16:GPL2* plants more strongly than in the wild type. Transcript levels of the pathogen response marker genes *PR1* and *PR5* were measured using real-time reverse transcription (RT)-PCR and were indeed increased over the wild-type levels in both types of plant (Fig. 2D). The observation that *PR* transcript levels are increased in the quadruple mutant suggests that *GeBP/GPL* genes are repressors of *PR* gene expression. An up-regulation of *PR* genes in both *gebp gpl1,2,3* and in *VP16:GPL2* plants is not surprising since *GPL2* can act as a transcriptional activator in the presence of the VP16 domain. Therefore the *VP16:GPL2* fusion should behave as a dominant negative gain-of-function allele on *GPL2* negatively regulated genes such as *PR* genes, a situation already described for *LEAFY:VP16* and its target gene *TERMINAL FLOWER1* (Parcy et al., 2002).

Taken together, these data indicate that the *GeBP/GPL* genes are likely involved in cell death and defense pathways but not in trichome development or endoreduplication, in contrast to *CPR5*.

Epistatic Relationship between *GeBP/GPL* and *CPR5* Pathways

To analyze the role of *GeBP/GPLs* within the *CPR5* pathway, we generated the *gebp gpl1,2,3 cpr5* quintuple mutant and looked for epistatic relationships. As shown in Figure 3A, the growth defect of the *cpr5* mutant was suppressed, at least partially, in the *gebp gpl1,2,3 cpr5* mutant, indicating that *gebp/gpl* mutations are epistatic to the *cpr5* mutation. Leaf area in the *gebp gpl1,2,3 cpr5* mutant was 62% larger, on average, than the *cpr5* leaf area (Fig. 3B). This leaf growth increase was mainly caused by a suppression of the *cpr5* premature growth arrest phenotype (Kirik et al., 2001), as leaf elongation of the *gebp gpl1,2,3 cpr5* quintuple mutant continued after the *cpr5* leaf elongation stopped (Fig. 3C).

To determine whether the suppression of the *cpr5* growth arrest phenotype by *gebp/gpl* mutations was due to the restoration of cell proliferation, we first analyzed the sensitivity of the wild type and mutants to the DNA replication-blocking agent aphidicolin, an inhibitor of early S phase (Fig. 4A). When grown in the presence of aphidicolin, wild-type plants showed a slight reduction of growth. While the quadruple *gebp gpl1,2,3* mutant displayed a similar degree of sensitivity, the growth of *cpr5* plants was completely inhibited, providing experimental indication for a *cpr5* defect in

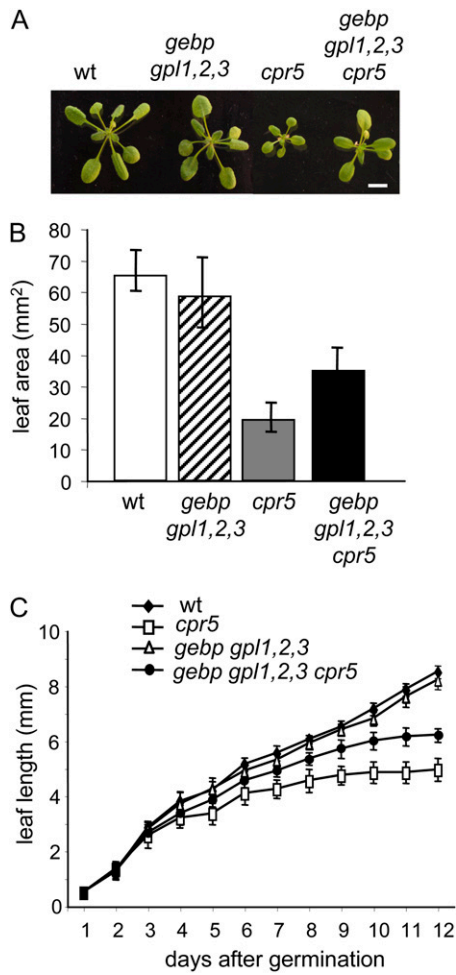


Figure 3. Vegetative growth of wild-type, *cpr5-2*, and *gebp gpl1,2,3 cpr5* mutants. **A**, Rosettes of wild type, *gebp gpl1,2,3* quadruple mutant, *cpr5-2*, and *gebp gpl1,2,3 cpr5* quintuple mutant grown in soil for 3 weeks. Scale bar: 3 mm. **B**, Leaf area of the wild type, *gebp gpl1,2,3* quadruple mutant, *cpr5-2*, and *gebp gpl1,2,3 cpr5* quintuple mutant. **C**, Leaf elongation rate in wild type, *cpr5-2*, *gebp/gpl* quadruple mutant, and *gebp/gpl cpr5-2* quintuple mutant. Plants were grown in soil, and measurements of the third leaf were taken at daily intervals. Initial growth rates were similar in all types of plant. [See online article for color version of this figure.]

the cell cycle or cell proliferation, although we cannot exclude that aphidicolin affects additional replication-independent processes. The growth of the *gebp gpl1,2,3 cpr5* quintuple mutant was similar to that of the *cpr5* single mutant, suggesting that *gebp/gpl* mutations do not restore cell proliferation in the *gebp gpl1,2,3 cpr5* quintuple mutant.

To reinforce this observation, we analyzed endoreduplication levels in leaf cells of wild-type and mutant plants using flow cytometry. The ploidy profiles of wild-type and quadruple *gebp gpl1,2,3* mutant plants were similar, with a majority of nuclei having an 8C DNA content. In the *cpr5* mutant, the ploidy profile was shifted toward lower values, with a main peak at

4C and a decrease of 8C and 16C populations. Similarly, in the *gebp gpl1,2,3 cpr5* quintuple mutant, the population of 4C nuclei was increased at the expense of 8C and 16C populations, confirming that the role of *CPR5* in endoreduplication is independent of *GeBP/GPL* genes. Finally, we introgressed the *VP16:GPL2* construct into lines expressing the cell cycle markers *PcycB1:GUS* and *PcdkA:GUS* and measured transcript levels of *cycD3* in wild type and *VP16:GPL2* (Supplemental Fig. S4). No changes in GUS expression patterns or in transcript levels were observed in the *VP16:GPL2* background, in agreement with our transcriptomic analysis, which detected no cell cycle genes that are known to be transcriptionally regulated.

Altogether, these results indicate that *GeBP/GPL* genes display a role in *CPR5*-dependent leaf expansion and plant growth but are not involved in control of the cell cycle or endoreduplication.

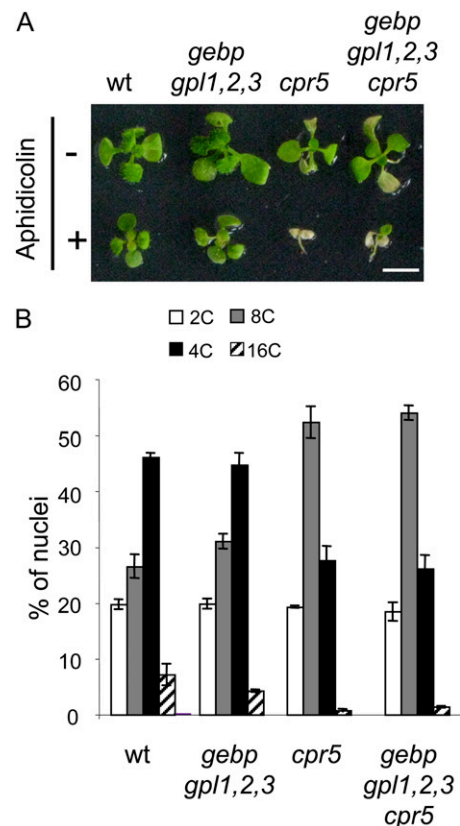


Figure 4. Aphidicolin sensitivity assay and DNA levels in wild type, *gebp gpl1,2,3* quadruple mutant, *cpr5-2*, and *gebp gpl1,2,3 cpr5* quintuple mutant. **A**, Aphidicolin sensitivity assay. Plants were grown in vitro for 3 weeks in the absence (–) or presence (+) of aphidicolin (12 $\mu\text{g mL}^{-1}$). Scale bars: 5 mm. **B**, Distribution of nuclei according to DNA content in cells of third rosette leaves. [See online article for color version of this figure.]

