DROOPY LEAF1 controls leaf architecture by orchestrating early brassinosteroid signaling

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Leaf architecture directly determines canopy structure, and thus, grain yield in crops. Leaf droopiness is an agronomic trait primarily affecting the cereal leaf architecture but the genetic basis and underlying molecular mechanism of this trait remain unclear. Here, we report that DROOPY LEAF1 (DPY1), an LRR receptor-like kinase, plays a crucial role in determining leaf droopiness by controlling the brassinosteroid (BR) signaling output in Setaria, an emerging model for Panicoideae grasses. Loss-of-function mutation in DPY1 led to malformation of vascular sclerenchyma and low lignin content in leaves, and thus, an extremely droopy leaf phenotype, consistent with its preferential expression in leaf vascular tissues. DPY1 interacts with and competes for SI BAK1 and as a result, causes a sequential reduction in SIBRI1–SIBAK1 interaction, SIBRI1 phosphorylation, and downstream BR signaling. Conversely, DPY1 accumulation and affinity of the DPY1–SIBAK1 interaction are enhanced under BR treatment, thus preventing SIBRI1 from overactivation. As such, those findings revealed a negative feedback mechanism that represses leaf droopiness by preventing an overresponse of early BR signaling to excess BRs. Notably, plants overexpressing DPY1 have more upright leaves, thicker stems, and bigger panicles, suggesting potential utility for yield improvement. The maize ortholog of DPY1 rescues the droopy leaves in dpy1, suggesting its conserved function in Panicoideae. Together, our study provides insights into how BR signaling is scrutinized by DPY1 to ensure the upward leaf architecture.

Significance

C4 cereals in subfamily Panicoideae typically produce large and long leaf blades for efficient capture of light and photosynthesis but the leaves drop downward, particularly at the adult stage, thus, adversely affecting canopy structure and grain yield. Identification of key regulators that control leaf droopiness is crucial to improve plant architecture in these crops. We showed that DPY1, a regulator of SIBRI1–SIBAK1 interaction, prevents BR signaling from overactivation in response to high doses of BRs to ensure that the long leaf blades grow upward in Setaria. Overexpression of DPY1 improves plant architecture with upright leaves. This study provides cellular and molecular insights into plant architecture control for cereal breeding.

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DPY1 Encodes a Plasma Membrane-Located Leucine-Rich Repeat Receptor Kinase (LRR-RK) and Functions Conservatively in the Panicoideae Subfamily. By map-based cloning and MutMap analysis, a point mutation was identified in the third intron of *Seita.5G121100*, resulting in a premature stop codon in the *dp1* transcript (Fig. 2 A and B and SI Appendix, Fig. S3). Thus, *Seita.5G121100* was locked in as a candidate for DPY1. To verify it *Seita.5G121100* is DPY1, the CRISPR/Cas9 system was used to knock out *Seita.5G121100* in WT plants. Two independently edited plants (CR-1 and CR-4) were generated (SI Appendix, Fig. S4A), which phenocopied *dp1*, showing conspicuously droopy leaves at the seedling stage and completely drooped leaves at the heading stage (Fig. 2 C and SI Appendix, Fig. S4B). The droopy leaves of *dp1* were rescued by transformation with a 7.0-kb *Seita.5G121100* genomic fragment fused to GFP (Fig. 2 C and SI Appendix, Fig. S4C). Thus, *Seita.5G121100* is responsible for DPY1. Overexpression of FLAG-tagged DPY1, under control of the strong maize Ubiquitin-1 promoter (Ubi::DPY1-3FLAG), resulted in more upright leaf architecture, a thicker stem, and more grains per panicle without a compromise in grain weight, compared to the WT (SI Appendix, Fig. S5), indicating the potential for crop improvement by manipulating DPY1 expression. Phylogenetic analysis revealed that DPY1, an ortholog of *Arabidopsis* NSP-interacting kinase 3 (NIK3), is a member of subfamily II of LRR-RKs and evolutionarily conserved in dicot and monocot species (SI Appendix, Fig. S6A). Two DPY1 homologs were found in maize (GRMZM2G010693/GRMZM2G067675) (SI Appendix, Fig. S6A). We cloned GRMZM2G010693 and overexpressed it under control of the Ubiquitin-1 promoter (Ubi::2zmDPY1-3FLAG) in *dp1*. The droopy leaf was fully rescued in the transgenic plants (Fig. 2 C and SI Appendix, Fig. S6B), suggesting the conserved function of DPY1 between rice and maize.

