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RESEARCH ARTICLE

# Transcriptome analyses of leaf architecture in *Sansevieria* support a common genetic toolkit in the parallel evolution of unifacial leaves in monocots

**Edward M. Golenberg** | **Aleksandar Popadić** | **Weilong Hao**

Department of Biological Sciences, Wayne State University, Detroit, Michigan, USA

**Correspondence**
Edward M. Golenberg and Weilong Hao, Department of Biological Sciences, Wayne State University, Detroit, MI, USA.
Email: golenberg@wayne.edu and haow@wayne.edu

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## Abstract

Planar structures dramatically increase the surface-area-to-volume ratio, which is critically important for multicellular organisms. In this study, we utilize naturally occurring phenotypic variation among three *Sansivieria* species (Asperagaceae) to investigate leaf margin expression patterns that are associated with mediolateral and adaxial/abaxial development. We identified differentially expressed genes (DEGs) between center and margin leaf tissues in two planar-leaf species *Sansevieria subspicata* and *Sansevieria trifasciata* and compared these with expression patterns within the cylindrically leaved *Sansevieria cylindrica*. Two *YABBY* family genes, homologs of *FILAMENTOUS FLOWER* and *DROOPING LEAF*, are overexpressed in the center leaf tissue in the planar-leaf species and in the tissue of the cylindrical leaves. As mesophyll structure does not indicate adaxial versus abaxial differentiation, increased leaf thickness results in more water-storage tissue and enhances resistance to aridity. This suggests that the cylindrical-leaf in *S. cylindrica* is analogous to the central leaf tissue in the planar-leaf species. Furthermore, the congruence of the expression patterns of these *YABBY* genes in *Sansevieria* with expression patterns found in other unifacial monocot species suggests that patterns of parallel evolution may be the result of similar solutions derived from a limited developmental toolbox.

**KEYWORDS**
abaxial, adaxial, cylindrical leaf, development, differential gene expression, flat leaf, laminar growth, planar development, *Sansevieria cylindrica*, *Sansevieria subspicata*, *Sansevieria trifasciata*, succulents, transcriptome

## 1 | INTRODUCTION

As all multicellular organisms increase in size, they encounter a common problem of a rapidly reduced surface to volume ratio. Demands for energy, nutrients, and oxygen, as well as waste production, are proportional to volume, whereas the capacity to transport material into and out is constrained by surface area. One common solution to this problem is to increase surface area by forming branches or reticulations as found in respiratory, renal, and digestive organs. An alternative solution is to reduce volume by forming flattened or laminar structures such as found in leaves, flatworms, and elephant ears. In each of these cases, the disproportionately large surface areas allow for the increased ability to capture light, absorb food and exchange gases, and disperse heat, respectively. In the case of non-smooth surfaces with repetitive invaginations or projections, localized growth and cell divisions can produce the nonuninform surface. In contrast, laminar structures require coordinated growth of top and bottom surfaces to maintain a planar structure except in circumstances of single cell thick organs such as bryophyte microphylls.

In land plants, true leaves or megaphylls are generally the primary light capturing, photosynthetic organs, and are hypothesized to have evolved from stems or branches (Beerling & Fleming, 2007). They have two differentially defined surfaces, the adaxial (analogous to dorsal) and abaxial (analogous to ventral). These surfaces are usually differentiated by cell shape and type. Similarly, the adaxial and abaxial regions are differentiated molecularly by region-specific expression of multiple genes (Satterlee & Scanlon, 2019). As inferred from studies in the monocot *Zea mays* and the dicot *Arabdopsis thaliana*, orthologs of *AS1*, *AS2* (*PHAN*), and *HD-ZIP III* are expressed adaxially (Bowman et al., 2002; Byrne et al., 2000; Garcia et al., 2006; Hay et al., 2006; Huang et al., 2006; Kim et al., 2003; Li et al., 2005; Ueno et al., 2007; Waites & Hudson, 1995), whereas *ETTIN*/*ARF3* and *KANADI* are expressed abaxially (Emery et al., 2003; Eshed et al., 2004; Izhaki & Bowman, 2007; Mallory et al., 2004; Moon & Hake, 2011; Tsiantis & Hay, 2003). The adaxial and abaxial defining proteins also negatively regulate the expression of the alternative genes, thereby maintaining the molecularly defined domains. It should be noted that these same gene sets are expressed in the stems and ovules where the adaxial genes are expressed centrally and the abaxial genes expressed marginally (Baker et al., 1997; Balasubramanian & Schneitz, 2002; Emery et al., 2003; Sieber et al., 2004). In the laminar organs, adaxial and abaxial surfaces come in contact at the edges of the lamina. Waites and Hudson (1995) proposed that the juxtaposition of the two surfaces and their molecular components creates a unique regulatory environment that controls the flat, marginal (mediolateral) growth of the leaf. Mutant analysis, most notably the work on *phan* and *phab* mutants, demonstrated that loss of either adaxial or abaxial identity can result in radial, cylindrical leaves in the most extreme phenotypes (McConnell et al., 2001; McConnell & Barton, 1998; Prigge et al., 2005; Waites & Hudson, 1995; Williams et al., 2005). However, similar phenotypes are not necessarily identified in different species with mutations in orthologous genes. These results emphasize that the genetic background may vary among species and can contextualize the effects of single genes.

As an alternative approach, we investigated leaf margin expression patterns that are associated with mediolateral growth by using naturally occurring phenotypic variation among congeneric species and attempt to identify genetic mechanisms dictating the development of leaf surface. Specifically, we generated and analyzed transcriptome data from leaf tissue of *Sansevieria subspicata*, *Sansevieria trifasciata*, and *Sansevieria cylindrica* (Figure 1). *Sansevieria* is a genus of succulent plants from subSaharan Africa. Leaf morphologies vary from pliable to rigid, flat to crescent to cylindrical, and thin to thick (Koller & Rost, 1988). Leaf development varies both among species and within species during development from bifacial, having both adaxial and abaxial polarities, to unifacial, having a single polarity (Stevenson, 1973). All species begin with a persistent radial leaf tip that is abaxialized. Flat and crescent shaped leaves develop an adaxial side beginning below the radial tip and extending basipetally (Stevenson, 1973). Submarginal meristem cell develops from the lateral sides of the medial adaxial meristem and can continue to divide even as the central adaxial meristem ceases activity basipetally (Stevenson, 1973). Depending of the species, cylindrical leaves may have an adaxial grove that extends up the leaf face or may be absent (Koller & Rost, 1988). Of particular note, even in flat, apparently bifacial leaves, vascular bundles are arranged with the phloem oriented to