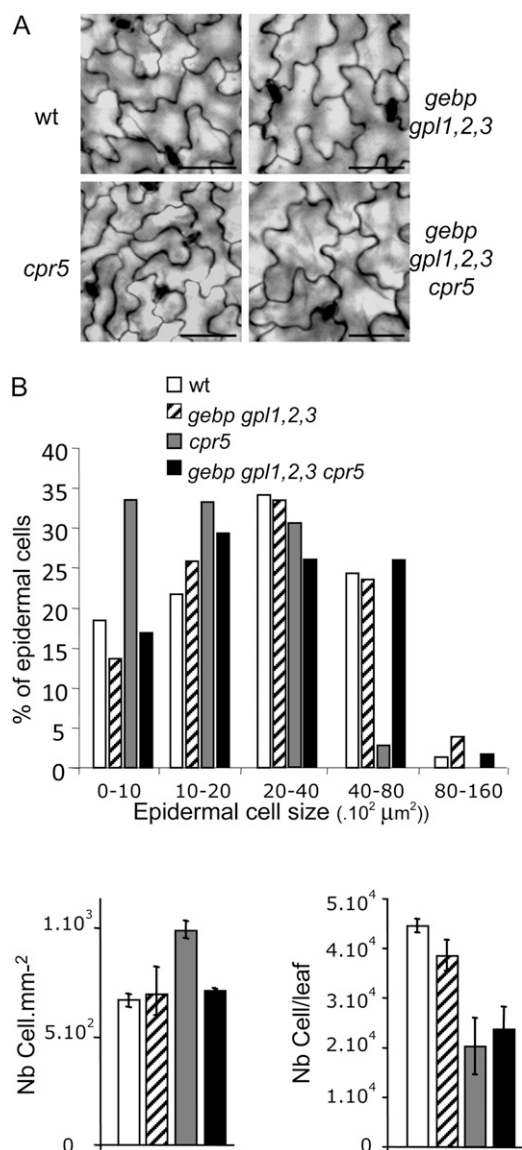


Figure 5. Size and number of adaxial pavement cells in leaves of wild-type and mutant plants. Epidermal cell size and number in wild type, *gebp gpl1,2,3* quadruple mutant, *cpr5-2*, and *gebp gpl1,2,3 cpr5* quintuple mutant. A, Adaxial pavement cell size of third leaves of 3-week-old plants grown in soil. Scale bars: 200 μm . B, Class distribution of epidermal cell size (top histogram), epidermal cell density (bottom left), and estimated epidermal cell number per leaf (bottom right). Class distribution of cell size was performed by measuring cell area from third leaves of 3-week-old plants grown in soil (Columbia, $n = 147$; quadruple mutant, $n = 149$; *cpr5*, $n = 294$; quintuple mutant, $n = 191$) using ImageJ software. The total number of epidermal cells per leaf was estimated by dividing the leaf area by the average cell area.

Epidermal Cell Size Is Controlled by GeBP/GPL Genes in the Absence of CPR5

To determine whether the suppression of *cpr5* growth defects in the quintuple mutant was due to an increase in cell expansion rather than in cell number, we looked at cell size in leaves of wild-type and

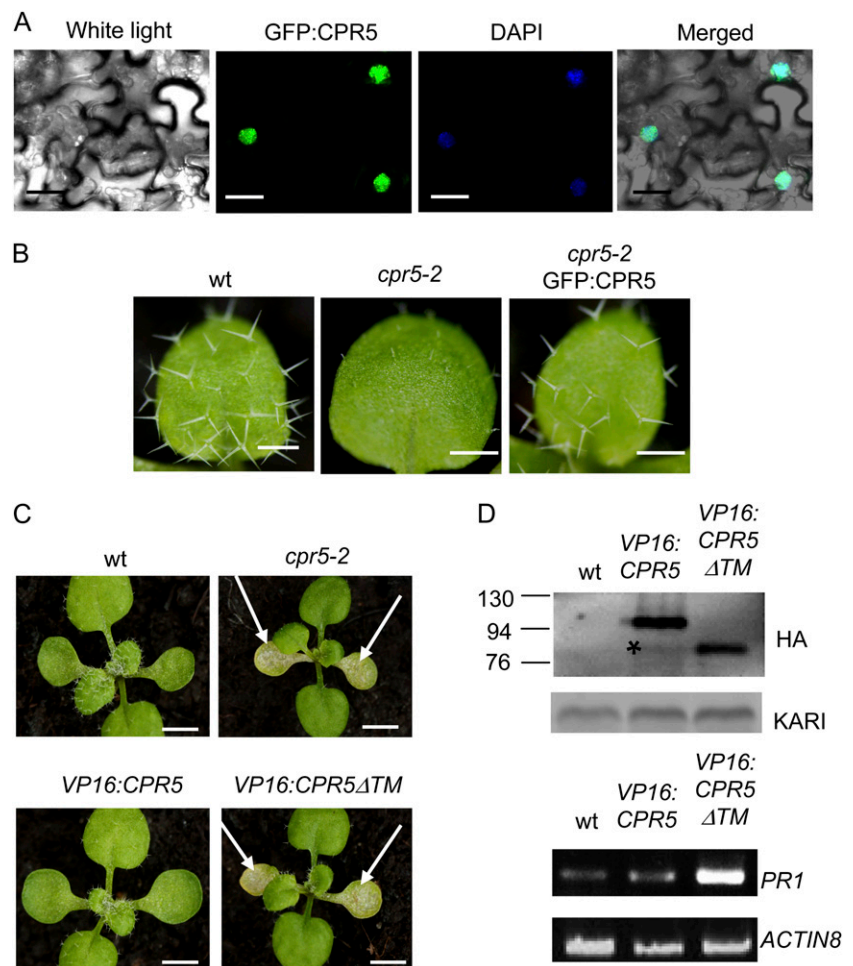
mutant lines. As previously reported for *cpr5* mutants (Kirik et al., 2001), pavement cells of *cpr5* were smaller compared to the wild type (Fig. 5A). Pavement cell size in the *gebp gpl1,2,3* quadruple mutant as well as the *gebp gpl1,2,3 cpr5* quintuple mutant was not distinguishable from the wild type. To quantify this observation, we looked at cell size distribution in wild type and mutants. Class distribution of pavement cell size indicated that *cpr5* had an excess of smaller cells and was deficient in larger cells (Fig. 5B) while the *gebp gpl1,2,3* mutant was similar to wild type. On the other hand, the *gebp gpl1,2,3 cpr5* quintuple mutant exhibited pavement cell sizes whose class distribution was similar to the wild type, indicating that the suppression of the *cpr5* growth defect in the quintuple mutant was mainly due to an increase in cell size. This increase did not occur in all cell types as palisade cells of the quintuple mutant had a size similar to palisade cells of *cpr5* (Supplemental Fig. S5). The estimated total number of epidermal cells in *cpr5* and quintuple mutant leaves were within the same range (Fig. 5B), reinforcing the conclusion that GeBP/GPL genes do not play a major role in cell proliferation.

CPR5 Is a Nuclear Protein Involved in Transcription

One of the key unsolved issues in understanding the function of CPR5 is its intracellular localization. To address this point, the GFP coding sequence was fused upstream of the full-length CPR5 cDNA under the control of the 35S promoter and transient transformation of tobacco (*Nicotiana tabacum*) cells was performed. As shown in Figure 6A, the GFP signal was present in the nuclei of plant cells. These results indicate that CPR5 is a nuclear protein. To determine whether the fusion was functional, this construct was introduced into a *cpr5* mutant background. As illustrated in Figure 6B, the full-length fusion complemented many aspects of the *cpr5* phenotype (dwarfism, spontaneous lesions, trichome development) except the early senescence of cotyledons, which was delayed but not completely abolished.

It has been proposed that CPR5 might function similarly to membrane-bound transcription factors that are kept in a dormant state when anchored in membranes and are released by proteolytic cleavage, enabling them to directly regulate downstream genes (Kirik et al., 2001; Seo et al., 2008). To determine whether CPR5 or its soluble portion might be directly involved in transcriptional mechanisms, the VP16 transcriptional activation domain was fused in frame to the coding sequence of wild-type CPR5 or a CPR5 deletion mutant (CPR5 Δ TM) lacking the transmembrane domain, and transgenic lines were generated. While all (23/23) VP16:CPR5 lines displayed a wild-type phenotype, nearly all (18/21) of the VP16:CPR5 Δ TM lines displayed a *cpr5* mutant phenotype with early senescing cotyledons, reduced trichome number, reduced growth (Fig. 6C), and up-regulation of *PR1* gene expression (Fig. 6D). Western-blot analysis

Figure 6. Intracellular localization and functional analysis of CPR5 and CPR5 Δ TM proteins. A, Subcellular localization of GFP:CPR5 protein in tobacco cells under confocal microscopy. Scale bars: 10 μ m. B, Trichome development in plants transformed with *35S::GFP::CPR5* on a *cpr5-2* mutant background. Young rosette leaves are shown. Scale bars: 1 mm. C, Phenotype of plants transformed with *35S::HA::VP16::CPR5* or *35S::HA::VP16::CPR5 Δ TM* on a wild-type background. Young rosettes are shown. White arrows indicate early senescing cotyledons. Scale bars: 3 mm. D, Detection of VP16 fusion proteins in western blots (two top sections) and transcript levels of *PR1* in wild-type and VP16 plants using semi-quantitative RT-PCR (two bottom sections) in wild-type and VP16 transgenic lines. The star indicates a weak band in the VP16:CPR5 lane corresponding to a protein with a size similar to that of the VP16:CPR5 Δ TM protein. Contrast has been increased to better visualize this band. VP16 fusions were detected with a monoclonal anti-HA antibody (Roche). The KARI protein used as a loading control was detected with a polyclonal anti-KARI antibody.



indicated that both fusion proteins were expressed at similar levels (Fig. 6D). In *VP16::CPR5* lines, we also detected very low levels of a protein with a molecular weight similar to the *VP16::CPR5 Δ TM* protein, suggesting that the full-length fusion can give rise to a transmembrane domain truncated form in vivo.