DPY1 protein structure analysis predicted that DPY1 contains a signal peptide at the N terminus, followed by five tandem LRRs, a transmembrane domain, and a serine/threonine protein kinase domain (SI Appendix, Fig. S6B), suggesting that DPY1 is likely a putative transmembrane kinase protein. Confocal imaging of foxtail millet leaf protoplasts transiently expressing 35S::DPY1-GFP showed clear plasma membrane localization of DPY1-GFP (Fig. 2D). qRT-PCR analysis showed that DPY1 had the highest expression in leaf blades (Fig. 2E). Histochemical analysis of 35S::GUS transgenic leaf blades (DPY1 promoter fused to β-glucuronidase) revealed vasculature-preferential GUS activity (Fig. 2F and G). This expression profile was recapurred in plants carrying DPY1-GFP fusion, driven by the DPY1 promoter (Fig. 2H), supporting the role of DPY1 in vascular tissue development.

DPY1 Directly Interacts with SIBAK1, but Not SIBRI1, in a Partial Phosphorylation-Dependent Manner. To study how DPY1 functions, an immunoprecipitation (IP) assay with an anti-FLAG antibody was performed using Ubi::DPY1-3FLAG transgenic foxtail millet, followed by liquid chromatography-tandem mass spectrometry (MS) analysis to isolate DPY1-interacting proteins. A list of putative DPY1-interacting proteins was identified, including XP_004972726, an ortholog of the BR coreceptor BAK1 (SI Appendix, Table S2). Since DPY1 may be involved in BR signaling, as indicated by the changed sensitivity of *dp1* to BL or BRZ treatment (Fig. 1I), we chose XP_004972726, named when grown in the dark. A stimulation effect of BL on coleoptile elongation was seen at a concentration as low as 0.01 nM and an inhibition effect at 10 nM in *dp1* (Fig. 1I), whereas the corresponding effects in the WT were seen at 100-fold higher concentrations, indicating enhanced sensitivity to BL in *dp1*. Conversely, when treated with BRZ (an inhibitor of BR synthesis), coleoptile elongation was inhibited starting at 5 nM in the WT, whereas a similar inhibition in *dp1* started at a 100-fold higher concentration (Fig. 1J). These findings suggest the involvement of DPY1 in BR signaling.

Results

dpy1 Shows Droopy Leaf Associated with Increased Sensitivity to BL Treatment. To investigate the developmental control of leaf architecture in foxtail millet, we performed ethyl methanesulfonate mutagenesis using the variety Yugu1 (wild type [WT]). We isolated a mutant that showed the most dramatically curved-down leaf blades, thus supporting the upright leaf architecture. Our findings fine tune a mechanism that governs BR1 activity in a tissue-specific manner and show possibility to use a BR signaling modulator for crop improvement.

KINASE1 (BAK1), forming a stable high-affinity complex through their leucine-rich repeat (LRR) domains (10–13). This allows proximity of the intracellular kinase domains that transphosphorylate each other (14–16), resulting in BR1 activation and BR1 kinase inhibitor (BK1) dissociation (17). Then, BR-activated BR1 modulates a cascade of kinases and phosphatases to transduce signaling from the cell membrane to the cytoplasm (8, 18, 19), where the key node transcription factors BRASSINAZOLE-RESISTANT1 (BZR1) and BR1-EMS-SUPPRESSOR1 (BES1/BZR2) are dephosphorylated, and move into the nucleus to activate global BR responses (20, 21). While intense signaling activity is presumably essential for the initiation of signaling transduction, several lines of evidence show that feedback regulation at the signal perception stage is crucial for proper signal output and normal plant development. For example, a SUPPRESSOR of BR1 (SB1)–Protein Phosphatase 2A (PP2A) complex located on the plasma membrane has been shown to negatively regulate early BR signaling by inactivating BR1 (22, 23). BR induces SB1 expression, which, in turn, promotes PP2A to associate with plasma membrane, where it selectively dephosphorylates/inactivates BR-activated BR1 to maintain a proper signal output. The importance of feedback regulation in early BR signaling is less documented in cereal plants, especially with regard to leaf blade development.

