Rapid report

Broad spectrum developmental role of Brachypodium AUX1

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Summary

- Targeted cellular auxin distribution is required for morphogenesis and adaptive responses of plant organs. In Arabidopsis thaliana (Arabidopsis), this involves the prototypical auxin influx facilitator AUX1 and its LIKE-AUX1 (LAX) homologs, which act partially redundantly in various developmental processes. Interestingly, AUX1 and its homologs are not strictly essential for the Arabidopsis life cycle. Indeed, aux1 lax1 lax2 lax3 quadruple knock-outs are mostly viable and fertile, and strong phenotypes are only observed at low penetrance.

- Here we investigated the Brachypodium distachyon (Brachypodium) AUX1 homolog Bdaux1 by genetic, cell biological and physiological analyses.

- We report that Bdaux1 is essential for Brachypodium development. Bdaux1 loss-of-function mutants are dwarfs with aberrant flower development, and consequently infertile. Moreover, they display a counter-intuitive root phenotype. Although Bdaux1 roots are agrativtropic as expected, in contrast to Arabidopsis aux1 mutants they are dramatically longer than wild type roots because of exaggerated cell elongation. Interestingly, this correlates with higher free auxin content in Bdaux1 roots. Consistently, their cell wall characteristics and transcriptome signature largely phenocopy other Brachypodium mutants with increased root auxin content.

- Our results imply fundamentally different wiring of auxin transport in Brachypodium roots and reveal an essential role of Bdaux1 in a broad spectrum of developmental processes, suggesting a central role for AUX1 in pooidae.

Introduction

Modulation of auxin activity through differential auxin distribution plays a central role in developmental and adaptive growth processes (Benjamins & Scheres, 2008; Zazimalova et al., 2010). It is largely achieved through plasma membrane-integral auxin efflux carriers, the PIN-FORMED (PIN) proteins, whose polar cellular localization can lead to asymmetric auxin secretion. Coordination of PIN polarity across cell files thus can promote targeted, so-called polar auxin transport at the tissue and organ level (Benjamins & Scheres, 2008; Zazimalova et al., 2010). In contrast to the carrier requirement for auxin efflux, cellular auxin influx can occur through diffusion, because in the acidic environment of the apoplast auxin is mostly protonated and thus lipophilic enough to cross the plasma membrane (Zazimalova et al., 2010). Nevertheless, dedicated auxin influx facilitators, AUX1 and the LIKE AUX1 (LAX) proteins that accelerate auxin uptake have been identified (Maher & Martindale, 1980; Bennett et al., 1996; Marchant et al., 2001). Mutants in the three AUX1 homologs, LAX-3, display either no, or less conspicuous phenotypes (Ugartechea-Chirino et al., 2010; Vandenbussche et al., 2010; Peret et al., 2012). Their differential expression, as well as often polar localization, can modulate polar auxin transport to reinforce or attenuate local auxin accumulations. Arabidopsis thaliana (Arabidopsis) mutants in the prototypical auxin influx facilitator AUX1 have been identified because of their root agravitropism (Maher & Martindale, 1980), which can be rescued by addition of the lipophilic auxin analog 1-naphthylacetic acid (1-NAA) (Swarup et al., 2001). Mutants in the three AUX1 homologs, LAX-3, display either no, or less conspicuous phenotypes (Ugartechea-Chirino et al., 2010; Vandenbussche et al., 2010; Peret et al., 2012). However, corresponding multiple mutants reveal (partially) redundant roles of AUX1 and LAX-1, for instance in phyllostaxy (Bainbridge et al., 2008) and embryogenesis (Robert et al., 2018). This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
Although mutant phenotypes are not always fully penetrant. Moreover, AUX1 and LAX1-3 proteins are not fully interchangeable in every cellular context (Peret et al., 2012).

