Expression of DOF genes identifies early stages of vascular development in Arabidopsis leaves

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ABSTRACT The sequence of events underlying the formation of vascular networks in the leaf has long fascinated developmental biologists. In Arabidopsis leaves, vascular-precursor procambial cells derive from the elongation of morphologically inconspicuous ground cells that selectively activate expression of the HD-ZIP III gene ATHB8. Inception of ATHB8 expression operationally defines acquisition of a typically irreversible preprocambial cell state that preludes to vein formation. A view of the constellation of genes whose expression is activated at preprocambial stages would therefore be particularly desirable; however, very few preprocambial gene expression profiles have been identified. Here, we show that expression of three genes encoding members of the DOF family of plant-specific transcription factors is activated at stages overlapping onset of ATHB8 expression. Expression of DOF genes is initiated in wide domains that become confined to sites of vein development. Congruence between DOF expression fields and zones of vein formation persists upon experimental manipulation of leaf vascular patterning, suggesting that DOF expression identifies consistently recurring steps in vein ontogeny. Our results contribute to defining preprocambial cell identity at the molecular level.

KEY WORDS: Arabidopsis thaliana, leaf development, vein patterning, procambium, auxin transport

Introduction

The vascular system of plants is composed of strands that extend and intersect throughout all organs (Esau, 1965). Vascular strands are responsible for long-distance transport of water and nutrients, and are source of signals that act locally, to direct the development of neighboring cells, and systemically, to coordinate the initiation of new shoot organs with that of new roots (Berleth and Sachs, 2001). Sites of vascular strand differentiation are defined during organ development by emergence of continuous lines of elongated vascular-precursor procambial cells (Esau, 1943).

Few natural phenomena have attracted more widespread attention than the patterned formation of vascular strands in the leaf. From a developmental standpoint, the process is particularly fascinating because it seems to be organized de novo in each leaf primordium. At early stages of leaf ontogeny, in fact, the cells located beneath the epidermis appear as a morphologically homogeneous population of tightly connected, polygonal, isodiametric cells (‘ground cells’) (Smith, 1934; Foster, 1936, 1952); during leaf development, complementary, anatomically inconspicuous subsets of ground cells will differentiate to generate procambial strands and the photosynthetic tissue of the leaf, the mesophyll.

The molecular details of the mechanism by which cells acquire procambial identity during organ development are not entirely clear, but transport and transduction of the plant signaling molecule auxin have long been implicated in defining paths of vascular differentiation (Berleth et al., 2000; Sachs, 1981). In leaf development, ground cells are directed towards procambial fate through induction of wide domains of expression of the PINFORMED1 (PIN1) auxin exporter and of the auxin response

Abbreviations used in this paper: CFP, cyan fluorescent protein; DAG, days after germination; DOF, DNA-BINDING WITH ONE ZINC FINGER; GFP, green fluorescent protein; HD-ZIP III, class III HOMEODOMAIN-LEUCINE ZIPPER; LUT, look-up table; NPA, 1-N-naphthylphthalamic acid; YFP, yellow fluorescent protein.

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transcription factor MONOPEROS (MP) (Donner et al., 2009; Hardtke and Berleth, 1998; Scarpella et al., 2006; Wenzel et al., 2007). Decay of PIN1 and MP expression and associated relapse to ground state occur in some of the cells, and domains of PIN1 and MP expression are eventually restricted to individual files of cells that will elongate into procambial cells (Donner et al., 2009; Scarpella et al., 2006; Wenzel et al., 2007).