Here, we used foxtail millet (Setaria italica), a new model species for C4 and the Panicoideae grasses (24, 25), to study leaf architecture control. We isolated a droopy leaf mutant, namely, droopy leaf 1 (*dpy1*). DPY1 represses early BR signaling to release BR inhibition on the proliferation of both clear cells and abaxial sclerenchyma cells in leaf veins and lignin deposition in leaf blades, thus supporting the upright leaf architecture. Our findings fine tune a mechanism that governs BR1 activity in a tissue-specific manner and show possibility to use a BR signaling modulator for crop improvement.
Transverse section of the midrib at 5 cm distal to the blade-sheath joint of an adult leaf, showing few clear cells and less organized veins in bimolecular fluorescence complementation (BiFC) assay in Arabidopsis (A) level detectable by a pull-down assay (D). The yeast growth results showed that DPY1 interacted with ubiquitin membrane-based yeast two-hybrid system (Fig. 3D). Interaction between DPY1 and SiBAK1 was also seen in a split-ubiquitin membrane-based yeast two-hybrid system (26), the KDs of DPY1 and SiBAK1 were purified and fused with His and MBP tag, respectively. A pull-down assay showed that SiBAK1-KD interacted with DPY1-KD (Fig. 3C), indicating that DPY1 and SiBAK1 directly interact through their KDs. Moreover, the interaction between DPY1 and SiBAK1 was also seen in a split-ubiquitin membrane-based yeast two-hybrid system (Fig. 3D). The yeast growth results showed that DPY1 interacted with SiBAK1, but not SiBRII (Fig. 3D). Introduction of a mutation into the kinase domain of DPY1 compromised the interaction to a level detectable by a pull-down assay (SI Appendix, Fig. S7), but not by the Y2H assay (Fig. 3D). These findings demonstrated that DPY1 kinase activity or phosphorylated status contributes to the interaction with SiBAK1.

DPY1 Represses BR Signaling and Prevents Leaf Droopiness by Competing with SiBRII for SiBAK1. Since the BR1–BAK1 association upon BR binding initiates early BR signal transduction and DPY1 physically interacts with SiBAK1 as shown above, we hypothesized that DPY1 represses BR signaling by diminishing the BR1–BAK1 interaction. An in vitro pull-down assay was then performed to examine the competition between DPY1 and SiBRII for SiBAK1. The results showed that SiBAK1 and SiBRII interacted with each other through their KDs, consistent with previous reports in other species (13), and such interactions were weakened by the addition of DPY1-KD in a dosage-dependent manner (Fig. 4A). Next, we transiently expressed 35S::SiBAK1-HA in foxtail millet leaf protoplasts of dpy1 and WT, respectively, precipitated protein complexes containing SiBAK1-HA with the anti-HA antibody, and examined the amount of coprecipitated SiBRII with the anti-SiBRII antibody that had been validated (SI Appendix, Fig. S8). When an equal amount of precipitated SiBAK1-HA was loaded, a remarkable increase in coprecipitated SiBRII was detected from dpy1, relative to that from WT (Fig. 4B and SI Appendix, Fig. S9B), demonstrating an enhanced in vivo SiBRII–SiBAK1 interaction in dpy1. We expected that enhancement of the SiBRII–SiBAK1 interaction in dpy1 would enhance transphosphorylation and, in turn, increase phosphorylated SiBRII. To test this, we immunoprecipitated endogenous SiBRII with anti-SiBRII antibody to detect the phosphorylation level of SiBRII by Western blotting with the anti-pThr antibody. The outcome showed that phosphorylated SiBRII was enhanced in dpy1 (Fig. 4C and SI Appendix, Fig. S9B). To further confirm elevated BR signaling in dpy1, the in vivo phosphorylation status of SiBZR1 (Seita.2G367800.1), a foxtail millet ortholog of BZR1 that is a key downstream component in transducing the BR signal upon dephosphorylation, was examined. We first confirmed the functionality of SiBZR1 by creating SiBZR1-overexpressing plants that displayed droopy leaves, a phenotype similar to that of dpy1 (SI Appendix, Fig. S10). The dephosphorylated SiBZR1 isofrom was significantly elevated in dpy1, to a level detected in the WT when treated with BL at 1 μM (Fig. 4D and SI Appendix, Fig. S8 B and C), indicating enhanced BR signaling in dpy1. Correspondingly, RNA-seq analysis revealed 5,276 differentially expressed genes (DEGs) in dpy1 and 4,108 DEGs in the BL-treated WT, compared with the mock-treated WT control (SI Appendix, Fig. S11 A and B), in which 2,009 and 1,498 putative BZR1 target genes, respectively, were identified. Roughly half of these BZR1 target genes (714 of 1,498) in the BL-treated WT
overlapped with that in dpy1 (714 of 2,009 DEGs) (Fig. 4E), and nearly 80% of them had similar expression trends in both groups (Fig. 4F and G). These findings support the repression of DPY1 in BR signaling in foxtail millet.