Compared to the well characterized roles of AUX1/LAX1-3 in Arabidopsis, little is known about the developmental role of auxin influx facilitators in monocotyledons (Balzan et al., 2014). Yet, AUX1 homologs can be readily identified, since they are highly conserved. For example, in rice (Oryza sativa) and the more distantly related panicoid grasses maize (Zea Mays L) and Setaria viridis (Setaria), five AUX1 homologs have been identified (Zhao et al., 2012; Huang et al., 2017). In maize, the closest AtAUX1 homolog has 73% sequence identity (Hochholdinger et al., 2000). Functional studies of mutants in AUX1 homologs in maize and Setaria demonstrated involvement of those genes in inflorescence development and root gravitropism (Huang et al., 2017). Also, the OsAUX1 gene has subsequently been implicated in lateral root formation and shoot elongation (Zhao et al., 2015), as well as seminal root elongation and root hair elongation (Yu et al., 2015). Although rice, maize and Setaria can be considered model systems for the grasses, it remains unclear whether findings from these species can be directly transferred to other groups within the poaceae. One such group is the pooidae, which comprise the major cereal crops wheat, rye and barley. The monocotyledon Brachypodium distachyon (Brachypodium) is a model species for these temperate cereals (Brkljacic et al., 2011; Girin et al., 2014). AUX1 homologs can be readily identified in the Brachypodium genome. However, unlike rice, maize or Setaria with five homologs, Brachypodium only possesses three AUX1 homologs, which display almost sequence identity with their Arabidopsis counterparts (Supporting Information Fig. S1). Nevertheless, slightly divergent N- and C-termini and the gene sequences allow the assignment of clear one-to-one homologies in sequence similarity analyses (Fig. 1a). Here we investigated the developmental role of the closest AUX1 homolog of Brachypodium, the Brachypodium distachyon AUX1 (BdAUX1) gene. We report that BdAUX1 loss-of-function results in counter-intuitive root phenotypes and reveals its essential role in a broad spectrum of developmental processes, suggesting a more central and diversified role for AUX1 in pooidae.

Materials and Methods

Plant materials, genotyping and growth conditions

The BdTar2<sup>PPo</sup> mutant has been described before (Pacheco-Villalobos et al., 2013). The Bdaux1 mutant line Jf5658 was obtained from a Brachypodium T-DNA insertion library (Bragg et al., 2012). RT-PCR was performed to verify that the T-DNA insertion indeed leads to a truncated Bdaux1 mRNA. To this end, the following oligonucleotides were used: F1 5′-ATG GTG CCG CGC GAG CAT G-3′, located at the start-codon; R1 5′-GCA TGA TCT CCA CGT TGA CG-3′, at the border of the T-DNA insertion; R2 5′-GTT GAA GCT GAG TAG CG-3′, located 285 bp before the STOP-codon; and R3 5′-GAT CGG GTA GTT GTG GAA GG-3′, located 160 bp before the T-DNA insertion (see Fig. S2A). Bdaux1<sup>CRISPP</sup> mutants were obtained directly as homozygotes from transformations (see below, 'Transformation') and could not be amplified due to their sterility. BdTar2<sup>PPo</sup> Bdaux1 double mutants were obtained by crossing. For tissue culture, seeds were sterilized as described (Bragg et al., 2012) and stratified for 3 d at 4°C before transfer to plates with half-strength Murashige-Skoog (MS) media (2.45 g l<sup>-1</sup> MS salts with vitamins, 0.3% sucrose, 1% agar, pH 5.7) placed vertically at a slight angle to prevent roots from growing into the media or the air. Unless indicated otherwise, analyses were performed on 2-d-old seedlings raised as previously described (continuous light of 100–120 μE intensity, 22°C, PhilipsF17T8/TL741 fluorescent light bulbs) (Pacheco-Villalobos et al., 2013). Roots that had grown into the media or the air were excluded from analysis. For gravitropism assays, seeds were grown for 1 d on vertically oriented plates, which were then rotated 90° and seedlings were left to grow for another 2 d. Root length was measured using Fiji software (https://imagej.net/Fiji?Downloads).

For auxin analysis, cell wall analysis and RNAseq, 1 cm seminal root segments harvested 2–3 mm above the root tip were used (Pacheco-Villalobos et al., 2016). Genotyping of BdTar2<sup>PPo</sup> was performed as described (Pacheco-Villalobos et al., 2013). For Bdaux1 genotyping, the wild type allele was monitored with primers 5′-GGT GAA GCT GAC GAG TAG CG-3′ and 5′-TCA CAA GAG CTT CGC AAT GG-3′, and the T-DNA insertion with 5′-GTG AAC TTT CCA CCT CAC TGA GC-3′ and 5′-CAA GAG CTG GGC AAT GG-3′, respectively.

