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px$_2$, the Newly Identified Gene in *Rhizobium leguminosarum,* Is Characterized to Enhance Its Adjacent *nodF* Expression

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***nodFEL* operon is one of the NodD-dependent inducible *nod* operons that is clustered on the symbiosis plasmid of *Rhizobium leguminosarum biovar viciae.* A recent study on the upstream regulatory region of *nodFEL* operon had identified a new promoter, which was responsible for the transcription of a 0.72 Kb RNA molecule, called *px*$_2$, in the opposite direction to *nodF*. This new promoter was further characterized to overlap with that of *nodF* and its *in vitro* transcription was inhibited by another newly identified *nod* regulator, Px. In this paper we report that the sequence analysis of the *px*$_2$ transcript revealed only one open reading frame (ORF$_{66}$), corresponding to a polypeptide of 66 amino acids. Moreover, the increase in *px*$_2$ copy numbers enhanced the *in vivo* inducible expression level of *nodF,* whereas the frame-shift mutation of ORF$_{66}$ eliminated such effect, providing evidence that *px*$_2$ is responsible for specifically upregulating *nodF* expression. This result also raises the big possibility that *px*$_2$ encodes this polypeptide. A model for coordinated expression of *px*$_2$ and *nodF,* transcribed divergently from each other, is proposed.** © 2000 Academic Press

Key Words: **promoter; transcription; ORF; upregulate; frame-shift mutation.**

The molecular basis of establishing productive symbiosis between *rhizobia* and its specific host legume is an exchange of signal molecules. Nodulation genes (*nod*, *nol*, *noe*) of *rhizobia*, expressed at the early stage of signal exchanging process, are responsible for synthesis of a type of special oligosaccharides, called nod factors, which act as *rhizobia's* specific signals to overcome the obstacle of host range and subsequently trigger the nodule formation program of the host plants (1–3). In general, the expression of *nod* genes are subject to NodD-dependent inducible activation, that is NodD, the *nod* regulatory gene product, activates the transcription of *nod* genes in a yet unknown mechanism upon perceiving the inducer secreted by host plant (4–6). It is clear that NodD is a DNA binding protein that interacts specifically with a conserved sequence ("*nod* box") within the promoters of the inducible *nod* operons (7, 8).

Thirteen *nod* genes have been identified on pRL1JI, Sym (symbiosis) plasmid of *R. leguminosarum biovar* (*bv.*) *viciae* and they lie in five transcription units, *nodO*, *nodMNT*, *nodFEL*, *nodD* and *nodABCIJ* (9–12). These *nod* operons all suffer the inducible activation of *nodD*, which is negative autoregulated (13, 14). Currently, we defined that the upstream regulatory region of *nodFEL* encompassed two overlapping but divergent promoters, one was corresponding to the expression of *nodFEL* operon, while another was a new promoter, responsible for the transcription of a 0.72 kb RNA molecule, called *px*$_2$. In addition, the study on the *px*$_2$ found that its transcription *in vitro* was inhibited by Px, a newly identified *nod* regulator, featuring binding to the specific sites within the promoter regions of *nod* genes and stimulating *in vitro* transcription of *nodD* (15, 16). In this paper, *px*$_2$ was further characterized to specifically upregulate the inducible expression of its adjacent *nodF*, and *px*$_2$ is more likely to function as a protein coding for 66 amino acids. The fact that *px*$_2$ and *nodF* share the common regulatory region, particularly together with that *px*$_2$ has positive effect on *nodF* expression, support the proposed model for coordinated expression of these divergently transcribed two genes.

MATERIALS AND METHODS

Strains, plasmids, primers and growth condition. Relevant strains, plasmids and primers used in this study were listed in Table 1. Growths of *E. coli* for cloning procedures were performed at 37°C in Luria Broth medium, whereas *Rhizobium leguminosarum* were grown at 28°C in TY medium (22). If required, appropriate antibiotics, ampicillin (Ap), stretomycin (Str), kanamycin (Km), tetracycline (Tc) were added. Method for tripartite conjugation was that of C.