Additionally, we found down-regulation of most cyclin-like genes that have been shown to contribute to sclerenchyma cell proliferation in rice leaf joints (3) and of cell wall organization-related genes in dpy1 and BL-treated WT plants (SI Appendix,
Regulation to Prevent SiBRI1 from Overactivation.

BL Treatment Enhances DPY1 Accumulation as Negative Feedback Regulation to Prevent SiBRI1 from Overactivation. YABBY genes that have been reported to regulate leaf droopiness in maize (1), were not differently expressed (fold change < 1.5; SI Appendix, Fig. S11E). Expression decline of those genes may account for the decreased sclerenchyma cell number and lignin deposition observed in dpy1 (Fig. 1 F and G and SI Appendix, Table S1).

Finally, the supply of exogenous BL to dpy1 seedlings resulted in severely curled leaf blades, while the treatment with BR synthesis inhibitor BRZ partially rescued its droopy leaf phenotype (Fig. 4 H–J). The differential responses from WT suggest enhanced BR signaling in dpy1. Collectively, these findings demonstrate that DPY1 prevents leaves from drooping by negatively regulating early BR signaling.

BL Treatment Enhances DPY1 Accumulation as Negative Feedback Regulation to Prevent SiBRI1 from Overactivation.

To further examine the negative modulation mechanism of DPY1, we conducted in vitro kinase activity assays to examine if DPY1, we conducted in vitro kinase activity assays to examine if DPY1 could phosphorylate kinase-dead SiBRI1 (mSiBRI1) but DPY1 could not phosphorylate kinase-dead SiBAK1 (mSiBAK1) (Fig. 5 B). The increased phosphorylation of DPY1 was detected in protoplasts isolated from Ubi::DPY1-3FLAG transgenic plants treated with BL (Fig. 5 E and SI Appendix, Fig. S13B). Additionally, with SiBAK1-HA transiently expressed in protoplasts isolated from Ubi::DPY1-3FLAG transgenic plants, protein stabilization of DPY1-FLAG was enhanced relative to that of protoplasts expressing the vector control (Fig. 5 C and D).

The differential phosphorylation of DPY1 is critical for its interaction with SiBAK1 (Fig. 5 D and SI Appendix, Fig. S14A). Furthermore, the increased phosphorylation of DPY1 was detected in Ubi::DPY1-3FLAG transgenic plants treated with BL (Fig. 5 E and SI Appendix, Fig. S14B). Since phosphorylation of DPY1 is critical for its interaction with SiBAK1 (Fig. 5 D and SI Appendix, Fig. S7B), we proposed that BL-stimulated DPY1 phosphorylation would promote the DPY1–SiBAK1 interaction. A Co-IP assay with protoplasts coexpressing SiBAK1-HA and DPY1-GFP showed that BL treatment promoted the DPY1–SiBAK1 interaction, as indicated by the increase of communoprecipitated SiBAK1 under BL treatment along with the same amount of immunoprecipitated DPY1 (Fig. 5 F and SI Appendix, Fig. S15A). Similar results were