Plasmid construction

To create a vector with kanamycin resistance, the nptII sequence was amplified with primers 5′-CCA TTC CCA CAC TAC GC-3′ and 5′-TCT GCA CCT AGT GCC AAC ATG-3′ using Gibson ligation. Bdaux1::NLS3xVENUS was created by insertion of amplified NLS3xVENUS into HindIII–PmI-digested pCAMBIA1305.1-nptII. Next, Bdaux1::BdAUX1 was amplified in three pieces from genomic DNA with primers 5′-CAT GAT TAC GAA TTC TGG CAG CTC ACT CCT CGT TAA CCT CGT CTC TCT CC-3′ for piece 1, 5′-CAG ATT GCC TAT AGC TCC TCT TCT CGT CCT CC-3′ for piece 2, and 5′-CAA TGC ACC TCA TGG TGA TCC CA-3′ for piece 3. Double mutants were genotyped with the same methodology for both T-DNA insertions.

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TCT AGA GGA TCC ATG GTG CCG CGC GAG CAT-3' and 5'-TTT TTC GGG TTA GAT TAA TTC-3', and GFP was amplified from pVec8GFP with primers 5'-ATG GTG AGC AAG GGC GAG G-3' and 5'-ATC CTC TAG AGT CGA CCT TGT ACA GCT CGT CCA TGC-3'. The three fragments were then combined into XmaI–PacI-digested pCAMBIA1305.1-nptII in a Gibson reaction. The BdAUX1 CRISPR/Cas9 cassette was created by amplifying the Zea mays UBIQUITIN (UBQ) promoter (Bragg et al., 2012) using primers 5'-GAG CTC CAG CTT GCA TGC CTG CAG TG-3' and 5'-GAG CTC TCT AGA GTC GAC CTG CAG AA-3' and ligation of the fragment into SacI-digested pCAMBIA1305.1. A Brachypodium-optimized Cas9 with FLAG-tag and nuclear localization signal (Methods S1), followed by a multiple cloning site, was synthesized and cloned.
behind the UBQ promoter after KpnI and BstXI digestion, to create vector p5Cas. Next, a 770 bp cassette containing a Brachypodium U6 promoter, BsaI restriction sites, tracrRNA, a rice U6 promoter, BtgZI restriction sites and tracrRNA was synthesized (see Methods S1) and cloned into BamHI–EcoRI-digested pDONR221. This allowed two sgRNA sequences to be added, using BsaI and BtgZI restriction sites, respectively. The Bdaux1 knock-out cassette was then assembled by annealing, phosphorylating and ligating the following primer pairs into the synthesized (see Methods S1) and cloned into U6 Brachypodium U6 create vector p5Cas. Next, a 770 bp cassette containing a paramomycin and 600 bp Brachypodium transformation (Pacheco-Villalobos et al., 2013) the closest homologs in Brachypodium (Fig. S2B).

**Microscopic**

For microscopic imaging, seminal roots of 2-d-old seedlings were fixed overnight at 4°C in 1% glutaraldehyde, 4% formaldehyde and 50 mM sodium phosphate buffer (pH 7.2). Roots were dehydrated for at least 1 h each in 15%, 30%, 50%, 70%, 85% and 100% ethanol (EtOH). Samples were pre-incubated and embedded in Technovit 7100 solution as described (Pacheco-Villalobos et al., 2013). 0.3-μm sections were obtained on a Leica RM2255 microtome. Sections were stained with 0.1% toluidine blue before visualization with a Leica DM5000 microscope. Cell numbers were counted in one representative image per root using the cell counter plugin of IMAGEJ software. (https://imagej.nih.gov/ij/plugins/cell-counter.html)