While onset of PIN1 and MP expression marks an unstable and reversible state in formation of leaf vascular strans (or ‘veins’), lines of PIN1 and MP-expressing ground cells that are stabilized towards procambial fate activate expression of the class III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP III) gene ATHB8 (Baima et al., 1995; Donner et al., 2009; Kang and Dengler, 2004; Sawchuk et al., 2007; Scarpella et al., 2004). Initiation of ATHB8 expression is directly controlled by MP (Donner et al., 2009), and identifies acquisition of a critical and typically irreversible ‘procambial’ cell state that accurately predicts sites of vascular differentiation (Alonso-Perale et al., 2006; Candela et al., 2001; Carland and Nelson, 2004; Cnops et al., 2006; Donner et al., 2009; Kang and Dengler, 2004; Koizumi et al., 2000; Petricka and Nelson, 2007; Pineau et al., 2005; Sawchuk et al., 2007, 2008; Scarpella et al., 2004, 2006). A view of the transcriptional landscape of cells at procambial stages would be particularly valuable as it might provide insight into the molecular pathways underlying vein formation. However, very few genes have been identified whose expression is initiated at stages prior to procambium formation [e.g., (Alonso-Perale et al., 2006; Baima et al., 1995; Carland and Nelson, 2009; Hardtke and Berleth, 1998; Kang and Dengler, 2004; Konishi and Yanagisawa, 2007; Scarpella et al., 2006; Wenzel et al., 2007)]. Therefore, to identify new preprocambial gene expression profiles, we interrogated an available global gene expression map of the Arabidopsis root (Birnbaum et al., 2003) to extract root vascular-specific gene expression datasets. We focused on genes encoding members of the DOF family of plant-specific transcription factors because their evolutionary diversification is associated with the compartmentalization of the plant body into separate organs (Shigyo et al., 2007). We found that nine of the 36 DOF genes in Arabidopsis (Lijavetzky et al., 2003; Yanagisawa, 2002) displayed a strong expression bias for root vascular cells (Fig. 1). Detailed expression data for five of these nine DOF genes are already available and support their vascular-specific expression in roots and other organs (Gualberti et al., 2002; Skirycz et al., 2006; Ward et al., 2005; Zhao et al., 2005), suggesting that preselecting vascular gene expression profiles based on root expression patterns from whole-genome microarray datasets may be an effective criterion. Here we investigated leaf expression of the remaining four DOF genes 2.1, 3.1, 4.6 and 5.3.

Expression of DOF genes in seedling organs

Because the upstream noncoding region is sufficient to recapitulate the endogenous transcript accumulation pattern in 80% of the cases for 44 Arabidopsis transcription factors (Lee et al., 2006), to visualize DOF gene expression patterns at high resolution, we employed transcriptional reporter gene fusions with an

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Root cap</th>
<th>Epidermis</th>
<th>Cortex</th>
<th>Endodermis</th>
<th>Stele</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOF1.1</td>
<td>At1g07640</td>
<td>16 ± 7.8</td>
<td>18 ± 8.9</td>
<td>33 ± 16.5</td>
<td>42 ± 20.8</td>
<td>114 ± 56.5</td>
</tr>
<tr>
<td>DOF2.1</td>
<td>At2g28510</td>
<td>22 ± 4.3</td>
<td>17 ± 3.4</td>
<td>69 ± 13.9</td>
<td>76 ± 15.2</td>
<td>123 ± 24.7</td>
</tr>
<tr>
<td>DOF2.2</td>
<td>At2g28810</td>
<td>28 ± 7.1</td>
<td>18 ± 4.6</td>
<td>35 ± 8.7</td>
<td>35 ± 8.9</td>
<td>104 ± 26.3</td>
</tr>
<tr>
<td>DOF2.5</td>
<td>At2g46590</td>
<td>11 ± 5.7</td>
<td>8 ± 4.4</td>
<td>29 ± 15.7</td>
<td>28 ± 14.8</td>
<td>60 ± 32.4</td>
</tr>
<tr>
<td>DOF3.1</td>
<td>At3g21270</td>
<td>35 ± 12.1</td>
<td>28 ± 9.7</td>
<td>39 ± 13.6</td>
<td>41 ± 14.4</td>
<td>121 ± 41.9</td>
</tr>
<tr>
<td>DOF3.6</td>
<td>At3g55370</td>
<td>2 ± 1.4</td>
<td>4 ± 3.3</td>
<td>9 ± 7.0</td>
<td>6 ± 4.3</td>
<td>36 ± 26.5</td>
</tr>
<tr>
<td>DOF3.7</td>
<td>At3g61850</td>
<td>26 ± 12.2</td>
<td>19 ± 8.6</td>
<td>74 ± 34.4</td>
<td>69 ± 32.2</td>
<td>289 ± 134.2</td>
</tr>
<tr>
<td>DOF4.6</td>
<td>At4g24060</td>
<td>15 ± 5.4</td>
<td>14 ± 4.9</td>
<td>78 ± 27.5</td>
<td>67 ± 23.5</td>
<td>170 ± 59.9</td>
</tr>
<tr>
<td>DOF5.3</td>
<td>At5g60200</td>
<td>3 ± 0.7</td>
<td>6 ± 1.5</td>
<td>26 ± 6.2</td>
<td>29 ± 7.1</td>
<td>168 ± 40.7</td>
</tr>
</tbody>
</table>

