Highly expressed cell wall genes contribute to robustness of sepal size

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Abstract
Reproducibility in organ size and shape is a fascinating trait of living organisms. The mechanisms underlying such robustness remain, however, to be elucidated. Taking the sepal of Arabidopsis as a model, we investigated whether variability of gene expression plays a role in variation of organ morphology. Previous work from our team identified cell-wall related genes as being enriched among the genes whose expression is highly variable. We then hypothesized that the variation of measured morphological parameters in cell-wall related single knockout mutants could be correlated with the variation in gene expression of the corresponding gene (the knocked-out gene) in wild-type plants. We analyzed sepal morphology from 16 cell-wall mutants and found that, unexpectedly, the higher a gene is expressed in wild type, the more variable the size of the corresponding mutant. These findings support a contribution of cell-wall related genes to the robustness of sepal size.

Keywords
Cell wall mutants, Morphological robustness, Arabidopsis sepal, Gene expression variability

Introduction
Organisms of the same species typically exhibit a remarkably reproducible morphological development despite high variability at the cellular level. The invariant expression of phenotype in the face of environmental and/or genetic perturbations, commonly referred to as “robustness” (Félix & Barkoulas, 2015), is indeed an important characteristic of living beings (Schmalhausen, 1949; Waddington, 1953, 1959). In plants, molecular mechanisms have been found to modulate morphogenetic robustness to environmental perturbations, as in the case of heat-shock proteins (Queitsch et al., 2002), and also to genetic changes such as whole genome duplications (Lachowiec et al., 2016). Robustness, however, also refers to developmental stability despite systemic internal noise (Alvarez-Buylla et al., 2008). Indeed, gene expression has an important stochastic component attributed to a combination of external and internal noise (Elowitz et al. 2002). In a multicellular context, such as that of Arabidopsis plants, gene expression appears to be extremely variable in time and space (Joseph et al., 2015; Araújo et al., 2017; Meyer et al., 2017). In fact, when measuring variability in gene expression at the whole-organism level, there are some genes that exhibit very high variability between individuals. This variability itself has been observed to differ between day and night, for example, in Arabidopsis seedlings (Cortijo et al. 2019) and between developmental stages in *C.*

elegans (Zalts & Yanai, 2017) and Drosophila (Liu et al., 2020). Development is, then, not only robust to gene expression variability, but also, possibly dependent on it.

In a recent paper, Hartasánchez et al. (2023) used the sepal of wild-type Arabidopsis plants to identify modules of co-expressed genes which co-vary with sepal morphology. Cell-wall related genes were found to be over-represented in two of these modules. In addition, highly variable genes were also enriched in cell-wall related genes. Building upon these results, we wanted to check if cell-wall related genes could be involved in the robustness of sepal morphology. We selected a sample of 16 genes and we studied the corresponding mutants to evaluate the variability of their sepal morphology in relation to the variability of expression of the corresponding gene in the wild-type sepal. Our results reveal a positive correlation between the level of expression of the genes in wild type and variability of morphology in the corresponding mutants. Altogether, our work supports a contribution of highly expressed cell-wall related genes to the robustness of sepal size.

Materials and methods

Plant Material

Col-0 Arabidopsis plants were grown on soil at 20°C in short day conditions (8 h light/16 h dark) for 20 days before being transferred to long day conditions (16 h light/8 h darkness). Sepals were dissected from secondary inflorescences after at least 10 siliques were formed. We assessed the final shape of sepals (stage 13, according to Smyth et al. 1990).

Mutants

Mutant seeds (from Col-0 background) were obtained from well-characterized stocks (Desnos et al., 1996; Bringmann et al., 2012; Sénéchal, 2013; Endler et al., 2015) or from SAIL and SALK collections maintained at NASC (McElver et al., 2001; Alonso et al., 2003) as described in Supplementary Table 1. Mutant plants were genotyped following O'Malley et al. (2015), except for csi1-3 and prc1 which have distinctive phenotypes.