**Results and Discussion**

To investigate the role of auxin influx facilitators in Brachypodium, we obtained a T-DNA insertion line in Bradi2g55340 (BdAUX1 hereafter), the closest homolog of Arabidopsis AUX1 (AtAUX1) in Brachypodium. In this BdAUX1 mutant allele, BdAUX1 is disrupted by an insertion in the 6th intron, which leads to a truncated mRNA (Figs 1b, S2). Plants that were homozygous for this insertion displayed agravitropic roots (Fig. 1c), similar to Ataux1 loss-of-function mutants (Maher & Martindale, 1980; Bennett et al., 1996). Thus, the T-DNA insertion apparently results in BdAUX1 loss of function. However, unlike Ataux1 mutants, BdAUX1 mutant roots were considerably longer than those of their wild type siblings or the corresponding Bd21-3 wild type background line (Figs 1c–e, S3A). Quantitative RT-PCR (qPCR) suggested that this phenotype was not due to possible (over)compensatory up-regulation of the two other AUX1 homologs in Brachypodium (Fig. S2B).
**Bdaux1** plants also displayed a dwarf shoot phenotype with aberrant flower development (Fig. 1f–g). *Bdaux1* mutants were thus sterile (Fig. 1h) and could not be maintained as homozygotes in practice. Both the root and shoot phenotypes could be complemented by introduction of transgenes that expressed either BdAUX1 or GFP-BdAUX1 fusion protein under control of the native BdAUX1 promoter (BdAUX1::BdAUX1 and BdAUX1::GFP-BdAUX1) into the *Bdaux1* background (Fig. S3B,C). Moreover, the mutant phenotypes were also observed in *Bdaux1* homozygous knock out plants that were generated by the CRISPR/Cas9 technique (BdAUX1^CRISPR). This included the severe shoot phenotype and infertility (Fig. S3C,D), which also precluded recovery of the lines. Therefore, *Bdaux1* loss-of-function was causative for the observed mutant phenotype.

A more detailed characterization of the mutants revealed that their increased root elongation could be attributed to increased mature cell length (Fig. 2a). Moreover, *Bdaux1* roots were markedly thinner than wild type roots (Fig. 2b). Although the number of cell files was significantly reduced in every tissue except xylem and phloem (Fig. 2c), this alone could not entirely account for the overall reduction in root thickness. Rather, cells generally appeared slightly smaller in radial sections (Fig. 2b), and at the same time, root hairs were markedly shorter, reduced in number and appeared later than in wild type (Fig. 2d). Therefore, the *Bdaux1* root elongation phenotype was apparently caused by overall higher cellular anisotropy. Interestingly, it thus resembles the roots of hypomorphic mutants in the Brachypodium TAR2-LIKE (TAR2L) gene (Pacheco-Villalobos et al., 2013). *Bdtar2l^ppp* mutants are partially impaired in a rate-limiting step of auxin biosynthesis, which results in higher cellular auxin levels in the root because of the particular regulatory wiring in Brachypodium (Pacheco-Villalobos et al., 2013, 2016). To further explore the similarity between *Bdaux1* and *Bdtar2l^ppp* mutant roots, we also determined cellular auxin levels in *Bdaux1* root tips. Indeed, we again observed increased auxin levels (Fig. 3a). This result was surprising, given the Arabidopsis precedent that AUX1 is needed for efficient shoot to root mobilization of auxin, and *Ataux1* mutants therefore have reduced, rather than increased, auxin levels in the root (Marchant et al., 2002). In *Bdtar2l^ppp* plants, the root phenotype was also associated with slight alterations in cell wall composition, notably a reduction in 1,3-galactosyl and 1,2-galactosyl residues, suggesting an altered arabinogalactan structure, and an increase in 1,4-glucosyl residues (Pacheco-Villalobos et al., 2016). Similar changes were observed in *Bdaux1* root tips (Figs 3b, S3E), again confirming similarity with *Bdtar2l^ppp* plants. Finally, a survey of the *Bdaux1* expressed genes, mostly in cell wall modifiers (Table S1), which are thought to be primary targets of auxin-induced cell elongation (Cosgrove, 2005). Confirming the qPCR analysis, no differential expression of the two other AUX1 homologs was observed in the *Bdaux1* transcriptome (Table S2). In summary, in many ways *Bdaux1* roots phenocopy *Bdtar2l^ppp* roots.