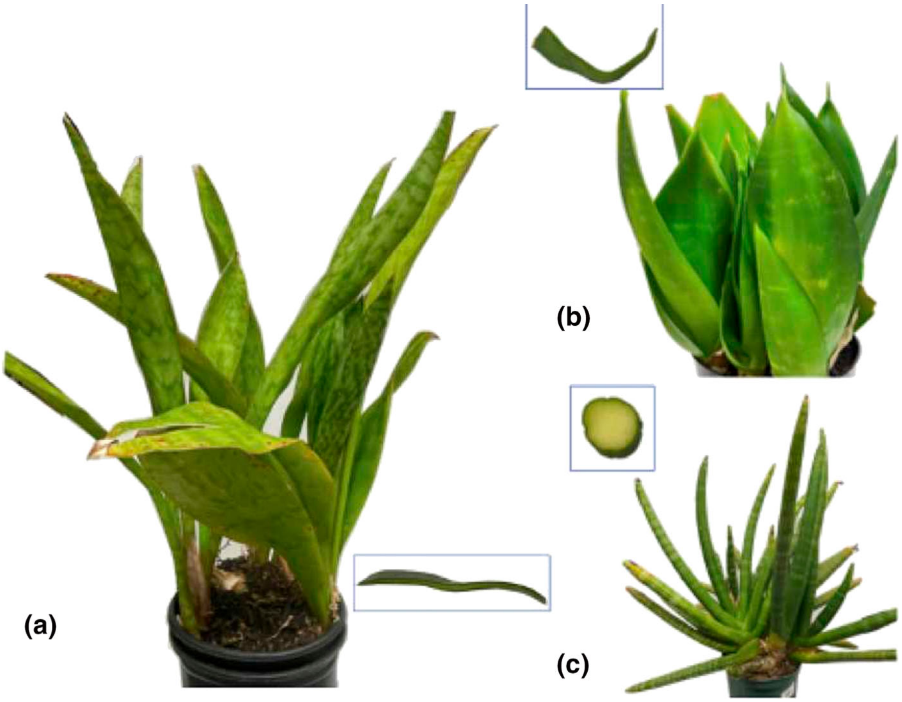

**FIGURE 1** Three *Sansevieria* species demonstrating different leaf morphologies. (a) *Sansevieria subspicata* has long, flat, and relatively narrow leaves. (b) *Sansevieria trifasciata* has broad, flat leaves. (c) *Sansevieria cylindrica* has long, cylindrical leaves. Inserts in all figures show cross-section of leaves from the associated species and are oriented adaxial side up.

either the closest surface or to the margins rather than being consistently oriented toward to abaxial pole (Koller & Rost, 1988). Among our sampled species, *S. subspicata* and *S. trifasciata* have crescent or flat leaves. *S. trifasciata* has broad, pliable leaves, whereas *S. subspicata* has narrower leaves. In contrast, *S. cylindrica* develops thick, round, cylindrical leaves. First, by comparing gene expression patterns between medial tissue and marginal tissue independently in the flat-leaved species and then exploring for overlapping patterns, we increase the potential for uncovering edge specific patterns while reducing the focus on species-specific patterns. Second, by then detecting congruence in gene expression pattern differences between each of the flat-leaved species, *S. subspicata* and *S. trifasciata*, and the cylindrical-leaved *S. cylindrica*, our analyses provide further characterization of genes associated with marginal growth emerges.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant material

Three individual plants of *S. subspicata* were purchased from Glasshouse Works, Stewart, OH, USA. Three plants of *S. trifasciata* and three plants of *S. cylindrica* were purchased from English Gardens, Dearborn Heights, MI, USA. Plants are maintained in a greenhouse at Wayne State University.

### 2.2 | RNA extraction and transcriptome sequencing

Fresh leaf tissue from all three species was harvested by cutting sections of young leaves with a razor blade. In the case of *S. subspicata* and *S. trifasciata*, the leaf sections were then cut to collect approximately 1 $cm^2$ of tissue from the center of the leaf and tissue from approximately 2 mm width at the margins of the leaf. All leaf center sections had samples of adaxial and abaxial epidermal tissue along with mesophyll tissue. In the case of the marginal tissue, all three tissue types, adaxial epidermal, abaxial epidermal, and some mesophyll tissue, were present, along with the unique edge tissue. In the case of the *S. cylindrica* leaf, a single sample was collected from each leaf consisting of the epidermal tissue and 2 mm of mesophyll. There was no tissue sample comparable with the central leaf blade tissue due to the shape of the leaf. Approximately 100–150 mg of tissue was used to extract RNA. In the case of *S. subspicata* and *S. cylindrica*, the tissue was ground in a mortar and pestle with liquid nitrogen, and RNA was extracted using Qiagen RNeasy Plant Mini Extraction Kit following the manufacturer's protocol. *S. trifasciata* proved to be more recalcitrant to extracting sufficient quantities and qualities of RNA. Successful RNA extractions from *S. trifasciata* were achieved using ZR Plant RNA MiniPrep Kits (Zymo Research, Orange, CA, USA) following manufacturer's protocol. cDNA libraries were constructed and sequenced by BGI (Hong Kong) using DNBseq platform producing 100-bp paired end reads.

### 2.3 | De novo assembly, differential expression analysis, and gene ontology (GO)

Raw RNA-seq reads (pair-end 100 bp) were processed using the FASTP program (Chen et al., 2018) to filter low quality data. Filtered sequencing reads were assembled into contigs using rnaSPAdes (Bushmanova et al., 2019). Initial de novo assembly was done individually on each sample. The samples with high coverage and N50 were used as the reference for subsequent mapping analysis. For *S. trifasciata* and *S. subspicata* references, samples of the center and marginal tissue from the same plant were merged to build reference contigs to include any tissue-specific transcripts. Open reading frames (ORFs) were predicted using the TransDecoder program in Trinity (Grabherr et al., 2011).

There are 53,380 predicted ORFs in *S. subspicata*, 38,571 in *S. trifasciata*, and 42,154 in *S. cylindrica*. Sequence similarity search was performed using the predicted ORFs against curated protein sequences from the UniProt database (Wu et al., 2006). This was done locally using the BLASTP option in the DIAMOND program (Buchfink et al., 2015). Homologs were defined using an *E*-value cutoff $<10^{-10}$, and homologs of reciprocal best hit (RBH) between species were deemed as orthologs. GO term was assigned following the best BLAST hit. Raw sequencing reads were mapped to the corresponding assembled contigs using Bowtie2 (Langmead & Salzberg, 2012). Read coverage for each annotated ORF was generated using BEDtools (Quinlan & Hall, 2010). Differential gene expression between center and margin tissues and between different species was determined using DEseq2 (Love et al., 2014). Genes of interest were subject to further phylogenetic analysis. Homologous protein sequences were aligned using MUSCLE (Edgar, 2004). Phylogenetic trees were constructed using PhyML (Guindon et al., 2010) under a $\mathrm{JTT} + \Gamma$ substitution model.