These results suggest that the CPR5 protein is located in the nucleus of plant cells, supporting the hypothesis that CPR5 can be involved in transcriptional processes.

DISCUSSION

GeBP/GPL Genes Are Involved in Stress Responses

Stress in plants can trigger a wide variety of physiological and molecular responses, leading to HR-like responses, reactive oxygen species production, hormonal disorders, and, ultimately, programmed cell death. We show here that *GeBP/GPLs* play a role in pathogen resistance and can induce HR-like responses when overexpressed as a constitutively active form. Our transcriptomic analysis indicates that *GeBP/GPL*

genes control a relatively small number of genes, part of which are involved in stress-related pathways. This suggests that *GeBP/GPL* genes have a specific function among mechanisms triggered by stress. We have shown previously that *GeBP/GPL* genes are involved in the indirect regulation of cytokinin response genes, such as *ARR6* (Chevalier et al., 2008), which was also highlighted in our microarray data. Several links between cytokinins and biotic and abiotic stresses have been described: Cytokinin treatment increases transcript levels of *PR* genes (Memelink et al., 1987) and can directly trigger cell death, and a genetic link between plant defense response and cytokinins has been shown by mutations in defense genes (Igari et al., 2008). Therefore, it is possible that *GeBP/GPL* genes have a specific role in the link between stress and the cytokinin pathway. In addition, the *cpr5* mutant shows reduced cytokinin sensitivity under conditions of stress, such as the presence of a high concentration of Glc (Aki et al., 2007).

Transcriptomic, phenotypic, and genetic relationships between *GeBP/GPLs* and *CPR5* demonstrate that they are involved in similar processes, although *GeBP/GPL* functions are a subset of *CPR5* functions. These relationships suggest that *GeBP/GPL* genes act

downstream of *CPR5* or independently of *CPR5*. Transcript levels of *GeBP/GPLs* are similar on wild-type and *cpr5* backgrounds (data not shown), suggesting that *CPR5* does not regulate *GeBP/GPL* transcript level.

A Role for *GeBP/GPL* Genes in Cell Expansion

The control of final organ size implies a complex network of both promoters and inhibitors of cell proliferation and cell expansion, some of which have been identified (Mizukami, 2001; Aufran et al., 2002; Hu et al., 2003; Churchman et al., 2006; Disch et al., 2006; Busov et al., 2008). Our genetic analysis shows that *GeBP/GPL* genes play a repressive role in final organ size determination by counteracting the positive role of *CPR5* in this process. *GeBP/GPLs* do not seem to regulate cell proliferation, as the estimated epidermal cell number in leaves of *gebp gpl1,2,3* and *gebp gpl1,2,3 cpr5* plants was similar to that of wild-type plants and *cpr5* plants, respectively. This observation is reinforced by the absence of modifications in the transcript levels of cell cycle marker genes in *gebp gpl1,2,3* and *VP16:GPL2* plants. In addition, DNA content measurements and aphidicolin sensitivity assays suggest that endoreduplication and cell cycle mechanisms are not controlled by *GeBP/GPLs*. Our phenotypic analysis revealed a role for *GeBP/GPLs* in cell size regulation, at least in epidermal cells. This role of *GeBP/GPLs* was observed on a *cpr5* mutant background, where cell number was reduced compared to the wild type. Compensatory effects between cell number and cell expansion in leaves have been reported in many studies using growth mutants or ecotypes (Tsukaya and Beemster, 2006; Ferjani et al., 2007; Tisné et al., 2008), ectopic expression of cell-cycle-related genes (Hemerly et al., 1995; Cockcroft et al., 2000; Wang et al., 2000; De Veylder et al., 2001), or environmental stresses (Granier et al., 2000; West et al., 2004; Aguirrezabal et al., 2006; Cookson et al., 2006, 2007). This compensatory system allows, for example, a decrease in cell number to be balanced totally or partially by an increase in cell size, so that the final size of the organ is maintained. Although mechanisms involved in this process are not well understood, a general consensus exists that cell-autonomous-like programming (e.g. the classical cellular theory; Francis, 1992, 2007; Granier et al., 2000) is not sufficient to systematically account for organ development. Several works support this view and suggest that an unknown, nonautonomous signal from the epidermal layer triggers plant growth in *Arabidopsis* (Savaldi-Goldstein et al., 2007; Savaldi-Goldstein and Chory, 2008; Kawade et al., 2010). It is possible that *GeBP/GPLs* play a role in the restriction of epidermal cell size under conditions of stress.

In addition, the opposite regulation of cell expansion by *GeBP/GPL* and *CPR5* genes could be due to more complex mechanisms. Previous work (Vanacker et al., 2001) has shown that salicylic acid whose level is

increased in *cpr5* mutants can influence cell expansion in opposite manner depending on the genetic background. Therefore we cannot exclude that the *GeBP/GPL* role in cell expansion might be different in other pathogen-resistant mutant backgrounds.

Based on the abnormal composition of cell walls in *cpr5* mutants, Brininstool et al. (2008) suggested that *CPR5* is involved in some cell-wall-related mechanism, as cell wall metabolism plays roles in cell expansion, pathogen response signaling, and cell death (Ellis et al., 2002; Somerville et al., 2004; Ahn et al., 2006). Several genes downstream of *GeBP/GPLs* and *CPR5* are known to play a role in cell elongation or cell wall metabolism, such as the expansin gene family, whose activation is sufficient to induce the entire process of leaf development. This supports the concept of cell-division-independent mechanisms controlling organ development (Pien et al., 2001). Therefore, the cell-wall-related pathways could be controlled by *CPR5* and *GeBP/GPL*, while other aspects, such as cell proliferation, could be regulated by *CPR5* only.

It is interesting to notice that the partial suppression of *cpr5* phenotype by *gebp/gpl* mutations concerns the growth defect phenotype while both *gebp/gpl* and *cpr5* mutants display a similar defense response phenotype. Thus, *GeBP/GPLs* and *CPR5* genes regulate plant and organ size in an opposite manner while they both act as repressors of defense response.

CPR5 Is a Nuclear Protein Involved in Transcription

CPR5 is predicted to be a type IIIa membrane protein with five transmembrane domains and a potential cytoplasmic N-terminal domain that contains a bipartite NLS. While the potential biochemical function of the *CPR5* protein remains speculative, two hypotheses have been proposed to account for the prediction that it represents a membrane protein with a NLS: (1) *CPR5* may be localized in membranes outside the nucleus, and its cytoplasmic domain may be proteolytically cleaved and transported into the nucleus (Kirik et al., 2001), a signaling process for which there is substantial precedent in plants (Seo et al., 2008); or (2) *CPR5* may be targeted to the inner nuclear membrane through an NLS-dependent mechanism (Brininstool et al., 2008), as demonstrated for some proteins in yeast (*Saccharomyces cerevisiae*; Lusk et al., 2007), a hypothesis that does not exclude the possibility that its nucleosolic domain is proteolytically cleaved and participates in transcriptional processes. Our work supports the latter hypothesis, as we show that the full-length *CPR5* protein is localized in the nucleus and the nonmembrane portion of the protein can induce a *cpr5* mutant phenotype when fused to the *VP16* activation domain. Although we cannot exclude that *CPR5* is also present outside the nucleus, this suggests that *CPR5* or its cleavage product might be directly required in the nucleus for the proper functioning of transcriptional processes. This does not imply that *CPR5* is a transcription factor

as it could participate to transcriptional complexes without being involved in transcription regulation per se.