Fig. 3. DPY1 interacts with SiBAK1, but not SiBRI1, in a partial kinase-dependent manner. (A) The association between DPY1 and SiBAK1 was detected in a Co-IP assay. Protein extracts from protoplasts transiently expressing 35S::DPY1-GFP and 35S::SiBAK1-HA were immunoprecipitated with GFP-Trap beads (IP: α-GFP antibody) and immunoblotted with an α-HA antibody (Co-IP). (B) Interaction between DPY1 and SiBAK1 detected in a BiFC assay using tobacco epidermal cells. SiCK4 (Seita.1G023400), a closely related protein with DPY1 in the LRRII-RKs subfamily (see SI Appendix, Fig. S6A), was used as a negative control. (Scale bar, 25 μm.) (C) An in vitro pull-down assay showing the interaction of DPY1 with SiBAK1 via kinase domains (KDs). The MBP or MBP-SiBAK1-KD proteins were incubated with the His-DPY1-KD protein and immunoblotted with an α-His antibody (Co-IP). (D) DPY1 interacted with SiBAK1, but not with either SiBRI1 or mDPY1 (a dead version of DPY1, also see SI Appendix, Fig. S7A), in a DUAL-membrane yeast two-hybrid assay. Yeast growth is presented at three dilutions.
DPY1 competes with SiBRI1 to bind SiBAK1 to repress BR signaling. (A) An in vitro pull-down assay indicated that DPY1 competes with SiBRI1 to bind to SiBAK1. The MBP-SiBAK1-KD (kinase domain) protein was incubated with the GST-SiBRI1-KD protein with various amounts of His-DPY1-KD and immunoprecipitated with MBP beads (IP) and immunoblotted with an α-GST antibody (Co-IP). MBP and GST were used as negative control proteins. Coomassie brilliant blue (CBB) staining of input proteins is presented at the Bottom. Asterisk indicates the band of His-DPY1-KD. (B) A Co-IP assay in the WT and dpy1 protoplasts revealed the increased interaction between SiBAK1 and SiBRI1 in dpy1. Protein extracts from the WT or dpy1 protoplasts transiently expressing 35S::SiBAK1-HA or the vector control were immunoprecipitated with an α-HA antibody (IP) and immunoblotted with an α-SiBRI1 antibody (Co-IP), which had been validated (see SI Appendix, Fig. S8A). ImageJ was used to quantify signal intensity. Values represent the mean ± SD (n = 3). (C) Enhanced phosphorylation of endogenous SiBRI1 in dpy1 plants. SiBRI1 was immunoprecipitated with an anti-SiBRI1 antibody (IP) and its phosphorylation status was determined by immunoblotting (IB) with an anti-pThr antibody. ImageJ was used to quantify signal intensity. Values represent mean ± SD (n = 4). (D) Immunoblots with an anti-OsBZR1 antibody that had been validated in foxtail millet (see SI Appendix, Fig. S8B and C). Images indicate the increase in dephosphorylated SiBZR1 versus phosphorylated SiBZR1(SiBZR1-p) as a verification of enhanced BR signaling in dpy1 due to more phosphorylated SiBRI1 observed in C. The WT plants were treated with 1 μM BL to simulate an enhancement of dephosphorylated SiBZR1 as a comparison. (E) Venn diagram of differentially expressed BZR1 target genes in dpy1 and BL-treated WT plants relative to expression levels in untreated WT plants. A fold-change >2 (P < 0.01) was used as the cutoff. (F) Number of coexpressed (i.e., expression change in the same direction) and non-coexpressed genes among the 714 differentially expressed BZR1 target genes identified in both the WT_BL vs. WT and dpy1 vs. WT comparisons. (G) Heatmap visualizing the expression patterns of the 555 differentially expressed BZR1 target genes that were coexpressed in the above two groups. (H) Responses to BR and BR synthesis inhibitor BRZ in WT and dpy1 plants. Plants were treated with 5 μM BL or BRZ for 3 d. (Scale bar, 1 cm.) (I) Diagram illustrating the proximal-distal distance (PDD) and full length (FL) of a leaf blade. (J) PDD/FL ratio of the second leaf from Top in WT and dpy1 seedlings treated with mock, BL and BRZ, respectively. Error bars indicate the SD (n = 15). Different letters indicate significant differences at P < 0.01 by one-way ANOVA analysis.
also seen in a reverse Co-IP assay (SI Appendix, Fig. S15B). The dynamics of DPY1 interacting with SiBAK1 is different from that of other negative regulators of BR signaling, such as BAK1–INTERACTING RECEPTOR-LIKE KINASE 3 (BIR3), which is released from BAK1 upon BR perception (27). Nevertheless, the BL-enhanced DPY1–SiBAK1 interaction may raise the threshold of SiBRI1–SiBAK1 complex formation at the membrane.