Fig. 1. Chart of DOF expression in root tissues. Heat map showing levels of DOF expression in different tissues of the Arabidopsis root. Data compiled in (Birnbaum et al., 2003) were interrogated with the AGI codes of the 36 DOF genes in Arabidopsis (Lijavetzky et al., 2003; Yanagisawa, 2002) through the Arabidopsis eFP browser tool (Winter et al., 2007), and profiles of the nine genes with biased expression in vascular cells (‘stele’) are represented. Values represent mean ± SD of gene expression levels at development stages I-III (see (Birnbaum et al., 2003) for more details). To visualize changes in gene expression, a light-to-dark blue look-up table (LUT) with 25-percentile color steps was adopted, in which darker shades represent progressively stronger expression.
DOF expression in vein formation

DOF expression in vein formation

DOF expression in first leaf development

DOF expression during leaf development

We first asked whether DOF promoter activity could recapitulate the root vascular-specific expression suggested by transcript profiling (Birnbaum et al., 2003). To address this question, we imaged expression of DOF5.3pro:mGFP4er (Lee et al., 2006), DOF2.1pro:HTA6:EYFP, DOF3.1pro:HTA6:EYFP and DOF4.6pro:HTA6:EYFP in the root of seedlings 4 days after germination (DAG). While transcriptional fusions of DOF2.1, DOF4.6 and DOF5.3 were expressed in root vascular cells, DOF3.1pro:HTA6:EYFP fluorescence was confined to the quiescent centre region (Fig. 2 A-D), suggesting that, at least in the root, patterns of DOF3.1 promoter activity may not reflect the gene’s transcriptional profile.

We next asked whether DOF gene expression was associated with sites of vascular strand formation in the leaf. To address this question, we visualized expression of DOF2.1pro:HTA6:EYFP, DOF3.1pro:HTA6:EYFP, DOF4.6pro:HTA6:EYFP and endoplasmic reticulum-targeted GFP (mGFP4er) (Haseloff et al., 1997) or a nuclear localized YFP (HTA6:EYFP) (Zhang et al., 2005).

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expression of **DOF** fusions occupied wider territories, which in **DOF**5.3pro:mGFP4er comprised two adjacent columns of cells and in **DOF**2.1pro:HTA6:EYFP encompassed nearly all subepidermal cells. Expression of fusions of **ATHB**8, **DOF**2.1 and **DOF**4.6 was additionally detected at the tip of the 2.5-DAG primordium. At 3 DAG, all fusions were strongly expressed at sites of midvein and first loop appearance; however, **DOF** promoters were active in broader domains than **ATHB**8pro:HTA6:EYFP, with only slightly expanded fields of **DOF**5.3pro:mGFP4er expression at the apical side of the developing first loops and weak **DOF**2.1pro:HTA6:EYFP fluorescence in almost all subepidermal cells (Fig. 3 F,J,N,R). At 4 DAG, activity of all fusions marked zones of formation of midvein, first and second loops, and higher-order veins, even though levels of midvein-associated **DOF**5.3pro:mGFP4er expression were considerably lower than those detectable in all other veins (Fig. 3 G,K,O,S). Further, while domains of **ATHB**8 promoter activity were equally narrow in all developing veins, expression of **DOF** fusions in prospective second loops and higher-order veins pervaded larger fields of cells than in the emerging midvein and first loops. Finally, at 5 DAG, all promoters directed expression in developing midvein, first, second and third loops, and in higher-order veins (Fig. 3 H,L,P,T), but fields of **DOF** fusion activity were wider in veins emerging in basal areas of the leaf, and **DOF**5.3pro:mGFP4er expression had subsided in midvein and first loops.