Although the *CPR5* gene has been isolated more than 10 years ago, we have provided here, to our knowledge, the first functional evidence that *CPR5* is a nuclear protein that might play a role in transcriptional processes. In addition, we have tackled the *CPR5*-dependent cell expansion pathway and provide a link between the *CPR5* and *GeBP/GPL* pathways.

In several organisms, highly pleiotropic genes such as *CPR5* participate in many biological processes through distribution of the protein in more cellular components and involvement in more protein-protein interactions compared to nonpleiotropic genes (He and Zhang, 2006; Zou et al., 2008). It will be of interest to determine whether *CPR5* behaves as a shuttling membrane-bound (co)transcription factor interacting with multiple partners and to characterize in detail the molecular link between *CPR5* and *GeBP/GPL* genes.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds were sown on soil or surface sterilized and grown in petri dishes on Murashige and Skoog basal salt mixture medium (Sigma). Plants were grown at 22°C in long-day photoperiod (16 h of 100 μ E light). The *Arabidopsis thaliana* Columbia-0 ecotype was the wild type used. *Agrobacterium tumefaciens* C58 pGV3121 was used for stable and transient transformation of *Arabidopsis* using the floral-dip technique (Clough and Bent, 1998) or transient expression in *Nicotiana benthamiana* leaves (Lavy et al., 2002). The Pcyb1:GUS line was provided by Dr. Murray (Cambridge, UK) and the PcdkA:GUS line was a gift of Dr. Faure (Versailles, France). For GUS staining, plants were vacuum infiltrated and incubated 2 to 12 h with GUS substrate at 37°C and destained as described (Gallagher, 1992).

Microarray Analysis

Plants used for microarray analysis were grown in vitro under long-day conditions for 21 d. Whole rosettes and roots were used. RNA samples were processed in triplicate from pooled samples (6–8 rosettes). Total RNA was prepared using the total RNA isolation kit (Qiagen). Hybridization on chips and data analysis were performed by Partnerchip. Enrichment analyses were done with the singular enrichment analysis tool at AgriGO (<http://bioinfo.cau.edu.cn/agriGO/analysis.php>). Comparative analysis with publicly available microarray data were performed with Genevestigator software (<https://www.genevestigator.com/gv/index.jsp>). Graphic outputs were made with MultiExperiment viewer software (<http://www.tm4.org/>).

The enrichment in Gene Ontology terms was calculated as the ratio of the proportion of genes involved in a pathway at the genome level over the proportion of genes involved in the same pathway in our microarray data. For instance the response-to-stress pathway (GO: 0006950) comprises 456 genes among the 31,819 genes of *Arabidopsis*. Of these 456, 13 genes are found among the 88 misregulated genes in the *gebp/gpl* quadruple mutant. Therefore the enrichment is calculated as the ratio of 13/88 over 456/31,819 = 10.30. A similar enrichment (10.47-fold) is found for the VP16:GPL2 line giving an average of 10.38 enrichment for both type of plants.

Data of *cpr5* microarrays were obtained at the NASCArray Web site (<http://affymetrix.arabidopsis.info/>) under the NASCArrays experiment reference number NASCARRAYS-355.

Real-time RT-PCR was performed as described (Curaba et al., 2003) using the following oligonucleotides: 5'-TCTTGTAGGTGCTCTGTCTTCC-3' and 5'-CAACCCTCTCGTCCCACTGC-3' for *PR1*, 5'-TTGAATTGACTCCAGGTGCTCC-3' and 5'-GCCAGAGTGACGGGAGGAAC-3' for *PR5*, and 5'-AGCCTTACAACGCTACTCTGTCTGTC-3' and 5'-CACCAGACATAGTAG-CAGAAATCAAG-3' for *TUBULIN*.

Semiquantitative RT-PCR for *PR1* was performed as previously described (Chevalier et al., 2008) with 26, 28, and 30 PCR cycles to ensure that the amplification was linear. Cycles 30 and 28 are shown for *PR1* and *ACTIN8*, respectively. The following primers were used: 5'-ATGAATTTACTGGC-TATTCTCGATTTTAAATCG-3' and 5'-TTAGTATGGCTTCTCGTTCACATA-ATTC-3' for *PR1* and 5'-AATCAGATGTGGATCTCTAAGGCA-3' and 5'-TCCGAGTTTGAAGAGGCTAC-AAAC-3' for *ACTIN8*.

Molecular Cloning

The *CPR5* cDNA, kindly provided by Viktor Kirik, was sequenced and cloned as an Acc65I-SalI fragment between the Acc65I and *XhoI* sites of pENTR4 vector (Invitrogen) in frame with attB sites to give p672 vector. The *CPR5* Δ TM version was made by cloning the Acc65I-BsrBI fragment of *CPR5* between the Acc65I and *EcoRV* sites of the pENTR4 vector to give p673. Translational fusions with GFP were made using the pK7WGF2.0 destination vector (Plant Systems Biology, Vlaams Interuniversitair Instituut voor Biotechnologie-Ghent University) using the attL-attR (LR) clone (Invitrogen) with entry vectors p672 and p673 to give p680 and p674, respectively. Translational fusions with the VP16 activation domain were made using Alligator1 (Bensmihen et al., 2004), kindly provided by François Parcy (Grenoble, France) using the LR clone with entry vectors p672 and p673 to give p677 and p681, respectively. Expression of VP16 fusion proteins in transgenic lines was assessed by western blot with a monoclonal anti-HA antibody (Roche) while the loading control ketol-acid reducto-isomerase (KARI) protein was detected with a polyclonal antibody (Dumas et al., 2001).

The N-terminal deletion in *GeBP* was done by deleting the *EcoRI-BamHI* region in the gateway entry vector p497 and ligating the adapter map by annealing the two following oligonucleotides: AATTCAAGAAGAAATTGG and GATCCAATTCTCTCTTG. This deletion removes the first 55 amino acids and was subsequently cloned in the VP16 destination vector as previously described (Chevalier et al., 2008).

Tissue Preparation and Microscopy

For confocal microscopy analysis, p680 and p674 expression vectors were introduced separately into *Agrobacterium* and independently infiltrated into tobacco (*Nicotiana tabacum*) leaves as previously described (Lavy et al., 2002) except that 4,6-diamidinophenylindole was added to the cell suspension at 1 μ g mL⁻¹ before infiltration. Observations were made with a Leica confocal microscope and data were analyzed with the Leica LCS 2.61 software. Laser excitation was done in the sequential mode in between frames first with an argon laser (515 nm) and then with a UV laser (351–364 nm). Spectra were analyzed to check the specificity of GFP emission.

Trichome nuclear DNA contents were measured as described previously by Brininstool et al. (2008). Images were captured with a SensiCam QE 12-bit, cooled CCD camera, and analyzed with Slidebook software from 3I. Care was taken when setting image capture parameters that the nuclei with the highest DNA content in a group of samples did not saturate the dynamic range of the images. Samples were normalized to a wild-type control, assuming a mean value of 32C for wild-type trichome nuclei. Nonparametric statistics (Kruskal-Wallis one-way ANOVA and Dunn's all pairwise multiple comparison) were performed using SigmaStat.

For flow cytometric analysis, the third pair of rosette leaves from 3-week-old plants grown on soil were used. Tissues were chopped with a razor blade in 900 μ L of 45 mM MgCl₂, 30 mM sodium citrate, 20 mM MOPS, pH 7, and 0.1% Triton X-100 supplemented with 1% polyvinylpyrrolidone 10,000, 5 mM metabisulfite, and 5 μ g mL⁻¹ RNase from a stock solution at 50 units/mg. Propidium iodide was added to the filtered supernatants at 50 μ g mL⁻¹. The nuclei were analyzed with the CyFlow SL cytometer using FloMax (Partec) software, or with an EPICS/ELITE cytometer and its software from Beckman-Coulter.

Trypan blue staining of cells was done as previously described (Lu et al., 2009). For aphidicolin sensitivity assay, plants were grown in vitro for 3 weeks in the absence or presence of aphidicolin at 12 μ g mL⁻¹ as described previously (De Schutter et al., 2007).

For leaf area measurements and cell number determination, leaves were harvested at 21 d after sowing, cleared overnight in ethanol, stored in lactic acid for microscopy, and observed with a microscope fitted with differential interference contrast optics (Leica). The total leaf blade area was determined from images taken with a digital camera (Zeiss) mounted on a binocular (Leica). Total number of pavement and guard cells, from which the average

cell area was calculated, were obtained from at least 40 pictures of the adaxial epidermis located 25% and 75% from the distance between the tip and the base of the leaf, halfway between the midrib and the leaf margin. The total number of cells per leaf was estimated by dividing the leaf area by the average cell area. Epidermal cell size measurement was done using ImageJ software. Palisade cells were treated as previously described (Horiguchi et al., 2006) and cell size measurement was done using ImageJ software.