### 2.4 | DNA extraction and PCR testing

Approximately 200 mg of leaf tissue from each species was disrupted with zirconium oxide beads in 1 ml of CTAB extraction buffer (2% CTAB, 100-mM Tris HCl pH 8.0, 20-mM EDTA pH 8.0, 1.4-M NaCl). The lysate was mixed with 1 volume phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged for 5 min. The aqueous layer was transferred to a clean tube and extracted once with chloroform/isoamyl alcohol (24:1). The aqueous layer was again transferred to a clean tube, and the DNA was precipitated with 2 volumes of 95% ethanol. The ethanol was removed, and the DNA was air-dried before reconstituting in 100-μl $H_2O$. Primers were designed from conserved sequences from *S. spicate* and *S. trifasciata* from the genes of interest. Primers are listed in Table S1. Polymerase chain reaction (PCR) reactions were generated using Promega 2X Go Taq Master Mix and 5-μM primers. PCR products were separated on a 1.5% synergel/agarose TBE gel and scored for presence/absence.

**TABLE 1** Basic information of the transcriptomic data.

| | Center tissue | Marginal tissue | Contig number | Contig length (bp) | N50 | GC% |
|---|---|---|---|---|---|---|
| *Sansevieria cylindrica* | 3* | | 162,525 | 107,536,784 | 1432 | 41.07 |
| *Sansevieria subspicata* | 3 | 6 | 221,617 | 152,095,569 | 1538 | 40.47 |
| *Sansevieria trifasciata* | 5 | 4 | 127,289 | 89,586,756 | 1541 | 42.47 |

*No separation between center tissue and marginal tissue in cylindrical leaves.

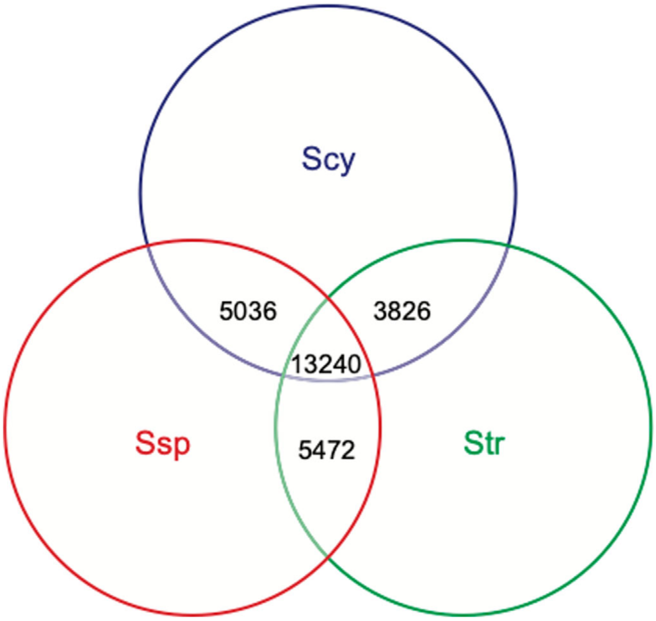

**FIGURE 2** Venn diagram of shared orthologs among *Sansevieria cylindrica* (Scy), *Sansevieria subspicata* (Ssp), and *Sansevieria trifaciata* (Str).

## 3 | RESULTS

### 3.1 | Sequences and de novo assembly

The total assembled transcripts are 107,536,784 bp in *S. cylindrica*, 152,095,569 bp in *S. subspicata*, and 89,586,756 bp in *S. trifasciata* (Table 1). The transcriptome assemblies of the laminar-bladed *S. subspicata* and *S. trifasciata* show a large difference in both contig number of total contig length, while N50 values are similar. Sequences with extreme, particularly low GC content are known to exhibit under-coverage in sequencing (Browne et al., 2020). This does not seem to be the case as the average GC content is comparable among the thee species, ranging from 40.47% in *S. subspicata* to 42.47% in *S. trifasciata*. Thus, the relatively low number of contigs and predicted genes in *S. trifasciata* cannot be simply explained by sequencing bias.

Shared orthologs were identified based on reciprocal best BLAST hits (see methods); 13,240 orthologous transcripts are shared among all three species. Orthologous transcripts shared by just two species are 5472 between *S. subspicata* and *S. trifasciata*, 5036 between *S. cylindrica* and *S. subspicata*, and 3826 between *S. cylindrica* and *S. trifasciata*. This shows that the two species with flat leaves share the most orthologs, despite the lowest number of total assembled transcripts in *S. trifasciata* (Figure 2).

### 3.2 | Differential gene expression between center and margin leaf tissues

All predicted genes were analyzed to identify genes differentially expressed between the center and marginal leaf tissues in *S. trifasciata* and *S. subspicata*, respectively. There are 53 differentially expressed genes (DEGs) between the center and margin leaf tissue samples in *S. subspicata* (Figure 3a). Among the 53 DEGs in *S. subspicata*, 15 DEGs are downregulated in margin tissue and 38 DEGs are upregulated in margin tissue. Forty of the 53 (75%) DEGs in *S. subspicata* have BLAST hits with *E* values < $10^{-10}$ in the UniProt database. These are listed in Table S1. Ten genes were differentially expressed in *S. trifasciata*. Among the 10 DEGs in *S. trifasciata*, six DEGs are downregulated in margin tissue, and four DEGs are upregulated in margin tissue (Figure 3b). Six DEGs in *S. trifasciata* have BLAST hits with *E* values < $10^{-10}$ in the UniProt database (Table S1). Among the 10 *S. trifasciata* DEGs, only one DEG (Str032566.p1) has RBH with *S. subspicata* DEG (Ssp023299.p1) (Table S1).

The Gene Ontogeny (GO) terms were exacted for the 40 DEGs that have matches in the UniProt database and visualized using the REViGO website (Supek et al., 2011). The enriched functions involve metabolic processes, cellular responses, ion transport, cell wall organization, and cell division (Figure 4).