In summary, expression of all **DOF** genes seemed to be tightly associated with regions of vascular strand formation throughout leaf development.

### Stage-specific **DOF** expression in vein formation

Comparison between **DOF** and **ATHB**8 expression profiles during leaf development (Fig. 3) suggests that expression of **DOF** genes is initiated as early as that of **ATHB**8, and that therefore **DOF** expression could be assigned to preprocambial stages of vein formation. An unambiguous criterion to test such a hypothesis, however, would be to visualize expression of individual **DOF** genes and **ATHB**8 within the same sample. We therefore tested the degree of colocalization between **DOF**2.1pro:HTA6:EYFP and **ATHB**8pro:ECFP-Nuc (Sawchuk et al., 2008), between **DOF**4.6pro:HTA6:EYFP and **ATHB**8pro:ECFP-Nuc, and between **DOF**5.3pro:mGFP4er and **ATHB**8pro:HTA6:EYFP.

Covisualization of **DOF** transcriptional fusion signals and inception of **ATHB**8promoter activity showed overlapping expression of the fluorescent reporters (Fig. 4 D-L), suggesting that **DOF** expression is initiated at preprocambial stages. However, at onset of
**DOF expression in vein formation**

*DOF* expression in vein formation

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ATHB8 promoter activity, expression domains of *DOF* fusions were always wider than those of *ATHB8*. Further, domains of *ATHB8* fusion expression were visible that did not extend throughout the full length of fields of *DOF* promoter activity (Fig. 4 D-F, J-L). Finally, we observed entire expression domains of *DOF* fusions that were totally devoid of *ATHB8* promoter activity (Fig. 4 G-I).

DOF expression in auxin transport-inhibited leaves

We finally asked whether expression of *DOF* genes remained associated with zones of leaf vascular strand formation upon experimental interference with vein patterning. Auxin transport has been shown to define sites of vein appearance in developing leaf primordia (Mattsson et al., 1999; Scarpella et al., 2006; Sieburth, 1999). Therefore, we grew seedlings harboring the *DOF* and *ATHB8* transcriptional fusions in the presence of the auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) and imaged fluorescent protein expression in first leaves at 3, 4, and 5 DAG.

Leaves of plants germinated and grown in the presence of auxin transport inhibitors are characterized by a number of distinct anomalies in vascular organization; most conspicuously, great numbers of broad vein loops fuse along the entire margin of the leaf, to give rise to a wide zone of vascular differentiation, and extend parallel at the centre of the leaf, to give rise to a laterally expanded midvein (Mattsson et al., 1999; Sieburth, 1999). As shown in Figure 5, domains of *ATHB8* pro:HTA6:EYFP expression retained their characteristically slender shape throughout development of auxin transport-inhibited leaves (Fig. 5 A-C). Expression of *DOF* transcriptional fusions, on the other hand, appeared extremely expanded in leaves with reduced auxin transport, such that *DOF* promoters were active in nearly all subepidermal cells at 3 DAG (Fig. 5 D,G,J). Nevertheless, quasi-ubiquitous expression of *DOF* fusions became resolved into more discrete domains, which were already visible in the apical half of 4-DAG leaves (Fig. 5 E,H,K) and became further restricted in leaves at 5 DAG (Fig. 5 F,I,L).

In conclusion, the association between *DOF* expression domains and regions of vein formation observed under undisturbed conditions persisted in auxin transport-inhibited leaves, suggesting non-circumstantial correlation between zones of *DOF* expression and sites of vein emergence.

Discussion

The molecular details of the mechanisms controlling the recruitment of ground cells in the leaf towards procambium formation are largely unknown. Available evidence, however, suggests that the selection process terminates with emergence of files of ground cells that have activated expression of the HD-ZIP III gene *ATHB8* and that will successively elongate into procambial cells (Alonso-Peral et al., 2006; Candela et al., 2001; Carland and Nelson, 2004; Cnops et al., 2006; Donner et al., 2009; Kang and Dengler, 2004; Koizumi et al., 2000; Petricka and Nelson, 2007; Sawchuk et al., 2007, 2008; Scarpella et al., 2004, 2006). Therefore, the events preceding acquisition of the *ATHB8* preprocambial cell state are crucial for vein formation.