Extraction of morphological parameters from mutant lines

We analyzed sepal contours and quantified area, length, width, and aspect ratio following Hong et al. (2016). Briefly, we flattened the sepals between two slides and took photographs with a black background under a dissecting microscope. We used Python scripts (Van Rossum & Drake, 2009) to segment and align sepals, and to extract morphological parameters (Hong et al., 2016).

Data analysis

Raw data consisting of measurements of length, width, area, and aspect ratio for each mutant and for control plants were analyzed (Supplementary Tables 2 & 3). The difference between mutant and wild type for each morphological parameter was calculated as: (mean(mutantParameter)-mean(controlBatchParameter))/mean(controlBatchParameter). The squared coefficient of variation for each parameter was calculated as: (sd(mutantParameter)/mean(mutantParameter))^2. The average gene expression for each mutant gene in wild type was obtained from Hartasánchez et al. (2023) (Supplementary Table 4). Source data for Figures 1 & 2 are shown in Supplementary Table 5.

Results

To explore the link between cell-wall related gene expression and robustness of sepal morphology, we decided to exploit an unpublished mutant dataset previously produced in our team (Dumond, 2017). This mutant dataset had been generated in the context of sepal phenotype exploration and contained data for 16 of the 718 cell-wall related genes expressed in sepal (Table 1). The mutants had been selected based on relatively higher expression of the corresponding genes in sepals compared to other floral organs at stage 12 (Schmid et al., 2005) and on availability of mutants with T-DNA insertions in exons (we considered one mutant allele for each of these genes). Because it is difficult to compare developmental stages between mutant and wild-type plants, we focused on sepals that had ceased growing (stage 13). For each of these mutants, we had obtained length, width, area, and aspect ratio from 39 to 90 sepals. For the level of expression of the corresponding genes in wild-type sepals, we reasoned that transcriptome of sepals at stage 11 would be a good predictor of the final size of sepals (stage 13) because stage 11 precedes growth cessation. Accordingly, we used the data generated by Hartasánchez et al. (2023) composed of transcriptomes of 27 individual sepals (stage 11) from Col-0 background generated by bulk RNA-seq.

Given that previous results show that cell-wall related genes are highly variable (Hartasánchez et al., 2023), we first hypothesized that the level of expression of a given gene in wild-type plants could predict the effect on morphology of the corresponding knockout mutant. We thus examined if knockout mutants of genes with higher expression in wild-type plants exhibited a stronger phenotype (difference in morphological parameters compared to wild type) than genes with lower expression (Figure 1 for area; Figure 2 for length, width and aspect ratio). Our data, however, shows no correlation between the morphology of mutant lines and the level of expression of the corresponding gene in wild-type plants (Figure 1, top right).

We then hypothesized that genes with higher variability in wild type would tend to be more important for robust morphogenesis, and hence, when knocked out, would have a stronger effect on sepal average morphology or on variability in morphology. Again, this was not supported by the data (Figure 1, left column). Finally, we hypothesized that knocking down genes highly expressed in wild type would prove more difficult to cope with than for genes with lower expression. Accordingly, mutant phenotypic variability would correlate with gene expression in wild type. Indeed, there is a significant correlation between the coefficient of variability (CV^2) of the mutant phenotypes (for area, length and width, but not aspect ratio) and the log mean expression in the wild type of the corresponding knocked out genes (Figure 1 bottom right and Figure 2), supporting the latter hypothesis. The correlations shown are quite robust to removal of one or several mutants from the dataset. Our results indicate that the expression level of a cell-wall related gene in wild type correlates with variability (and not average effect) in area, length and width of the corresponding mutant.