### 3.3 | Comparative gene expression analysis of DEG orthologs in other *Sansevieria* species

The expression profiles of DEG orthologs were compared among the three species. Among the 53 DEGs in *S. subspicata*, 27 of them have orthologs in *S. trifasciata*. We identified the fold change in expression of the 27 genes in each species and compared them between species. The fold change between center and margin leaf tissues is significantly correlated between *S. subspicata* and *S. trifasciata* ($r = .92$, $p$ value $= 1.24 \times 10^{-11}$) (Figure 5a). In comparison, the correlation coefficient ($r$) in all shared orthologs ($n = 18{,}712$) between *S. subspicata* and *S. trifasciata* is .35, which is significantly lower than that in the orthologous pairs of DEGs (Figure 5b,c).

We further compared the expression of DEG orthologs ($n = 15$, Table S2) shared by all three species. Given that *S. cylindrica* lacks a

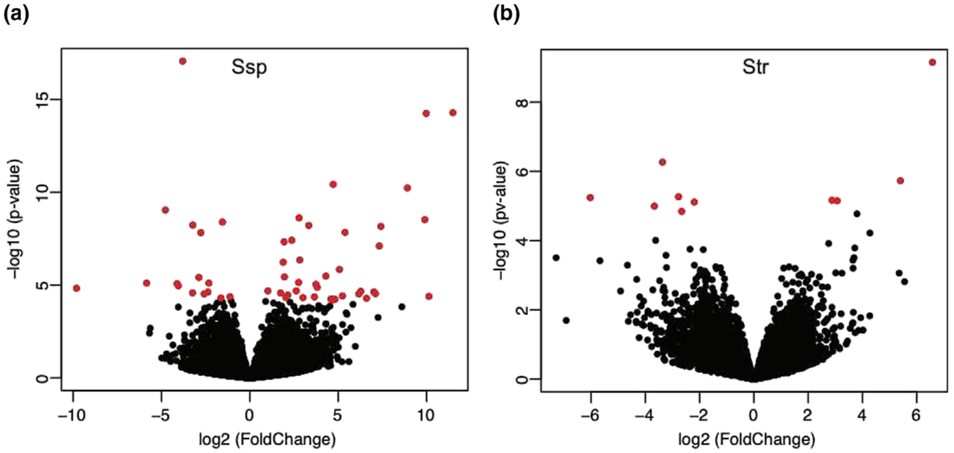

**FIGURE 3** Volcano plot showing differentially expressed genes (DEGs) between center and margin leaf tissues (a) in *Sansevieria subspicata* (Ssp) and (b) in *Sansevieria trifaciata* (Str). Fold change is the comparison of margin tissue against center tissue. Red dots indicate significant DEGs, $n = 53$ in Ssp and $n = 10$ in Str.

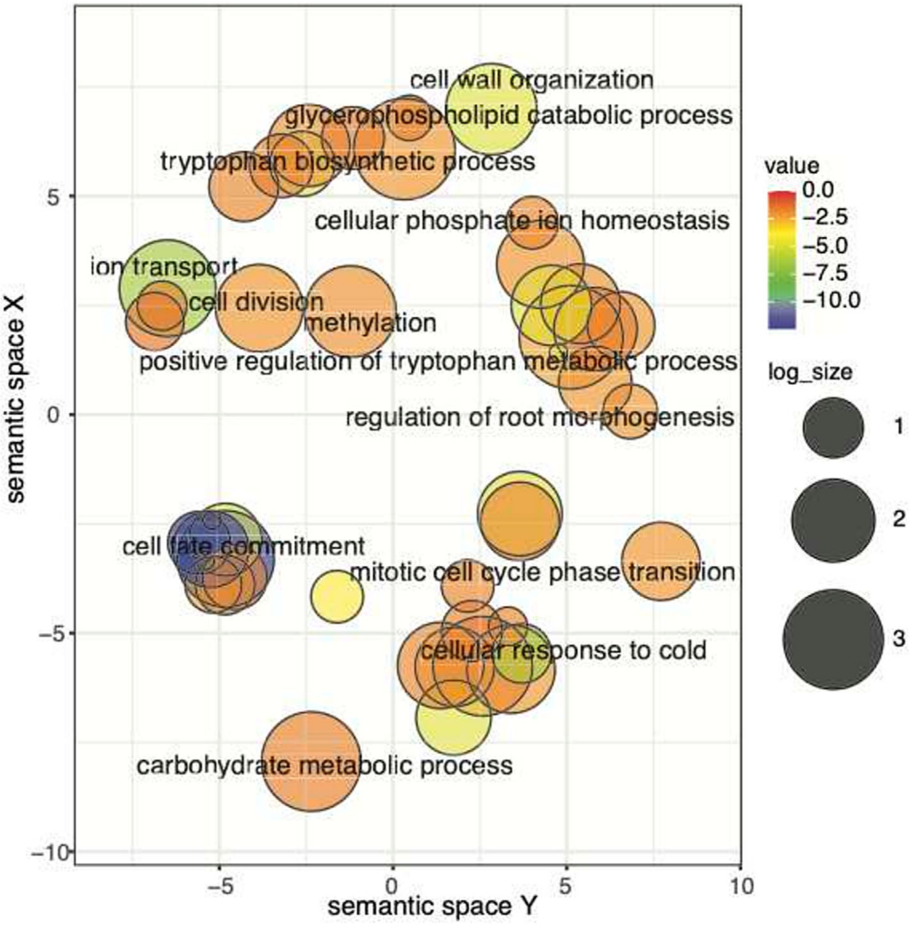

**FIGURE 4** REViGO semantic similarity scatterplot of the Gene Ontogeny (GO) enrichment of significantly expanded orthogroups in *Sansevieria subspicata*. Color indicates *p* values from the differential expression analysis, and bubble size indicates the relative frequency of the GO terms in the differentially expressed genes.

flat blade margin, we wished to test whether or not the *S. cylindrica* margin transcriptome was comparable in DEGs to center tissue of the two flat-leaved congeners. There is significant correlation between the fold change between center and margin leaf tissues in *S. subspicata* and the fold change between *S. cylindrica* and the margin leaf tissue in either *S. subspicata* or *S. trifasciata* (Figure 6). The expression profile of DEG orthologs in *S. cylindrica* is center-leaf tissue like. These results indicate that the margin tissue in *S. cylindrica* is analogous to the central leaf tissue in the two congeneric species and suggest that the loss of flat-leaf development is consistent with the loss of marginal tissue expression patterns found in flat leaves.