Pathogen Assay

The *Pst* DC3000 was grown at 29°C on King's B medium supplemented with rifampicin 50 µg mL⁻¹. Four- or 5-week-old plants were used for bacterial inoculation. For this objective, they were kept at high humidity 12 h before experiments for syringe infiltration facilities and then grown under daylight under the following conditions: 9-h light/15-h dark and 40%/70% humidity. Leaves without lesions from plant developing lesions were infiltrated with a bacterial suspension of 2 × 10⁵ colony forming units mL⁻¹. Determination of in planta bacterial growth was performed as previously described (Lorrain et al., 2004).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Overlaps between *cpr5*, *gebp/gpl1,2,3*, *VP16:GPL2*, and stress-induced transcriptomic alterations.

Supplemental Figure S2. A stable form of the founding member *GeBP* fused to the *VP16* domain mimics the *VP16:GPL2* phenotype.

Supplemental Figure S3. DNA level in trichomes of wild-type, *cpr5*, and *VP16:GPL2* plants.

Supplemental Figure S4. Expression of cell cycle markers in the *VP16:GPL2* background.

Supplemental Figure S5. Size of palisade cells in leaves of wild-type and mutant plants.

Supplemental Table S1. List of misregulated genes in *gebp gpl1,2,3* quadruple mutant.

Supplemental Table S2. List of misregulated genes in *VP16:GPL2* plants.

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LITERATURE CITED

- Aguirrezabal L, Bouchier-Combaud S, Radziejwoski A, Dauzat M, Cookson SJ, Granier C (2006) Plasticity to soil water deficit in Arabidopsis thaliana: dissection of leaf development into underlying growth dynamic and cellular variables reveals invisible phenotypes. *Plant Cell Environ* 29: 2216–2227
- Ahn JW, Verma R, Kim M, Lee JY, Kim YK, Bang JW, Reiter WD, Pai HS (2006) Depletion of UDP-D-apiose/UDP-D-xylose synthases results in rhamnogalacturonan-II deficiency, cell wall thickening, and cell death in higher plants. *J Biol Chem* 281: 13708–13716
- Aki T, Konishi M, Kikuchi T, Fujimori T, Yoneyama T, Yanagisawa S (2007) Distinct modulations of the hexokinase1-mediated glucose response and hexokinase1-independent processes by HYS1/CPR5 in Arabidopsis. *J Exp Bot* 58: 3239–3248
- Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, et al (2000) Gene ontology: tool for the unification of biology. *Nat Genet* 25: 25–29
- Autran D, Jonak C, Belcram K, Beeemster GT, Kronenberger J, Grandjean O, Inzé D, Traas J (2002) Cell numbers and leaf development in Arabidopsis: a functional analysis of the STRUWELPETER gene. *EMBO J* 21: 6036–6049
- Bensmihen S, To A, Lambert G, Kroj T, Giraudat J, Parcy F (2004) Analysis of an activated ABI5 allele using a new selection method for transgenic Arabidopsis seeds. *FEBS Lett* 561: 127–131
- Boch J, Verbsky L, Robertson L, Larkin J, Kunkel B (1998) Analysis of resistance gene-mediated defense responses in Arabidopsis thaliana plants carrying a mutation in CPR5. *Mol Plant Microbe Interact* 11: 1196–1206
- Bowling SA, Clarke JD, Liu Y, Klessig DE, Dong X (1997) The *cpr5* mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell* 9: 1573–1584
- Bowling SA, Guo A, Cao H, Gordon AS, Klessig DE, Dong X (1994) A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. *Plant Cell* 6: 1845–1857
- Brininstool G, Kasili R, Simmons LA, Kirik V, Hülskamp M, Larkin JC (2008) Constitutive Expressor Of Pathogenesis-Related Genes5 affects cell wall biogenesis and trichome development. *BMC Plant Biol* 8: 58
- Busov VB, Brunner AM, Strauss SH (2008) Genes for control of plant stature and form. *New Phytol* 177: 589–607
- Calikowski TT, Meulia T, Meier I (2003) A proteomic study of the Arabidopsis nuclear matrix. *J Cell Biochem* 90: 361–378
- Chevalier F, Perazza D, Laporte F, Le Héaniff G, Hornitschek P, Bonneville JM, Herzog M, Vachon G (2008) GeBP and GeBP-like proteins are noncanonical leucine-zipper transcription factors that regulate cytokinin response in Arabidopsis. *Plant Physiol* 146: 1142–1154
- Churchman ML, Brown ML, Kato N, Kirik V, Hülskamp M, Inzé D, De Veylder L, Walker JD, Zheng Z, Oppenheimer DG, et al (2006) SIAMESE, a plant-specific cell cycle regulator, controls endoreplication onset in Arabidopsis thaliana. *Plant Cell* 18: 3145–3157
- Clarke JD, Volko SM, Ledford H, Ausubel FM, Dong X (2000) Roles of salicylic acid, jasmonic acid, and ethylene in cpr-induced resistance in Arabidopsis. *Plant Cell* 12: 2175–2190
- Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J* 16: 735–743
- Cockcroft CE, den Boer BG, Healy JM, Murray JA (2000) Cyclin D control of growth rate in plants. *Nature* 405: 575–579
- Cookson SJ, Chenu K, Granier C (2007) Day length affects the dynamics of leaf expansion and cellular development in Arabidopsis thaliana partially through floral transition timing. *Ann Bot (Lond)* 99: 703–711
- Cookson SJ, Radziejwoski A, Granier C (2006) Cell and leaf size plasticity in Arabidopsis: what is the role of endoreduplication? *Plant Cell Environ* 29: 1273–1283
- Curaba J, Herzog M, Vachon G (2003) GeBP, the first member of a new gene family in Arabidopsis, encodes a nuclear protein with DNA-binding activity and is regulated by KNAT1. *Plant J* 33: 305–317
- De Schutter K, Joubès J, Cools T, Verkest A, Corellou F, Babiychuk E, Van Der Schueren E, Beeckman T, Kushnir S, Inzé D, et al (2007) Arabidopsis WEE1 kinase controls cell cycle arrest in response to activation of the DNA integrity checkpoint. *Plant Cell* 19: 211–225
- De Veylder L, Beeckman T, Beeemster GT, Krols L, Terras F, Landrieu I, van der Schueren E, Maes S, Naudts M, Inzé D (2001) Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis. *Plant Cell* 13: 1653–1668
- Disch S, Anastasiou E, Sharma VK, Laux T, Fletcher JC, Lenhard M (2006) The E3 ubiquitin ligase BIG BROTHER controls Arabidopsis organ size in a dosage-dependent manner. *Curr Biol* 16: 272–279
- Dumas R, Biou V, Halgand F, Douce R, Duggleby RG (2001) Enzymology, structure, and dynamics of acetohydroxy acid isomeroreductase. *Acc Chem Res* 34: 399–408
- Ellis C, Karafyllidis I, Wasternack C, Turner JG (2002) The Arabidopsis mutant cev1 links cell wall signaling to jasmonate and ethylene responses. *Plant Cell* 14: 1557–1566
- Ferjani A, Horiguchi G, Yano S, Tsukaya H (2007) Analysis of leaf development in fugu mutants of Arabidopsis reveals three compensation modes that modulate cell expansion in determinate organs. *Plant Physiol* 144: 988–999
- Francis D (1992) The cell cycle in plant development. *New Phytol* 122: 1–20