Discussion

Although some signaling pathways regulating organ size and shape have been identified (reviewed in Powell & Lenhard, 2012), the mechanisms behind morphological robustness have remained elusive. More recently, a screen for mutants that disrupted the robustness of sepal size and shape led to the identification of genes involved in reproducibility of sepal morphogenesis (Hong et al., 2016; Zhu et al., 2020; Xu et al., 2024). This work revealed that spatio-temporal averaging of cellular variability, precise timing of organ initiation, and growth balance between cell layers, are required for precision in organ size and shape. The mechanisms underlying organ robustness, however, remain largely enigmatic.

Robustness of organ size and shape is thought to be the result of the complex interaction of genes within gene regulatory networks and environmental cues. Gene expression variability can be considered, *a priori*, as a factor affecting robustness. Hong et al. (2016) reported that cell-to-cell variability of growth is required for robustness of sepal shape and size, opening the question of the role of variability in gene expression in this process. Trinh et al. (2023) found that an increase in variability of gene expression impaired robustness of sepal shape and size. To further assess the link between gene expression variability and robustness, we have evaluated sepal morphology in cell-wall related mutants. Therefore, we used mutants, not to search for candidate genes involved in morphology, but to test the way in which knockout mutants of genes putatively involved in robustness, such as cell-wall related genes, affected morphology. Our results show that knockouts of highly expressed genes are associated with an increased phenotypic variability. In particular, highly-expressed cell-wall related genes in wild-type plants exhibit larger morphological variation when knocked out than less expressed genes, although levels of active proteins do not necessarily correlate with RNA levels.

We can speculate that the effect caused by knocking out highly expressed cell-wall genes is more difficult to buffer, compared to buffering the effects of lowly expressed cell-wall genes. There is knowledge about capacitors of phenotypic variation, such as heat-shock protein HSP90 (Queitsch et al., 2002), which limits the manifestation of cryptic genetic variation allowing for developmental stability (Sangster et al., 2008). Additionally, the effect of the mutation of a gene can be compensated by changes in the expression of other genes (e.g. Sénéchal et al., 2015; Hocq et al., 2020), possibly masking the mutation. Our work suggests that the function of genes can be revealed by analyzing variability of mutant phenotype instead of their average phenotype. Our observation that cell-wall related genes appear to be involved in buffering mechanisms that allow for developmental robustness, adds to evidence of cell-wall related genes being important for organ size and proportions (Weiss et al., 2005).

This work contributes to the understanding of how gene regulatory networks are related to developmental robustness. In particular, it opens questions regarding the role played by the level of expression of cell wall genes in the robustness of sepal size. Our results point towards genes with higher expression levels being more relevant, not for morphology, but for morphological robustness.

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Declaration of interest statement

The authors report there are no competing interests to declare.

Data availability

Raw data for mutant sepal measurements and their corresponding controls; Supplementary Tables 1-5; and the R scripts to analyze the raw data, to produce Supplementary Tables 2-5 and Figures 1 & 2 are publicly available at Zenodo (https://doi.org/10.5281/zenodo.13918462).