## 3.4 | Presence of genes in developmental axes among all three *Sansevieria* species

Many genes in developmental axes in plants (adaxial/abaxial, proximal/distal, and medial/lateral) have been discovered (Satterlee &

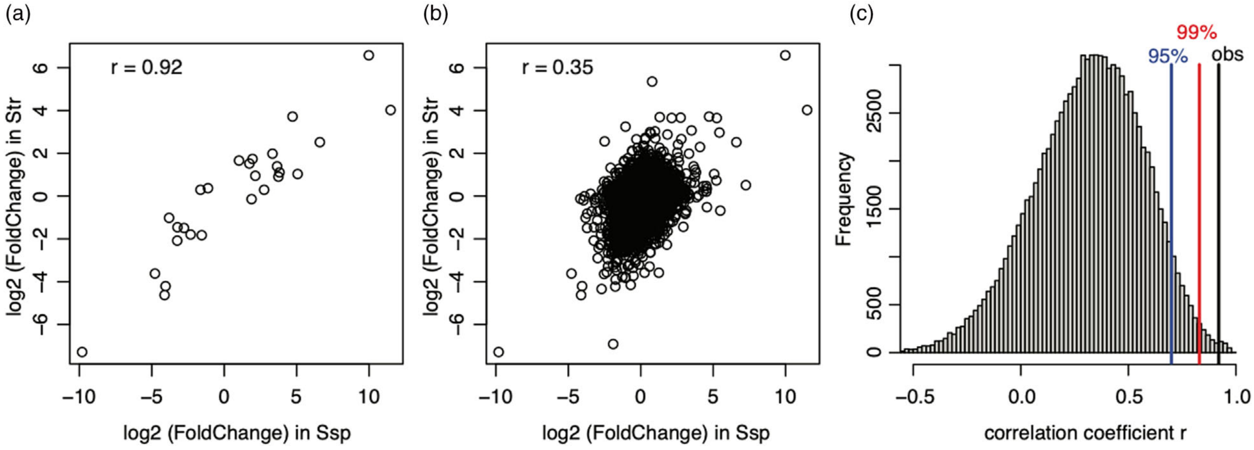

**FIGURE 5** Strong correlation of center-versus-margin fold changes (in log values) between DEGs in *Sansevieria subspicata* (Ssp) and their orthologs in *Sansevieria trifasciata* (Str). (a) Plot of log fold change between differentially expressed gene (DEG) orthologous pairs ($r = .92$, $p$ value $= 1.2 \times 10^{-11}$). (b) Plot of log fold change between all shared orthologs between Ssp and Str ($r = .35$, $p$ value $< 2.2 \times 10^{-16}$).
(c) Distribution of correlation coefficient values for 10,000 randomly selected gene sets with 26 orthologs in each set. The observed $r = .92$ is labeled.

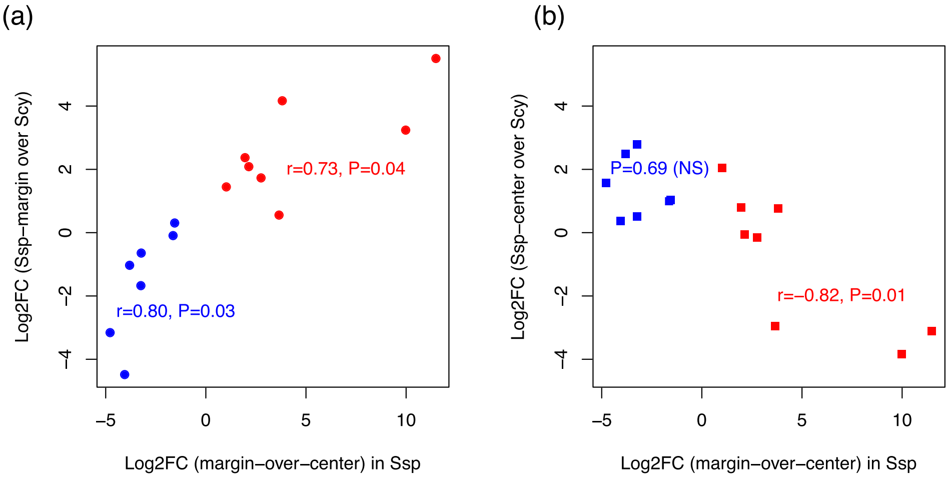

**FIGURE 6** Comparison of fold-change of Ssp differentially expressed genes (DEGs) against their Scy orthologs. (a) Comparison of fold change between Ssp-margin over Scy. (b) Comparison of fold change between Ssp-center over Scy. Correlation analyses were done separated for the high margin-over-center DEGs (in red) and low margin-over-center DEGs (in blue). The three DEGs that have noticeable low Ssp-center expression are Ssp019630.p1 (*PLT5*), Ssp023299.p1 (*SAP*), and Ssp038533.p1 (*MIK2*).

Scanlon, 2019). We have searched the known protein genes in developmental axes against the *Sansevieria* transcriptomes (Table 2). All searched genes have BLASTP matches in each of the three *Sansevieria* transcriptomes, and the *E* values are remarkably similar among the three species. Thus, there is no evidence that the loss of flat leaf morphology in *S. cylindrica* is due to the loss of genes in developmental axes.

## 3.5 | Absence of DEG orthologs in *S. cylindrica*

We next sought to determine whether homologs of all DEGs identified in *S. subspicata* are found in the transcriptomes of the two other *Sansevieria* species. All 53 DEGs in *S. subspicata* have BLASTP matches in the *S. trifasciata* transcriptome. All, but one, DEGs in *S. subspicata* have BLASTP matches in the *S. cylindrica* transcriptome. The sole *S. subspicata* DEG lacking BLASTP matches in *S. cylindrica* does not have detectable BLASTP matches in the UniProt database. Additional search in the NCBI nr database found its top BLASTP match to the *TAC1* (Tiller Angle Control 1) protein in date palm, *Phoenix dactylifera* with an *E* value of 5e-14. The *TAC1* protein is known to be associated with the development of leaf angle (Waite & Dardick, 2018).

As genes identified as homologs in BLASTP sequence searches may either be strict orthologs or paralogs, we conducted phylogenetic analyses on the subset of 40 DEGs that have matches in the UniProt database. *Arabidopsis thaliana* was included as an outgroup for identification of orthologous sequences. There are five DEGs that do not have detectable orthologs in the *S. cylindrica* transcriptome (Figure 7b and Figure S2). As an illustration, three *MAC* proteins (Scy039505.p1, Str026404.p1, and Ssp020447.p1) cluster within a single clade in Figure 7a. In contrast, in Figure 7b, Str032196.p1 and Ssp034423.p1 cluster, but no transcript was found in *S. cylindrica*. These DEGs include known plant-specific transcription factor *Crabs Claw* (*CRC*) and *RAX2*. *CRC* belongs to the *YABBY* gene family, whose members have been

**TABLE 2** BLASTP *E* values of genes in developmental axes against transcriptomes of *Sansevieria subspicata* (Ssp), *S. trifasciata* (Str), and *S. cylindrica* (Scy).