Similarities with *Bdtar2l* mutants could also be observed in the shoot. In mutants of the hypomorphic *Bdtar2l^ppp* allele, the root phenotype is accompanied by a slight reduction in leaf blade length and width (Pacheco-Villalobos et al., 2013). However, in mutants of the null allele *Bdtar2l^null*, the root phenotype is weaker and transient, while the shoot displays a dwarf phenotype that is accompanied by severely reduced fertility (Pacheco-Villalobos et al., 2013). Thus, the shoot phenotype of *Bdtar2l^null* plants is similar to *Bdaux1* plants. The strongly reduced fertility of *Bdaux1* appeared to be due to delayed development of anthers as compared to gynoecia as well as poor pollen viability (Fig. 1g). Nevertheless, because plants heterozygous for *Bdaux1* were similar to wild type, we could create double mutants with the *Bdtar2l^ppp* allele. Overall, the phenotype of these *Bdaux1 Bdtar2l^ppp* double mutants appeared to be additive as compared to their segregating single mutants and wild type siblings (with the caveat that background loci might modulate the phenotypes to some degree because the two single mutants had different wild type parents). The dwarfism of *Bdaux1* plants was more exaggerated in *Bdaux1 Bdtar2l^ppp* double mutants (Fig. S3B,F), and the double mutant roots were thinner than in either single mutant and longer than in *Bdtar2l^ppp* alone (Fig. S3G). This could be attributed to an even higher mature cell length, and an additional reduction in cell files (Fig. S2H). However, unlike the single mutants, the double mutants displayed a reduced root meristem size that was accompanied by slight changes in root meristem organization, such as an apparently smaller quiescent center (Fig. S3J). Overall, the data suggest parallel impacts of *BdAUX1* and *BdTAR2L* mutation that reinforce each other. This is also consistent with the absence of significant expression changes in rate-limiting auxin biosynthesis genes in *Bdaux1* (Table S2).

The *BdAUX1::GFP-BdAUX1* plants, as well as *BdAUX1::NLS-3XVENUS* plants, allowed us to assess the expression pattern of *BdAUX1* in the root. *AtAUX1* is expressed specifically in the Arabidopsis root protophloem, epidermis and root cap-columella (Marchant et al., 2002). *BdAUX1* transcriptional and translational reporters displayed similar expression patterns, with the exception of expression in the root cap. Moreover, unlike *AtAUX1*, *BdAUX1* was also expressed throughout the stele and in the outer cortex layers (Fig. 4a–c). Thus, the expression pattern of *BdAUX1* encompasses the combined domains of *AtAUX1*, *AtLAX2* and *AtLAX3* (Peret et al., 2012) with the exception of the root cap, and therefore, possibly, their combined functions in these tissues. Consistent with its homology to *AtAUX1*, GFP-BdAUX1 protein was localized at the plasma membrane, in a typically polar fashion (Fig. 4d,e). In the stele, the orientation was generally shootward (Fig. 4e), while in the outer cell layers, BdAUX1 polar localization appeared mostly rootward (Fig. 4f). However, in the later epidermis, BdAUX1 was detected on both the apical and basal sides of the cell, as well as facing inside (Fig. 4g). In summary, the localization is consistent with a role of BdAUX1 in promoting auxin transport from the shoot to the root tip, and in evacuating auxin from the tip via the epidermis. Notably, despite the increased auxin level in
Bdaux1 root tips (Fig. 3a), the Bdaux1 root agravitropism could be somewhat rescued by application of 1-NAA (Fig. 5a), similar to Ataux1 (Swarup et al., 2001). However, 1-NAA levels that rescued agravitropism did not restore normal root elongation (Fig. 5b), which was always higher in Bdaux1 than in Bd21-3, indicating that the roles of BdAUX1 in cell elongation and gravitropism are physiologically separable.