- Francis D (2007) The plant cell cycle—15 years on. *New Phytol* **174**: 261–278
- Gagnot S, Tamby JP, Martin-Magniette ML, Bitton F, Tacconat L, Balzergue S, Aubourg S, Renou JP, Lecharny A, Brunaud V (2008) CATdb: a public access to Arabidopsis transcriptome data from the URGV-CATMA platform. *Nucleic Acids Res (Database issue)* **36**: D986–D990
- Gallagher SR (1992) Quantitation of GUS activity by fluorometry. In SR Gallagher, ed, *GUS Protocols Using the GUS Gene as a Reporter of Gene Expression*. Academic Press, Inc., San Diego, pp 47–59
- Granier C, Turc O, Tardieu F (2000) Co-ordination of cell division and tissue expansion in sunflower, tobacco, and pea leaves: dependence or independence of both processes? *J Plant Growth Regul* **19**: 45–54
- He X, Zhang J (2006) Toward a molecular understanding of pleiotropy. *Genetics* **173**: 1885–1891
- Hemerly A, Engler JdeA, Bergounioux C, Van Montagu M, Engler G, Inzé D, Ferreira P (1995) Dominant negative mutants of the Cdc2 kinase uncouple cell division from iterative plant development. *EMBO J* **14**: 3925–3936
- Horiguchi G, Fujikura U, Ferjani A, Ishikawa N, Tsukaya H (2006) Large-scale histological analysis of leaf mutants using two simple leaf observation methods: identification of novel genetic pathways governing the size and shape of leaves. *Plant J* **48**: 638–644
- Hu Y, Xie Q, Chua NH (2003) The *Arabidopsis* auxin-inducible gene ARGOS controls lateral organ size. *Plant Cell* **15**: 1951–1961
- Igari K, Endo S, Hibara KI, Aida M, Sakakibara H, Kawasaki T, Tasaka M (2008) Constitutive activation of a CC-NB-LRR protein alters morphogenesis through the cytokinin pathway in *Arabidopsis*. *Plant J* **55**: 14–27
- Jing HC, Anderson L, Sturre MJ, Hille J, Dijkwel PP (2007) *Arabidopsis* CPR5 is a senescence-regulatory gene with pleiotropic functions as predicted by the evolutionary theory of senescence. *J Exp Bot* **58**: 3885–3894
- Jing HC, Dijkwel PP (2008) CPR5: a jack of all trades in plants. *Plant Signal Behav* **3**: 562–563
- Jing HC, Hebel R, Oeljeklaus S, Sitek B, Stühler K, Meyer HE, Sturre MJ, Hille J, Warscheid B, Dijkwel PP (2008) Early leaf senescence is associated with an altered cellular redox balance in *Arabidopsis* cpr5/old1 mutants. *Plant Biol (Stuttg) (Suppl 1)* **10**: 85–98
- Jing HC, Sturre MJ, Hille J, Dijkwel PP (2002) *Arabidopsis* onset of leaf death mutants identify a regulatory pathway controlling leaf senescence. *Plant J* **32**: 51–63
- John PC, Qi R (2008) Cell division and endoreduplication: doubtful engines of vegetative growth. *Trends Plant Sci* **13**: 121–127
- Kawade K, Horiguchi G, Tsukaya H (2010) Non-cell-autonomously coordinated organ size regulation in leaf development. *Development* **137**: 4221–4227
- Kirik V, Bouyer D, Schöbinger U, Bechtold N, Herzog M, Bonneville JM, Hülskamp M (2001) CPR5 is involved in cell proliferation and cell death control and encodes a novel transmembrane protein. *Curr Biol* **11**: 1891–1895
- Lavy M, Bracha-Drori K, Sternberg H, Yalovsky S (2002) A cell-specific, prenylation-independent mechanism regulates targeting of type II RACs. *Plant Cell* **14**: 2431–2450
- Lorrain S, Lin B, Auria MC, Kroj T, Saindrenan P, Nicole M, Balagué C, Roby D (2004) Vascular associated death1, a novel GRAM domain-containing protein, is a regulator of cell death and defense responses in vascular tissues. *Plant Cell* **16**: 2217–2232
- Lu H, Salimian S, Gamelin E, Wang G, Fedorowski J, LaCourse W, Greenberg JT (2009) Genetic analysis of *acd6-1* reveals complex defense networks and leads to identification of novel defense genes in *Arabidopsis*. *Plant J* **58**: 401–412
- Lusk CP, Blobel G, King MC (2007) Highway to the inner nuclear membrane: rules for the road. *Nat Rev Mol Cell Biol* **8**: 414–420
- Memelink J, Hoge JH, Schilperoort RA (1987) Cytokinin stress changes the developmental regulation of several defence-related genes in tobacco. *EMBO J* **6**: 3579–3583
- Mizukami Y (2001) A matter of size: developmental control of organ size in plants. *Curr Opin Plant Biol* **4**: 533–539
- Parcy F, Bomblies K, Weigel D (2002) Interaction of LEAFY, AGAMOUS and TERMINAL FLOWER1 in maintaining floral meristem identity in *Arabidopsis*. *Development* **129**: 2519–2527
- Pien S, Wyrzykowska J, McQueen-Mason S, Smart C, Fleming A (2001) Local expression of expansin induces the entire process of leaf development and modifies leaf shape. *Proc Natl Acad Sci USA* **98**: 11812–11817
- Rotter A, Usadel B, Baebler S, Stitt M, Gruden K (2007) Adaptation of the MapMan ontology to biotic stress responses: application in solanaceous species. *Plant Methods* **3**: 10
- Savaldi-Goldstein S, Chory J (2008) Growth coordination and the shoot epidermis. *Curr Opin Plant Biol* **11**: 42–48
- Savaldi-Goldstein S, Peto C, Chory J (2007) The epidermis both drives and restricts plant shoot growth. *Nature* **446**: 199–202
- Seo PJ, Kim SG, Park CM (2008) Membrane-bound transcription factors in plants. *Trends Plant Sci* **13**: 550–556
- Somerville C, Bauer S, Brininstool G, Facette M, Hamann T, Milne J, Osborne E, Paredez A, Persson S, Raab T, et al (2004) Toward a systems approach to understanding plant cell walls. *Science* **306**: 2206–2211
- Sugimoto-Shirasu K, Roberts K (2003) “Big it up”: endoreduplication and cell-size control in plants. *Curr Opin Plant Biol* **6**: 544–553
- Thimm O, Bläsing O, Gibon Y, Nagel A, Meyer S, Krüger P, Selbig J, Müller LA, Rhee SY, Stitt M (2004) MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *Plant J* **37**: 914–939
- Tisné S, Reymond M, Vile D, Fabre J, Dauzat M, Koornneef M, Granier C (2008) Combined genetic and modeling approaches reveal that epidermal cell area and number in leaves are controlled by leaf and plant developmental processes in *Arabidopsis*. *Plant Physiol* **148**: 1117–1127
- Tsukaya H, Beemster GT (2006) Genetics, cell cycle and cell expansion in organogenesis in plants. *J Plant Res* **119**: 1–4
- Vanacker H, Lu H, Rate DN, Greenberg JT (2001) A role for salicylic acid and NPR1 in regulating cell growth in *Arabidopsis*. *Plant J* **28**: 209–216
- Wang H, Zhou Y, Gilmer S, Whitwill S, Fowke LC (2000) Expression of the plant cyclin-dependent kinase inhibitor ICK1 affects cell division, plant growth and morphology. *Plant J* **24**: 613–623
- West G, Inzé D, Beemster GT (2004) Cell cycle modulation in the response of the primary root of *Arabidopsis* to salt stress. *Plant Physiol* **135**: 1050–1058
- Xia Y, Yu K, Navarre D, Seebold K, Kachroo A, Kachroo P (2010) The *glabra1* mutation affects cuticle formation and plant responses to microbes. *Plant Physiol* **154**: 833–846
- Yoshida S, Ito M, Nishida I, Watanabe A (2002) Identification of a novel gene HYS1/CPR5 that has a repressive role in the induction of leaf senescence and pathogen-defence responses in *Arabidopsis thaliana*. *Plant J* **29**: 427–437
- Zeller G, Henz SR, Widmer CK, Sachsenberg T, Ratsch G, Weigel D, Laubinger S (2009) Stress-induced changes in the *Arabidopsis thaliana* transcriptome analyzed using whole-genome tiling arrays. *Plant J* **58**: 1068–1082
- Zou L, Sriswasdi S, Ross B, Missiuro PV, Liu J, Ge H (2008) Systematic analysis of pleiotropy in *C. elegans* early embryogenesis. *PLoS Comput Biol* **4**: e1000003