| Information of query sequences | | | | | |
|---|---|---|---|---|---|
| **Gene** | **Full name** | **Accession** | **Ssp** | **Str** | **Scy** |
| *STM* | Shootmeristemless | Q38874 | 2.86E-40 | 2.26e-36 | 6.36e-37 |
| *BP* | Brevipedicellus | P46639 | 6.35E-40 | 4.73e-33 | 3.50e-33 |
| *KNAT2* | - | P46640 | 7.35E-36 | 5.73e-36 | 2.69e-36 |
| *KNAT6* | - | Q84JS6 | 4.37E-36 | 1.87e-32 | 1.60e-32 |
| *AS1* | Asymmetric Leaves1 | O80931 | 5.93E-138 | 1.02e-138 | 2.91e-132 |
| *AS2* | Asymmetric Leaves2 | O04479 | 1.69E-58 | 1.56e-58 | 5.41e-39 |
| *PHB* | Phabulosa | O04291 | .0 | .0 | .0 |
| *REV* | Revoluta | Q9SE43 | .0 | .0 | .0 |
| *PHV* | Phavoluta | O04292 | .0 | .0 | .0 |
| *ARF3* | Ettin/Auxin Response Factor3 | O23661 | .0 | .0 | .0 |
| *ARF4* | Ettin/Auxin Response Factor4 | Q9ZTX9 | .0 | .0 | .0 |
| *MP* | Monopteros | P93024 | 7.05e-163 | 9.56e-163 | 3.18e-163 |
| *KAN* | Kanad | Q93WJ9 | 4.55e-62 | 1.19e-63 | 3.36e-63 |
| *PIN1* | Pin-Formed1 | Q9SL42 | 3.22e-66 | 2.54e-66 | 2.56e-66 |
| *PRS* | Pressed Flower | Q9SIB4 | 1.96e-12 | 1.32e-12 | 1.38e-12 |
| *WOX1* | Wuschel-Like Homeobox1 | Q6X7K0 | 4.49e-14 | 3.60e-14 | 2.39e-14 |
| *CUC2* | Cup-Shaped Cotyledon2 | O04017 | 1.74e-82 | 4.08e-83 | 2.30e-59 |
| *TCP* | Teosinte Branched1/Cincinnata/Proliferating Cell Factor | Q93WI2 | 3.72e-16 | 2.70e-16 | 2.65e-16 |
| *NGA* | Ngatha | O82799 | 6.17e-71 | 5.31e-72 | 1.50e-52 |
| *FIL* | Filamentous Flower | O22152 | 1.06e-53 | 3.75e-84 | 1.91e-53 |
| *YAB3* | YABBY3 | Q9XFB1 | 2.22e-56 | 4.39e-83 | 4.75e-56 |
| *YAB5* | YABBY5 | Q8GW46 | 1.14e-68 | 7.22e-68 | 3.48e-68 |
| *BOP1* | Blade On Petiole1 | Q9M1I7 | 7.20e-135 | .0 | .0 |
| *BOP2* | Blade On Petiole2 | Q9ZVC2 | 3.92e-140 | .0 | .0 |

associated with laminar leaf development (Sarojam et al., 2010; Yamaguchi et al., 2012). *RAX2* (*Regulator of Axillary Meristems* 2) belongs to the *MYB* family and is associated with the establishment of stem cells in axillary regions (Keller et al., 2006; Müller et al., 2006). Therefore, the lack of these DEG orthologs in its transcriptome may play an important role in the development of cylindrical leaf shape in *S. cylindrica*.

To test whether the absence of an *S. cylindrica* transcript in each of these five genes is due to a deletion of the gene or a lack of transcription, PCR primers were designed for each gene based on sequences that are absolutely conserved between *S. subspicata* and *S. trifasciata*. We found PCR products from all five genes in independent replicate DNA extractions from each of the three species. On the basis of these results, we proposed that these genes are present in the genomes of all three species but are not activated in *S. cylindrica*.

## 4 | DISCUSSION

Land plants and angiosperms in particular display an impressive range of phenotypic variation in their vegetative organs. Much of this variation may occur within a single individual as the organism responds to environmental cues to change the morphology of newly emerging organs by alternative gene expression patterns. This inherent developmental flexibility can be the basis for fixed phenotypic variation when elements in gene expression modules are modified through mutation and being, themselves, fixed in the population through selection or drift. Changes in single upstream regulator elements can lead to changes in whole expression module profiles without further widespread mutational variation. Therefore, comparative transcriptome studies within closely related clades displaying striking morphological variation can prove to be highly informative of universal developmental processes and can be viewed as complementary to classic mutant analyses.

The genus *Sansevieria* is native to tropical Africa and grows in dry and rocky habitats. All species within the genus are considered to be succulents having relatively thick, leathery leaves. In particular, mesophyll structure in *Sansevieria* characterized by the absence of photosynthetic cells and the extensive organization of dead, water retaining and living, non-photosynthetic network cell. The extent and thickness of this water-retention tissue is correlated with the aridity of the