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Table 1

GENE	GENE ID	RELATED TO	INVOLVED IN or ENABLES
BETA GLUCOSIDASE 42 (BGLU42)	AT5G36890	CELLULOSE	cellulose catabolic process
CELLULOSE SYNTHASE-INTERACTIVE PROTEIN 1 (CSI1)	AT2G22125	CELLULOSE	cellulose biosynthetic process
COMPANION OF CELLULOSE SYNTHASE 1 (CC1)	AT1G45688	CELLULOSE	linking cellulose synthase complex and microtubules
CELLULOSE SYNTHASE 6 (CESA6)	AT5G64740	CELLULOSE	cellulose biosynthetic process
CELLULOSE SYNTHASE-LIKE D5 (CSLD5)	AT1G02730	CELLULOSE	cellulose biosynthetic process
CELLULOSE SYNTHASE-LIKE G3 (CSLG3)	AT4G23990	CELLULOSE	cellulose biosynthetic process
EXPANSIN A15 (EXPA15)	AT2G03090	CELLULOSE/ HEMICELLULOSE	cell wall organization
PECTIN METHYLESTERASE 32 (PME32)	AT3G43270	PECTIN	pectin catabolic process
PECTIN METHYLESTERASE INHIBITOR 3 (PMEI3)	AT5G20740	PECTIN	negative regulation of pectin catabolic process
POWDERY MILDEW RESISTANT 6 (PMR6)	AT3G54920	PECTIN	pectin catabolic process
MURUS 4 (MUR4)	AT1G30620	PECTIN/ HEMICELLULOSE	galactose metabolic process for cell wall biogenesis
CELLULOSE SYNTHASE-LIKE C8 (CSLC8)	AT2G24630	HEMICELLULOSE	cell wall organization
XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 15 (XTH15)	AT4G14130	HEMICELLULOSE	xyloglucan metabolic process
XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 6 (XTH6)	AT5G65730	HEMICELLULOSE	xyloglucan metabolic process
XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9 (XTH9)	AT4G03210	HEMICELLULOSE	xyloglucan metabolic process
GLYCOSYLTRANSFERASE (GALT29A)	AT1G08280	ARABINOGALACTAN PROTEINS	protein glycosylation