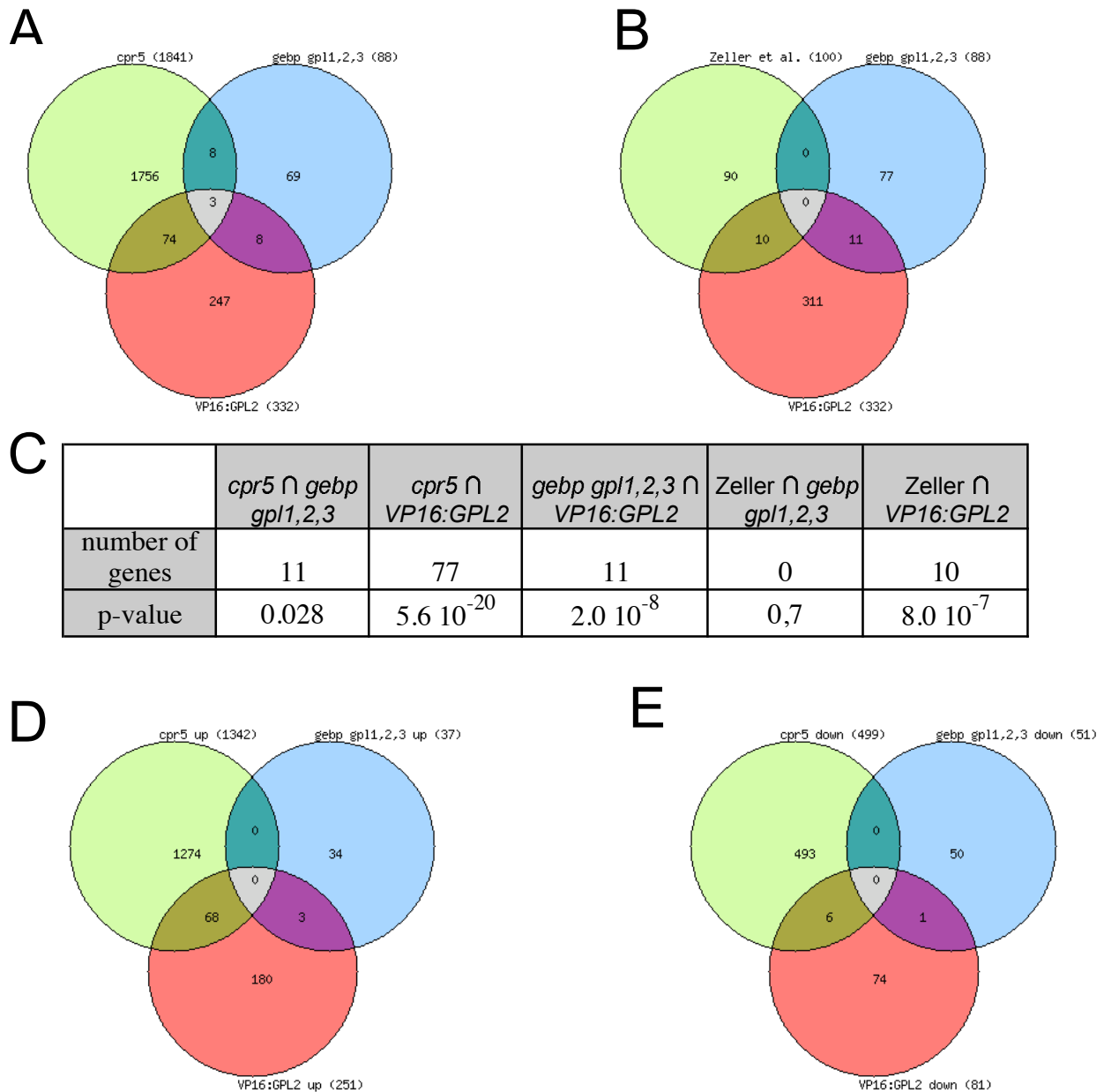


Figure S1: Overlaps between *cpr5*, *gebp/gpl1,2,3*, *VP16:GPL2* and stress-induced transcriptomic alterations. A, Overlaps between *cpr5*, *gebp/gpl1,2,3* and *VP16:GPL2* transcriptomic alterations. B, Overlaps between *gebp/gpl1,2,3*, *VP16:GPL2* and stress-induced transcriptomic alterations. The top-100 genes involved in multiple abiotic stress-induced transcriptomic alterations were defined in Zeller et al. (2009). C, Hypergeometric test *p*-values of the Venn intersections. Intersections between *cpr5* and *gebp/gpl* or *VP16:GPL2* data have lower *p*-values than intersections between universal stress genes (Zeller et al.) and *gebp/gpl* or *VP16:GPL2* data. This indicates that the *GeBP/GPL* pathway is closer to the *CPR5* pathway than to a general stress-related pathway. D and E, Overlaps between up (D) and down (E)-regulated genes assessing the opposite regulation between *cpr5* and *gebp/gpl* mutant. Three-way Venn diagrams were generated online (<http://www.pangloss.com/seidel/Protocols/venn.cgi>).

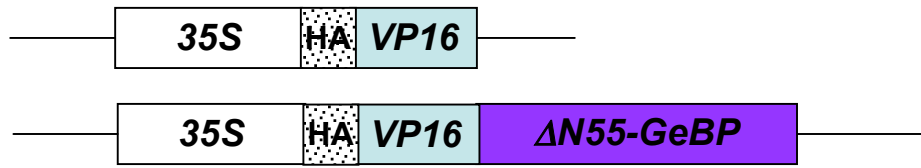
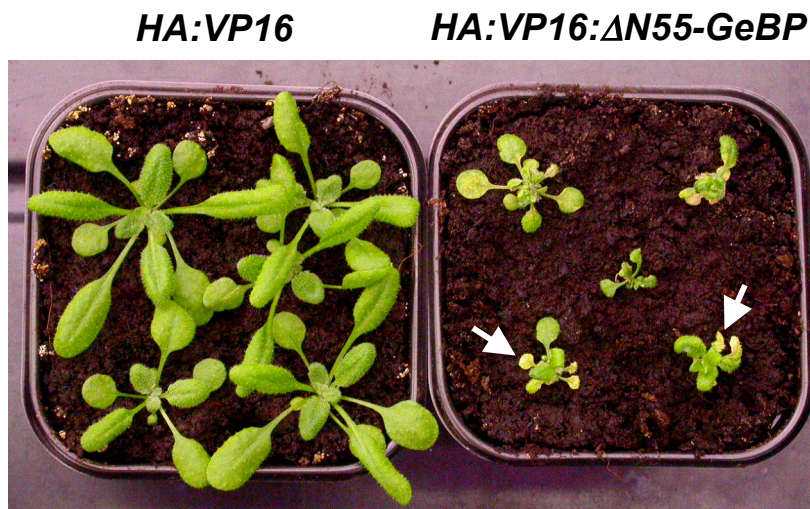
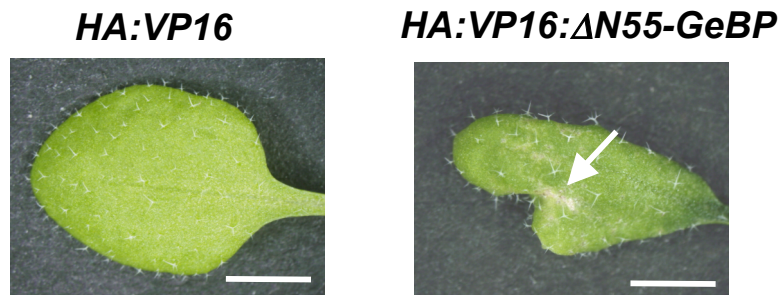
A**B****C**

Figure S2: A stable form of the founding member *GeBP* fused to the *VP16* domain mimics the *VP16:GPL2* phenotype. A, Schematic representations of the empty *VP16* construct and of the *VP16:ΔN55-GeBP* construct. The N-terminal 55 amino acids deleted in the $\Delta N55$ -*GeBP* protein show several potential post-translational modification sites (data not shown) while the *GPL2* protein does not. B, Phenotype of transgenic lines. Plants expressing the *VP16:ΔN55-GeBP* protein showed strong developmental defects similar to *VP16:GPL2* lines such as dwarfism, necrotic lesions and early senescence (arrows). The expression of the *VP16:ΔN55-GeBP* protein was assessed in western blots using an antibody against the HA epitope (data not shown). Transgenic lines were grown on soil in long days for 25 days. C, Necrotic lesions on *VP16:ΔN55-GeBP* plants. Fourth leaves of 2-week-old seedlings are shown. Necrotic lesions (arrow) and abnormal development are observed on *VP16:ΔN55-GeBP* leaves. Scale Bars: 2.5 mm.