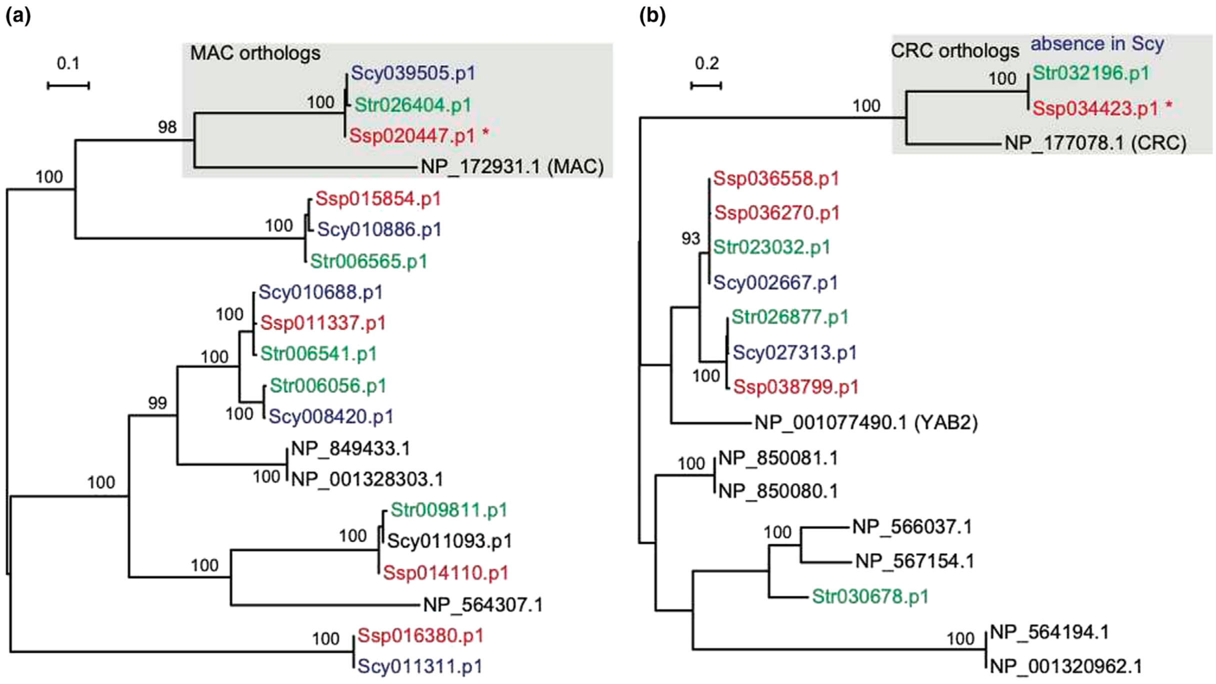

**FIGURE 7** Phylogenetic trees of gene families of two differentially expressed genes (DEGs) in Ssp (labeled with *). The three *Sansevieria* species are in different colors, and homologs in *Arabidopsis thaliana* are in black. (a) Genes in the *MAC*/perforin domain-containing protein family show the presence of DEG Ssp020447.p1 orthologs in all three *Sansevieria* species. (b) Genes related to *CRC* (*CRABS CLAW*) in the plant-specific transcription factor *YABBY* family miss the ortholog of DEG Ssp034423.p1 in Scy. Only bootstrap values >70 are shown.

individual species native habitat (Koller & Rost, 1988). As such, the variant morphology of *S. cylindrica* may be considered to be an extreme, water-conserving phenotype as its cylindrical leaves reduce the surface to volume ratio found in its close relatives while increasing its water retention ability. In this study, we focused on its loss of edge in comparison with its laminar-bladed relatives.

Within monocots, radial, unifacial leaf development has evolved independently multiple times and appears to follow similar development steps (Sajo & Rudall, 1999; Yamaguchi et al., 2010; Yamaguchi et al., 2012). The leaves can be ensiform or laminar; however, the vascular bundles are generally radially arranged with the xylem oriented to the center of the leaf reflecting an abaxial identity (Yamaguchi et al., 2010). *Sansevieria* species develop leaves that initiate with a radial, abaxial tip (Koller & Rost, 1988; Stevenson, 1973). Stevenson (1973) does describe a distribution of dividing cells on the adaxial side but not on the abaxial side in *S. trifasciata*. However, in flattened leaved *Sansevieria* species, there is no differentiation in stomatal distribution on either side of the leaf, there is no distinct adaxial/abaxial differentiation in the mesophyll, and the vascular bundles are arranged such that the phloem faces the adjacent leaf surface or the leaf margin (Koller & Rost, 1988). As such, even in laminar *Sansevieria* species, there is no strong adaxial/abaxial differentiation as is found in other monocot and eudicot leaves.

Previous seminal studies on leaf development identified key axis-polarity genes that distinguished adaxial and abaxial domains (Bowman et al., 2002; Canales et al., 2005; Kim et al., 2003; McConnell et al., 2001; McConnell & Barton, 1998; Prigge et al., 2005; Waites et al., 1998; Williams et al., 2005). Leaf primordia originate when the *MYB* protein *PHAN* suppresses *KNOX* protein suppression of cell differentiation and establishes adaxial polarity on the primordium. The adaxial domain and fate are determined and maintained by the expression of the class III homeodomain-leucine zipper (HD-ZIPIII) *PHAB* (McConnell et al., 2001; McConnell & Barton, 1998; Prigge et al., 2005) Abaxial identity is determined by the expression of *KANADI* and *ARF3/ETT* genes and appears to be the default identity. The adaxial and abaxial components suppress the expression of the alternative component, such that a dominant *phb* mutant adaxializes the leaf resulting in a tubular organ containing a vascular cylinder with xylem surrounding a phloem core (Waites & Hudson, 1995), whereas a recessive *phan* mutant produces tubular organs with a vascular cylinder having phloem surrounding a xylem core (Bowman et al., 2002). These observations led Waites and Hudson to hypothesize that laminar or planar growth results from the juxtaposition of the adaxial and abaxial components (Waites & Hudson, 1995). Subsequently, lateral expansion was associated with several YABBY genes that are activated at the margins (Bowman et al., 2002; Eshed et al., 2004).

As listed in Table 2, the major adaxial/abaxial genes appear to have homologs that are expressed in all three *Sansevieria* species. It is noteworthy that few of these genes are identified among the DEGs reported here. By comparing tissue samples from the blade center to leaf margin in both *S. trifasciata* and *S. subspicata* independently and then comparing these with the expression pattern in *S. cylindrica*, we may have either eliminated or repressed detection of differential expression in canonical adaxial/abaxial genes as both sets of genes

**TABLE 3** Clustering of annotated genes by related biological function.

| Biological function | Gene |
|---|---|
| Cell wall structure and remodeling | |
| | *MIK2* |
| | *OCT3* |
| | *E13B* |
| | *WAT1* |
| | *COMT1* |
| Pathogen resistance | |
| | *MIK2* |
| | *GATA* |
| | *WRKY* |
| | *GLDH* |
| | *NPR* |
| | *MACP1* |
| | *E13B* |
| | *PK4B* |
| | *EIX1* |
| Hormone response | |
| | *GATA* |
| | *WRKY* |
| | *GLDH* |
| | *NPR* |
| | *MACP1* |
| | *LHW* |
| | *LAX2* |

are expected to be found in the leaf center tissue and near-margin tissue sampled in both flat-leaved species. However, two *YABBY* family genes (listed as Ssp034423 and Ssp038799 in Table S2) are among the annotated DEGs found in *S. subspicata* that are downregulated in the leaf margin. These genes are orthologous to *Oryza sativa* genes *DROOPING LEAF* (*DL*) and *FILAMENTOUS FLOWER* (*FIL*), respectively.