In this study, we have sought gene expression profiles that were initiated at preprocambial stages. We have found that expression of *DOF2.1*, *DOF4.6* and *DOF5.3*, which encode members of the *DOF* family of plant-specific transcription factors (Lijavetzky et al., 2003; Yanagisawa, 2002), reproducibly identifies morphologically inconspicuous cell states in the process that culminates into onset of *ATHB8* expression.

Cell state transitions in vein formation

Transcription of *DOF2.1*, *DOF4.6* and *DOF5.3* could be con-
trolled by regions other than the upstream noncoding sequences used here to monitor their expression, and abundance of transcripts of DOF2.1, DOF4.6 and DOF5.3 could be regulated at the post-transcriptional level. However, our results are in good agreement with expression profiles extracted from publicly accessible large-scale microarray data sets (Birnbaum et al., 2003; Schmid et al., 2005; Winter et al., 2007), suggesting that expression patterns of DOF2.1, DOF4.6 and DOF5.3 can be accurately visualized by transcriptional fusions.

During leaf development, DOF2.1, DOF4.6 and DOF5.3 were expressed in seemingly overlapping subepidermal domains and with amazingly comparable dynamics. At early stages of leaf development, very low levels of DOF2.1 expression embraced all ground cells; within these fields, however, broad domains of maximum expression of DOF2.1 were distinguishable that became associated with sites of vein emergence, as identified by ATHB8 expression. During subepidermal tissue ontogeny, weak DOF2.1 expression became extinguished from subsets of ground cells, leaving only the intense vein-associated expression domains. Expression of DOF4.6 and DOF5.3 was initiated in wide domains that seemed to coincide with peaks of DOF2.1 expression, but their expression never incorporated all surrounding ground cells. Expression of DOF2.1 and DOF4.6 was sustained at all stages of vein formation, while that of DOF5.3 became terminated during procambium differentiation. Areas of DOF expression overlapped with sites of initiation of ATHB8 expression, suggesting that DOF2.1, DOF4.6 and DOF5.3 are expressed at procambial stages. However, that discrete DOF expression domains were visible that were partially or completely free of ATHB8 expression may suggest that expression of DOF2.1, DOF4.6 and DOF5.3 is initiated prior to acquisition of the ATHB8 procambial cell state. Alternatively, or in addition, this observation may point to transient appearance of DOF expression in cells that will never activate expression of ATHB8.

If congruence between expression of DOF2.1, DOF4.6 and DOF5.3 and sites of vein formation is more than a coincidence, one would expect to observe such association even under conditions of manipulated leaf vascular patterning. Expression of DOF genes in leaves with reduced auxin transport, which dramatically changes the shape of vein networks (Mattsson et al., 1999; Sieburth, 1999), retained dynamics comparable to those observed under undisturbed development. Furthermore, all aspects of DOF expression, including onset, intensity, decline, relation to ATHB8 expression and association with vein-forming cells under all experimental conditions proved to be highly reproducible. We therefore suggest that expression of DOF2.1, DOF4.6 and DOF5.3 identifies regularly recurring steps in procambial development.

Unlike ATHB8, DOF expression was always initiated in wide domains, and ATHB8 expression appeared within broad DOF expression domains. Furthermore, initially-wide fields of DOF expression became laterally confined over time, while ATHB8 expression domains are always narrow at inception and progress longitudinally during vein formation. In this respect, expression of DOF2.1, DOF4.6 and DOF5.3 resembles that of genes that have been functionally implicated in selection of ATHB8-expressing procambial cells [e.g., (Alonso-Perera et al., 2006; Candela et al., 2001; Carland and Nelson, 2004; Donner et al., 2009; Hardtke and Berleth, 1998; Petricka and Nelson, 2007; Sawa et al., 2005; Sawchuk et al., 2007; Scarpella et al., 2006)]. However, DOF2.1, DOF4.6 and DOF5.3 are not expected to be directly involved in this process because preprocambial expression of ATHB8 is under the immediate control of MP through a noncanonical auxin response element located in the ATHB8 promoter (Donner et al., 2009).