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TABLE 1

Strain, plasmid and primers	Relevant characteristics and primers sequences	Reference of source
R. leguminosarum 248	*R. leguminosarum bv. viciae* wide type strain of *R. phaseoli* lacking its symbiotic	17
Rhizobium strain 8401	plasmid Δ (lacZYA-arg) supE thi reCA1 lacZΔM15	18
E. coli DH 5 α F′		Promega
pRK2013	IncColE1, helper plasmid for tripartite mating	18
pRL1JI	*R. leguminosarum bv. viciae* native symbiotic plasmid	19
pKT230	Broad host-range cloning vector	20
pIJ1518	1.7 Kb *Bcl*I fragment of pRL1JI cloned into pKT230	21
pBluescrip SK(+)	2.9 Kb phagemid derived from pUC19	Gibco BRL
pMP220	IncP vector with promterless *lacZ*	7
pUCWZ	0.3 Kb *Eco*RI-*Pst*I fragment containing *nodAD* (12) intergenic region cloned into pUC19	This work
pUC19F	DNA fragment containing the upstream regulatory region of *nodFEL* was cloned into *BamH*I site of pUC19	This work
pMP220F	*Eco*RI-*Pst*I fragment of pUC19F cloned into pMP220	This work
pMP220D	*Eco*RI-*Pst*I fragment of pUCWZ cloned into pMP220	This work
pTvecpx_2	Fragment F_5 cloned into pBluescrip SK(±)	This work
pKT230px_2(±)	*BamH*I fragment of pTvecpx_2 cloned into pKT230 derivative of pKT230px_2(+)	This work
pKT230px_2(M+)	with *Sac*II site located within the px_2 impaired	This work
primer[a,b]		
P_{-104}	5′-CGCGGATCCC CGGCTCGTCG TGCG-3′	This work
P_{730}	5′-TTGAATTCCA AGAGGCGTAT TGAG-3′	This work

[a] The numbers, indicating the locations of primers, were relative to the transcription start point of px_2.
[b] The restriction sites introduced for cloning were underlined.

Shearman. A (23) using *E. coli* DH5α F′ (pRK2013) as the help strain.

DNA technique and DNA fragments generation. Genomic DNA of *R. leguminosarum* 248 was obtained as described in (24). Recombinant plasmid DNA from *E. coli* was isolated by alkaline lysis technique (24). Restriction endonucleases and modifying enzymes were from Promega and were used as recommended by the supplier. The PCR conditions of thirty cycles of amplification were denaturation at 94°C for 30 s, annealing at 47°C for 45 s, and extension at 72°C for 45 s. Double-stranded DNA sequencing was performed by dideoxy chain termination method of Sanger (25) using a Bio-Rad kit following the manufacture instructions. 835bp DNA fragment F_1 was the PCR product using P_{-104}-P_{730} primers and *R. leguminosarum* 248 genomic DNA as the template. PCR products was further purified through high pure PCR product purification kit (Boehringer Mannheim) and quantified on GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech). In search of ORFs, DNA sequence was analyzed by GENEPRO software.

β-galactosidase activity assay. Assays for β-galactosidase activity, using 100 nM naringenin as the *nodF* gene inducer, were performed as previously described (26, 27) using an automated microplate reader (Bio TEK) to measure the cell A_{540nm} and the hydrolysis of *o*-nitrophenyl galacotose at A_{405nm}. Each test was done in duplicate, three times.

Frame-shift mutagenesis. The 856-bp *BamH*I-digested fragment from pKT230px_2(+) was inserted into pUC19F, the vector lacking *Sac*II site. The resulting pUC19px_2 plasmid DNA was digested with *Sac*II, followed by treating with T4 DNA polymerase and religating the blunt ends. DNA sequencing with the resulting pUC19 (px_2M) plasmid DNA confirmed that the *Sac*II restriction site was impaired. Then *BamH*I-digested fragment from pUC19 (px_2M) was subcloned into pKT230, giving rise to pKT230 (px_2M). The inserted orientation was determined after digesting with restriction endonucleases.