Collectively, the above results revealed DPY1-mediated negative feedback regulation in early BR signaling. To further elucidate how such the regulation responses to BL treatment, we examined the SiBRI1–SiBAK1 interaction in the WT and dpy1 in response to BL treatment by employing a Co-IP assay using WT or dpy1 protoplasts expressing SiBAK1-HA treated with or without BL. The BL-stimulated SiBRI1–SiBAK1 interaction was more profound in dpy1 than in the WT (Fig. 5G and SI Appendix, Fig. S16). Consequently, BL treatment led to more phosphorylation of SiBRI1 in dpy1 (Fig. 5H and SI Appendix, Fig. S17). This is consistent with the observation that high-dose BL treatment results in more serious leaf droopiness in dpy1, relative to the WT (Fig. 4H–J). Additionally, we detected much higher levels of endogenous BL content in foxtail millet leaves (SI Appendix, Table S3) than in other cereal crops (28, 29). This finding may highlight the importance of DPY1 in preventing BR signaling from overactivation caused by a high BL level in foxtail millet (SI Appendix, Fig. S18).

Discussion

In this study, we identified a member of the subfamily II of LRR-RKs, DPY1, which supports the upward architecture of leaves by regulating proper output of early BR signaling in S. italica. In a DPY1 loss-of-function mutant, BR signaling is enhanced, and as a result, dpy1 plants develop droopy leaves and are more susceptible to pathogen attack (SI Appendix, Fig. S2). Our findings highlight the trade-off between BR-mediated growth and plant innate immunity.

We have demonstrated that DPY1 competes with SiBRI1 for SiBAK1 interaction, and that BL treatment up-regulates DPY1 transcripts and DPY1 protein level (SI Appendix, Fig. S12 and Fig. 5 A and B). Therefore, there should be two layers of negative feedback regulation to keep normal BR activity from over-activation. On one hand, DPY1 expression was promoted by BRs, particularly after 1 h of treatment (SI Appendix, Fig. S12), supporting the existence of a feedback loop from downstream signaling to DPY1 transcription. On the other hand, our results also suggest the existence of another feedback loop that stabilizes DPY1 and enhances DPY1–SiBAK1 affinity in the presence of excess BRs (Fig. 5 B and F). Further studies are needed to better understand the possible feedback mechanisms. Combining our data with the knowledge from the current literature, we hypothesize that DPY1 has a higher affinity than SiBRI1 to SiBAK1, which prevents SiBAK1 from interacting with SiBRI1 in the absence of BRs (SI Appendix, Fig. S18), as previously reported for BIR LRR receptor. 
such as FLS2 homologs, but are more susceptible to bacterial
pathogen attack, we propose that DPY1 mediates disease resistance
independent of the SiBAK1-dependent immune pathway, at least
for broad leaf disease. It would be interesting to investigate if
DPY1-overexpressing plants also gain disease resistance, in
addition to the observed improvement of leaf architecture and
potential yield increase.

We have demonstrated that DPY1-overexpressing plants ex-
hibit an improved plant architecture with upright leaf blades,
increased stem width, and yield per plant (SI Appendix, Fig. S5).
Thus, DPY1 may be relevant for cereal crop breeding. Moreover,
a maize DPY1 homolog fully rescues leaf droopiness of the
dpy1 mutant (Fig. 2C), suggesting that the regulatory function of DPY1
regarding leaf droopiness is conserved among species. Therefore,
DPY1 represents a candidate gene for the breeding of crops with
an enhanced plant architecture and yield. Our study data provide
cellular and molecular insights related to the plant architecture
in cereals.

Materials and Methods
A detailed description of plant materials, growth condition, map-based cloning of
DPY1, bioinformatics analysis of DPY1, paraffin section, vector construction,
plant transformation, RNA-seq analysis, protein–protein interaction assays, and
associated references is available in SI Appendix, Materials and Methods.

Data Availability. RNA sequencing raw data were deposited into the Euro-
pean Nucleotide Archive under accession number PRJEB31229. The primers
used in this study are listed in Dataset S1. Other study data are included in the
article and supporting information.

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