In summary, our detailed analyses of Bdaux1 mutants revealed phenotypes that are counterintuitive with respect to the expectations set by the precedent of corresponding Arabidopsis mutants. However, interestingly, an exaggerated root elongation phenotype has also been described for Osaux1 mutants (Yu et al., 2015), although it has not been noticed by others working with the same lines (Zhao et al., 2015). Moreover, Osaux1 mutants also display slightly reduced shoot organ elongation (Zhao et al., 2015). Yet, compared to the Bdaux1 mutants, these phenotypes appear relatively mild, and no flower development or reproductive phenotypes were reported. Likewise, AUX1 mutants in maize and Setaria also display apparently milder inflorescence and root phenotypes than BdAUX1 (Huang et al., 2017). Possibly, this reflects partial genetic redundancy in rice, maize and Setaria, which contain two more AUX1 homologs than Brachypodium, including close OsAUX1, ZmAUX1 and SvAUX1 homologs (Zhao et al., 2012, 2015; Huang et al., 2017). Thus, the auxin uptake facilitator
network in Brachypodium might be less complex than in other grasses, confirming once more that the regulatory wiring of auxin biosynthesis or transport can vary between species, and thus can trigger distinct physiological and morphological consequences if tampered with (Pacheco-Villalobos et al., 2013; O'Connor et al., 2014). In summary, our data suggest that in Brachypodium, BdAUX1 primarily assures correct local auxin accumulation and has a broad role in root and shoot development. This role is apparently broader than the role of AtAUX1 in Arabidopsis, and could potentially encompass activities of AtLAX homologs (Marchant et al., 2002). However, a detailed analysis of the other Brachypodium AUX1 homologs will be required to conclusively resolve whether this is indeed the case.

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**Fig. 4** Brachypodium *BdAUX1* expression. (a) Confocal microscopy of a 2-d-old Bd21-3 root meristem after ClearSee and calcofluor staining (white), illustrating background fluorescence (yellow) in the VENUS channel. Please note that autofluorescence of Brachypodium roots cannot be fully eliminated (see the Materials and Methods section). (b) Expression pattern of a *BdAUX1* transcriptional reporter (nuclear-localized VENUS fluorescence, yellow). (c) Expression pattern of a GFP-*BdAUX1* translational reporter fusion protein (plasma membrane-localized green fluorescence). (d) Expression level and cellular localization of GFP-*BdAUX1* fusion protein (magenta fluorescence) in different parts of a 2-d-old root meristem (arrows point towards root tip). (e) Cellular localization of GFP-*BdAUX1* fusion protein (magenta fluorescence) in the stele, showing shootward polar accumulation of BdAUX1 (arrowhead) (arrow points towards root tip). (f) Cellular localization of GFP-*BdAUX1* fusion protein (magenta fluorescence) in the early epidermis, showing rootward polar accumulation of BdAUX1 (white arrowhead) and absence from inward facing side (yellow arrowhead) (arrow points towards root tip). (g) Cellular localization of GFP-*BdAUX1* fusion protein (magenta fluorescence) in the late epidermis, showing both rootward (white arrowhead) and shootward pole accumulation (green arrowhead), as well as inward facing localization (yellow arrowhead) of BdAUX1 (arrow points towards root tip). (a–c) are composite images.

**Fig. 5** Rescue of Brachypodium *Bdaux1* gravitropism. (a) Response of indicated genotypes to a 90° change in the gravity vector (3-d-old roots, plates were turned when they were 1-d-old), in the absence or presence of 1-NAA. (b) Root length of indicated genotypes in the absence or presence of 1-NAA. Box plots display second and third quartiles, maximum, minimum and mean (white dot). Statistically significant differences are indicated (Student’s *t*-test: a, *P* < 0.001; b, *P* < 0.01; c, *P* < 0.05).
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Author contributions
A.v.d.S. and C.S.H. designed the study and wrote the paper. A.v.d.S., C.V., K.L., M.P. and C.S.H. designed experiments. A.v.d.S. and C.V. performed experiments. J.B. and J.V. provided crucial reagents.

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Supporting Information
Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

Fig. S1 Clustal protein sequence alignment of Arabidopsis and Brachypodium AUX1 homologs.

Fig. S2 Expression analysis of Brachypodium BdAUX1 and other AUX1 homologs.

Fig. S3 Various genetic and physiological analyses of Brachypodium BdAUX1.

Table S1 List of differentially expressed genes in Bd aux1 root segments (P<0.01).

Table S2 Comparison of RNAseq analyses of Bd aux1 and Bd21-3 root segments.

Methods S1 DNA sequences of oligonucleotides and the CRISPR/Cas9 cassette used in this study.

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