Figures

Figure 1

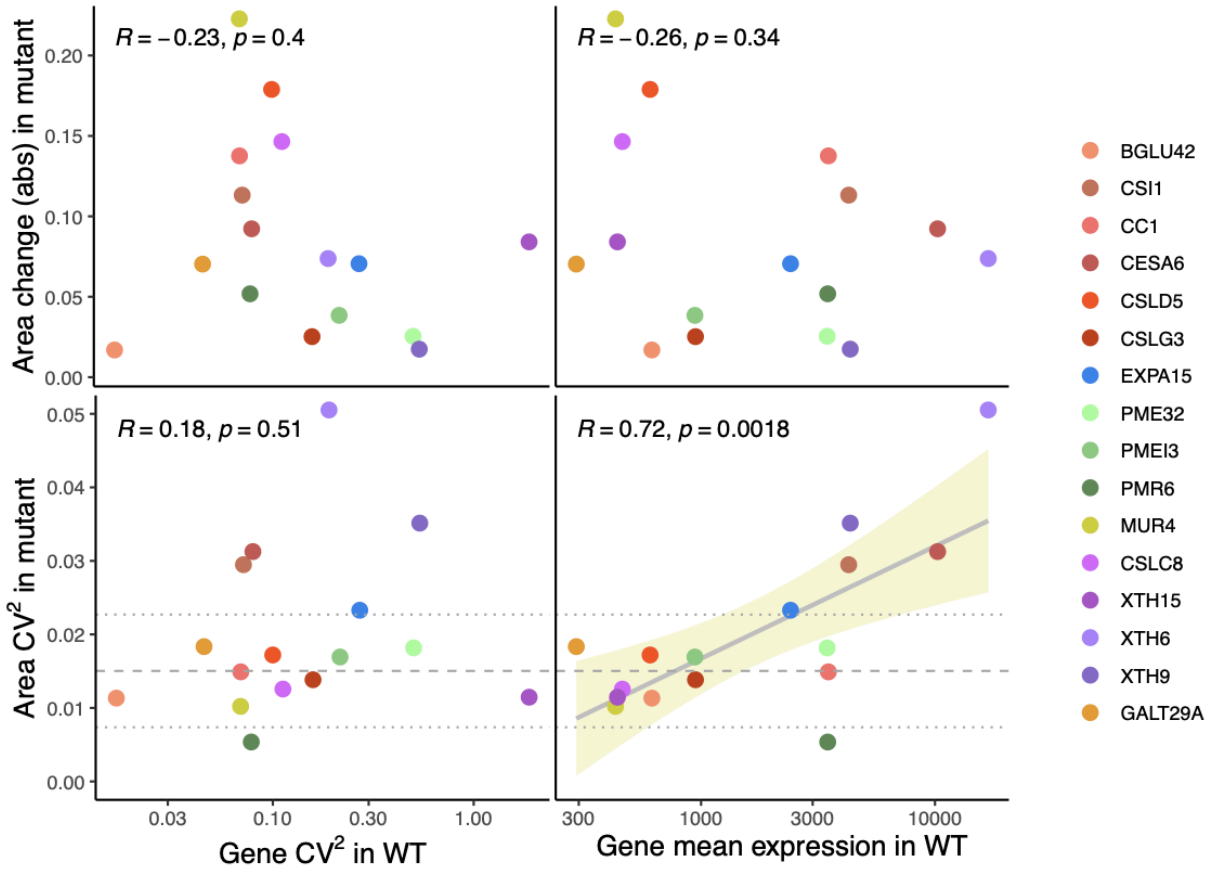

Figure 2

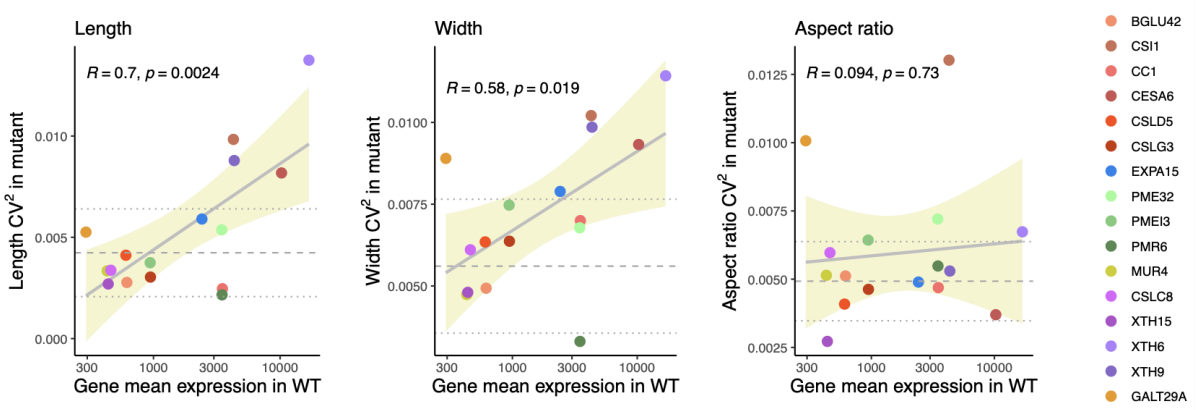

Figure Captions

Figure 1: Sepal area for 16 cell-wall related mutants (mutant effect compared to wild type and variability) against gene expression (mean and variation) of the corresponding gene in wild type. Top row y-axis: effect of the mutation on sepal area, corrected by sepal area in wild-type plants for each batch. Bottom row y-axis: squared coefficient of variation of sepal area in knockout mutants. Left column x-axis: coefficient of variation of each of the corresponding mutant genes in wild-type Col-0 plants. Right column x-axis: mean gene expression of the corresponding mutant genes in wild-type Col-0 plants. Each point corresponds to one gene. Pearson correlation coefficient R and p-values are shown on the top left corner in each plot. Gray solid line shows linear model adjustment with standard error in tan shade. Gray dashed lines correspond to average CV^2 in area measurements in wild-type Col-0 control batches with dotted lines showing average plus/minus one standard deviation.

Figure 2: Squared coefficient of variation of sepal length, width and aspect ratio in knockout mutants against mean gene expression of the corresponding mutant genes in wild-type Col-0 plants. Each point corresponds to one gene. Pearson correlation coefficient R and p-values are shown on the top left corner in each plot. Gray solid line shows linear model adjustment with standard error in tan shade. Gray dashed lines correspond to average CV^2 in length, width and aspect ratio measurements in wild-type Col-0 control batches with dotted lines showing average plus/minus one standard deviation.