*DL* controls the formation of the midrib through enhancing cell proliferation and is involved in proper formation of the auricle in grass leaves (Ishikawa et al., 2009; Kang et al., 2022; Nagasawa et al., 2003; Ohmori et al., 2011; Strable et al., 2017; Yamaguchi et al., 2004). It is homologous to the *Arabidopsis* gene *CRABS CLAW* that is expressed abaxially in carpels (Eshed et al., 1999). While also functioning in the formation of flower organs in rice (Yamaguchi et al., 2004), its role in midrib formation is shared in several grasses (Ishikawa et al., 2009). In *Juncus prismatocarpus*, the unifacial leaf blade is flattened due to the expression of *DL* that results in flattening along the adaxial/abaxial plane rather than the medial/marginal plane (Yamaguchi et al., 2010). As described above, in all *Sansevieria* species, the central mesophyll tissue is composed of dead, water storage cells within a network of living parenchymal cells (Koller & Rost, 1988). The thickness of the mesophyll corresponds with the aridity of the species' natural environment. It is reasonable to speculate that *DL* expression in *Sansevieria* is functioning to enhance mesophyll development as part of specific development of succulent leaf morphology. Therefore, our results in *Sansevieria* suggest that the role of *DL* in thickening the mesophyll is conserved with other monocots. However, it must be noted that whether the downregulation of *DL* in *Sansevieria* leaf margins reflects upregulation of cell proliferation in the mesophyll or is the result of increased mesophyll-specific sampling from center leaf tissue cannot be distinguished in our results.

The second *YABBY* family gene, *FIL*, has also been demonstrated to confer abaxial identity in meristems. The gene's function was initially identified primarily by flower phenotypes (Chen et al., 1999; Sawa et al., 1999). However, it has been shown to play a role in abaxial identity in meristems and leaves. Ectopic expression of *FIL* results in filamentous leaves with randomly arrange cells at the leaf margins (Sawa et al., 1999). In *Tropaeolum majus*, expression of *FIL* contributed to the abaxialization of the upper leaf surface, peltate leaf development, and radialization of the petiole (Gleissberg et al., 2005). These and other studies (Vosnakis et al., 2012) lead us to suggest that the reduced marginal expression of *FIL* reflects a strong abaxialization of unifacial leaves in *Sansevieria* and that radical modification of *FIL* expression plays a role in the loss of laminar leaf development in *S. cylindrica*.

Auxin regulatory genes have also been associated with leaf margin-specific growth (reviewed in Satterlee & Scanlon, 2019). *YUCCA* genes are involved in the biosynthesis of auxin and are upregulated in leaf margins (Wang et al., 2011). Two auxin pathway-related genes are identified among our DEGs, homologs of the *LHW* and *LAX* genes. *LHW*, the homolog of which is upregulated in *Sansevieria* leaf margins, interacts with *TOM5* and upregulates *YUCCA* expression in *Araidopsis* (Ohashi-Ito et al., 2019). It also modulates expression of auxin movement proteins such as *PIN1* and *MP* (Ohashi-Ito et al., 2013). In contrast, the *LAX2 Sansevieria* homolog is downregulated in the leaf margin. The *LAX2* protein is an auxin influx protein and, together with *PIN1*, pumps auxin to localizations of high concentrations (Swarup & Péret, 2012). Thus, these results appear to be inconsistent, although they may reflect differential roles for genes within the protein families that allow for patterns of spatial differentiation.

Further perusal of the DEG list shows that many of the DEGs that were annotated appeared to cluster in three functional groups: cell wall formation and remodeling, pathogen resistance, and salicylic acid (*SA*) or related hormone response (Table 3). These functions are not independent. For example, *MIK2* is a leucine rich receptor kinase that interacts with Serine-rich Endogenous Peptide (*SCOOP*) associated with fungal species *Fusarium* and bacterial family *Comamonadaceae* (Hou et al., 2021) and therefore may be part of the immune response. *MIK2* is also involved in response to cellulose wall deposition inhibition and in JA accumulation and lignin deposition (Ma et al., 2009; Stahl et al., 2022; Van der Does et al., 2017). Van der Does et al. (2017) suggest that the root angle phenotype of the *MIK2-1* mutant may reflect a loss of sensitivity to neighboring cell wall to cell wall feedback loop. We speculate that similar cell wall to cell wall communication may be at play at the juncture of adaxial and abaxial cells at the leaf margin. Additional genes that are involved in cell wall structure include *COMT1* (affects lignin deposition-upregulated) (Ma, 2009; Stahl et al., 2009), *WAT1* (responds to auxin levels, fungal pathogen resistance-down regulated) (Denancé et al., 2010; Hanika et al., 2021; Molina et al., 2021; Ranocha et al., 2013), *E13B* (glucan endo-1,3-beta-D-glucosidase-downregulated) (Hrmova & Fincher, 2001), and *OCT3* (interacts with ethylene response factors to downregulate cell wall modifications-upregulated) (Küfner & Koch, 2008). Thus, cell wall modification, pathogen resistance, and hormone response, all of which are represented in the DEGs, may all be responding to identical regulatory signals.

Lastly, our analyses uncovered five gene transcripts, with orthologs in *S. subspicata* and *S. trifasciata* for which we did not detect any transcripts in *S. cylindrica*. Surveying the genomic DNAs of *S. cylindrica*, *S. subspicata*, and *S. trifasciaa* by PCR detected identically sized fragments in all three species. This indicates that orthologs are present in all three genomes. If so, then these five gene are active in the margin determination pathway and are all suppressed or not activated in cylindrical leaves. Hence, these genes may be important downstream, modeling factors in leaf margin formation and may be worthy of further study.

Our results at least partially support the Waites and Hudson hypothesis that margin-specific regulatory factors at their juxtaposition of leaf faces generate novel regulatory modules to promote growth at the margins. However, *Sansevieria* species develop leaves that are initially, if not continuously, unifacial. In these species, it appears that the *YABBY* family genes are critical in promoting increased mesophyll development and abaxial identity in the leaf center. Indeed, the cylindrical leaf in *S. cylindrica* demonstrates comparable gene expression patterns to the center leaf tissue of *S. trifasciata* and *S. subspicata*. The congruence of these center leaf developmental patterns with other monocot species suggests that patterns of parallel evolution may be the result of similar solutions derived from a limited developmental toolbox. Here, we hypothesize that these parallel developmental patterns may reflect more universal downstream factors that are either directly or indirectly controlled by a limited number of major regulatory elements.

## AUTHOR CONTRIBUTIONS

EMG contributed to the design of the research, acquisition of the data, analysis of the data, interpretation of the results, and writing of the manuscript. AP contributed to the design of the research and interpretation of the results. WH contributed to the design of the research, analysis of the data, interpretation of the results, and writing of the manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

## DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available in the Sequence Read Archive (SRA repository), under the Accession number PRJNA925460 at NCBI.

## ORCID

*Edward M. Golenberg* https://orcid.org/0000-0002-0048-4776
*Aleksandar Popadić* https://orcid.org/0000-0001-7691-2901
*Weilong Hao* https://orcid.org/0000-0002-6337-7169

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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