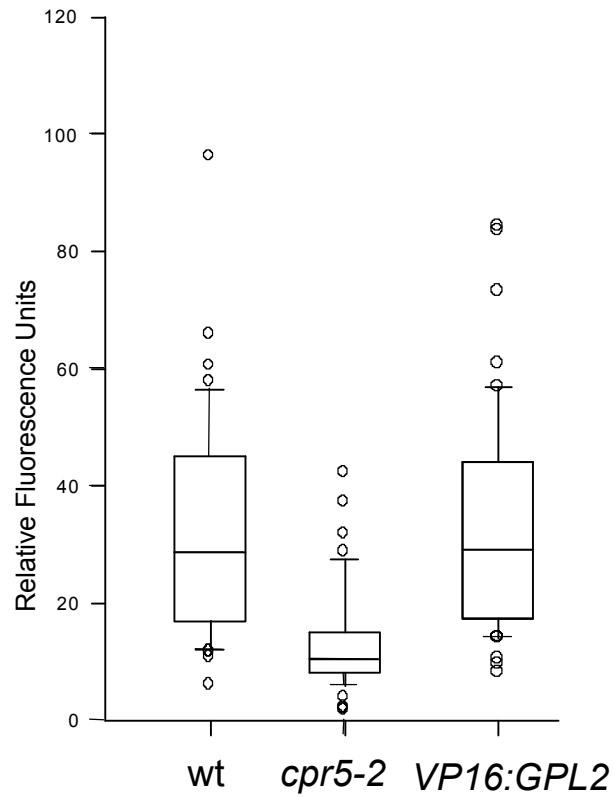


Figure S3: DNA level in trichomes of wild-type, *cpr5-2* and *VP16:GPL2* plants. Each genotype tested is represented as a box according to the Repeated Measures ANOVA on Ranks test. The ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles. *VP16:GPL2* trichomes are indistinguishable from wild-type trichomes while *cpr5-2* trichomes displays a lower DNA content as already reported.

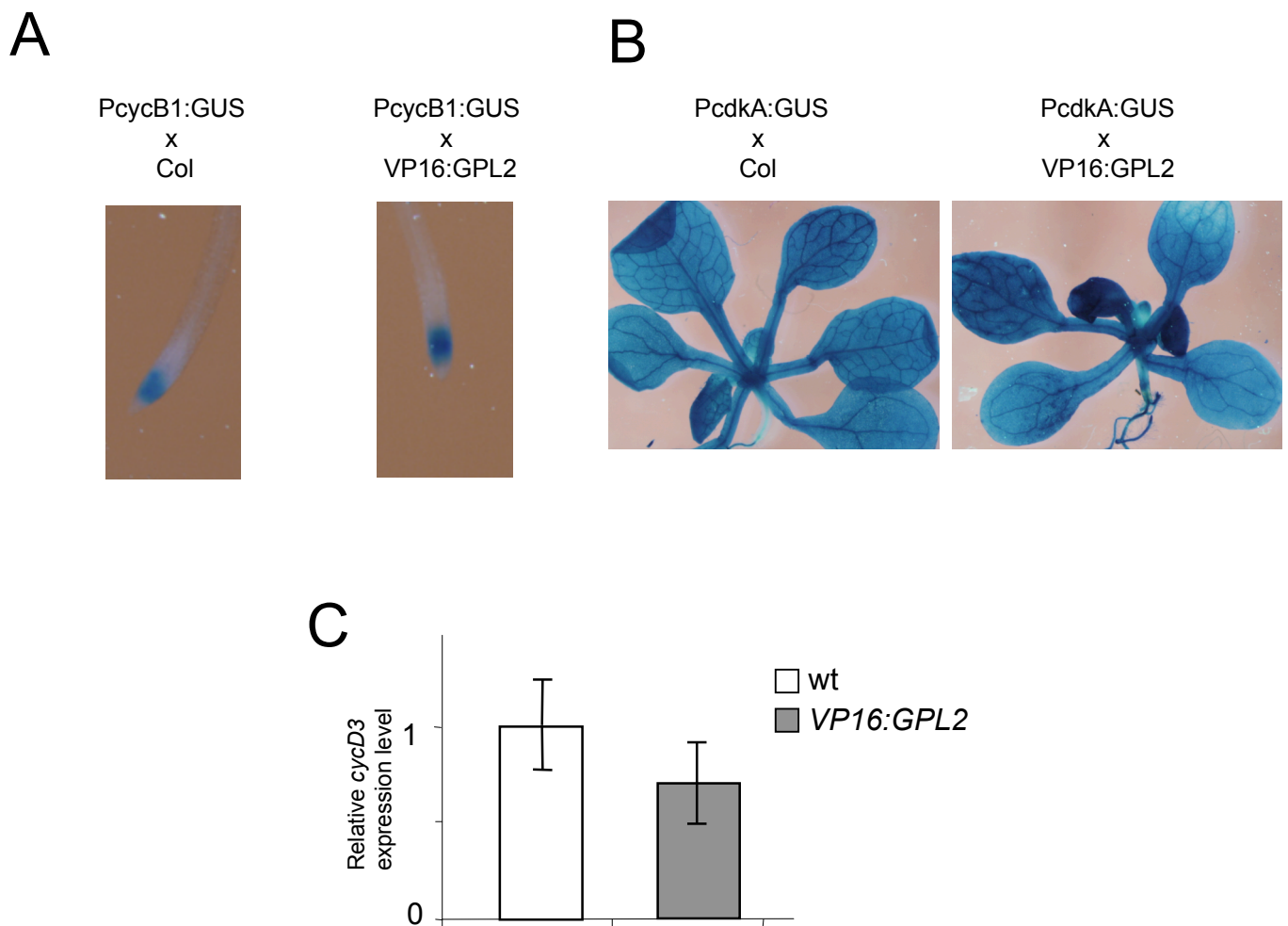
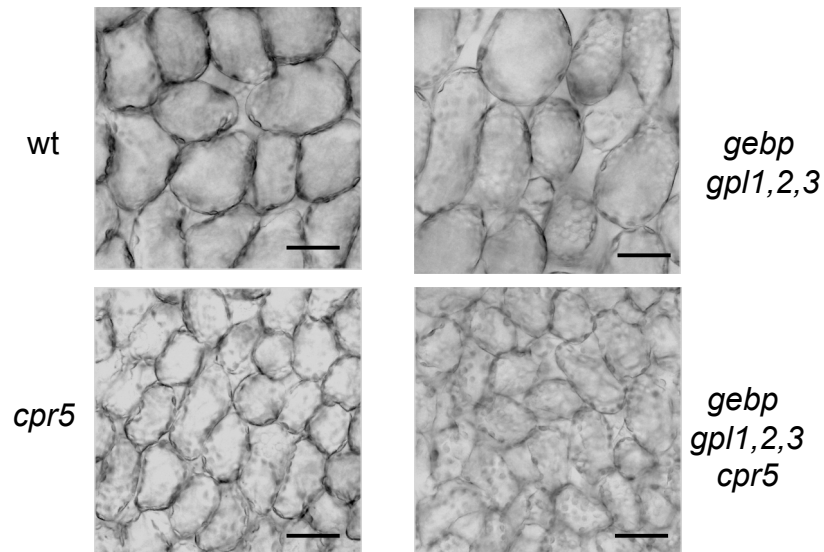


Figure S4: Expression of cell cycle marker genes in the *VP16:GPL2* background. A, Homozygous lines carrying transcriptional fusions between *uidA* and *cycB1* promoter were crossed to wild-type (left) or *VP16:GPL2* plants (right). B, Homozygous lines carrying transcriptional fusions between *uidA* and *cdkA* promoter were crossed to wild-type (left) or *VP16:GPL2* plants (right). The slightly more intense staining in the *VP16:GPL2* background observed in A and B panels is presumably due to the slower developmental rate compared to wt. C, Relative transcript level of *cycD3* in Col and *VP16:GPL2* lines. Similar transcript levels were observed in both backgrounds. Mean levels from three replicats are shown. All plants were grown *in vitro* for 18 days.

A



B

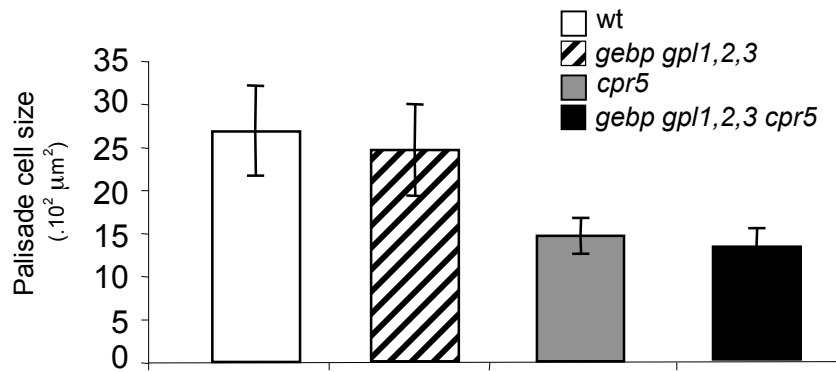


Figure S5: Size of palisade cells in leaves of wt and mutant plants.

A, Palisade cells of wt and mutants. Scale bars: 50 μm . B, Measurement of palisade cell size (Col, n=160; quadruple mutant, n=160; *cpr5*, n=140; quintuple mutant, n=140). Samples were prepared as previously described (Horiguchi et al., 2006) from third leaves of 3-week-old plants grown on soil.