Vascular expression profiles of DOF genes

The DOF genes whose leaf expression was investigated here were selected because of their biased expression for root vascular cells in an Arabidopsis transcription map (Birnbaum et al., 2003), and because their leaf expression had not previously been reported. Root vascular expression was recapitulated by patterns of promoter activity for three of the four DOF genes, and all of the three root vascular-specific promoters were also active at early stages of vascular strand formation in the leaf. Preselecting vein-associated gene expression profiles based on root expression patterns proved to be a valuable strategy, but this does not exclude the possibility that other DOF genes may be expressed in leaf vascular strands. Indeed, DOF5.8, which is negligibly expressed in root microarray datasets, displays prominent vein-associated expression (Konishi and Yanagisawa, 2007). Tissue-specific expression patterns are available for 13 of the 36 Arabidopsis DOF genes (Fornara et al., 2009; Gardner et al., 2009; Gualberti et al., 2002; Imaizumi et al., 2005; Konishi and Yanagisawa, 2007; Skirycz et al., 2006, 2007, 2008; Ward et al., 2005). These 13 genes sample the diversity of the DOR family, yet all of them appear to be expressed in vascular strands. While it will be interesting to understand the significance of the association between the expression of at least a large fraction of DOF genes and vascular cells, our study already contributes to the characterization of a largely unexplored class of plant-specific transcription factors.

Materials and Methods

Terminology

We apply the generic term ‘subepidermal’ to all positions of the leaf beneath the epidermis. We refer to ‘ground cells’ as polygonal, isodiametric, subepidermal cells of the leaf. We use the terms ‘procambial’ and ‘procambium’ to indicate morphologically identifiable vascular cell precursors. We designate as ‘preprocambial’ all stages of vein development prior to procambium formation.

Vector construction

To generate DOF transcriptional fusions, the genes’ entire noncoding regions were amplified from Arabidopsis (Arabiopsis thaliana) ecotype Col-0 genomic DNA using Finnzymes Phusion high-fidelity DNA polymerase (New England Biolabs Inc., Ipswich, MA, USA) and gene-specific primers (Supplementary Table S1), integrated into pDONR221 (Invitrogen, Carlsbad, CA, USA) with BP clonase II (Invitrogen), sequence-checked, and recombined into the Gateway-adapted pFYTAG binary vector, which contains a translational fusion between the coding region of histone 2A (HTA6; AT5g59870) and that of the enhanced YFP (EYFP) (Zhang et al., 2005), using LR clonase II (Invitrogen).

Plant material and growth conditions

The origins of the ATHB8pro:HTA6:YFP, DOF5.3pro:mGFP4er and ATHB8pro:EYFP-Nuc have been described (Lee et al., 2006; Sawchuk et al., 2007, 2008). Seeds were sterilized and germinated, and seedlings and plants were grown, transformed and selected as described (Sawchuk et al., 2007, 2008).
2007, 2008). For DOF2:1pro:HTA6:EYFP, DOF3:1pro:HTA6:EYFP and DOF4:8pro:HTA6:EYFP, the progeny of 11 to 18 independent transgenic lines were inspected to identify the most representative expression pattern. Successive expression analysis was performed on the progeny of at least four lines per construct, which were selected because of strong YFP expression that was emblematic of the expression profile observed across the entire series of transgenic lines and that resulted from single insertion of the transgene. For DOF5:3pro:mGFP4er, expression analysis was performed on the progeny of two lines per construct (JYB818.3, ABRC stock number: CS70640; JYB821.1, ABRC stock number: CS70641). In genetic crosses, the progeny of at least two independent lines per construct were examined. For auxin transport inhibition, seeds were germinated on growth medium supplemented with 2.5 μM NPA (Chem Service Inc., West Chester, USA). We define ‘days after germination’ (DAG) as days following exposure of imbibed seeds to light.

Microscopy and image processing

Dissected seedling organs were mounted and imaged as described (Donner et al., 2009; Sawchuk et al., 2007, 2008). Brightness and contrast were adjusted through linear stretching of the histogram in ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij). Signal levels and colocalization were visualized as described (Sawchuk et al., 2008).

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