RESULTS

Overexpression of px_2 Enhances the Expression of Its Adjacent nodF

It has been proved previously that RNA polymerase of *R. leguminosarum bv. viciae* initiated a transcription from a new promoter overlapping with that of *nodF*, while this new promoter was responsible for the transcription of a RNA molecule, called px_2, in the opposite direction to *nodF* (Fig. 1) (16). In an effort to gain an insight into px_2's function, we increased copies of px_2 *in vivo* by inserting the fragment F_1 containing the intact px_2 into the *BamH*I site of multiple copy vector pKT230 (20). Accordingly, two clones were obtained. One in which the orientation of px_2 was as same as that of *Str* gene on the vector, was designated as pKT230px_2(+), while the other was termed as pKT230px_2(−) with opposite direction in respect to the former. Then, these two derivatives of pKT230, together with the empty vector pKT230 as control, were mobilized into *Rhizobium* strain 8401 (pRL1JI) (kindly presented by J. A. Downie) separately through tripartite conjugation. Since the inserted fragment contained the native promoter of px_2, px_2 was expressed regardless of its orientation, resulting in increased expression of px_2 in those strains harboring pKT230px_2(+) or pKT230px_2(−). To quantify the effect of overexpression of px_2 on the expression of *nodF* or *nodD, nodF-lacZ* and *nodD-lacZ* transcriptional fusions were constructed, with the promoter of *nodF* or *nodD* fused to the upstream of the

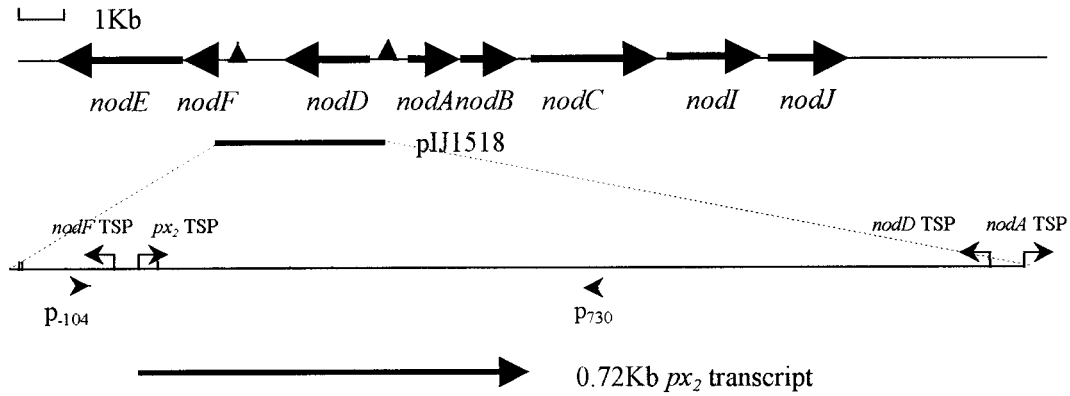

FIG. 1. Some *nod* genes of *R. leguminosarum* Sym plasmid pRL1JI. pIJ1518 were constructed by cloning 1.7 Kb *Bcl*I fragment of pRL1JI into pKT230, containing an intact *nodD* gene expressed from vector promoter (21). The TSPs (transcription start point) for *nodF*, px_2, *nodD* and *nodA* on the pIJ1518 were indicated, and the locations of primers used in this work were illustrated. The triangles represent the conserved "*nod box*". The bottom solid bar indicates the 0.72 Kb px_2 transcript.

promoterless *lacZ* gene on reporter vector pMP220 (7). The resulting plasmid pMP220F or pMP220D was transferred to *Rhizobium* strain 8401 (pRL1JI) harboring pKT230px_2(+), pKT230px_2(−) or pKT230, and the β-galactosidase activity expressed form *nodF* or *nodD* was monitored. The results revealed in Fig. 2 were fairly well in agreement with the typical expression patterns of *nod* genes in that the expression of *nodD* was constitutive, while for *nodF*, only its basic transcription level was observed in normal growth media, whereas upon exposing to the Naringenin, an apparent increase in *nodF* expression occurred, indicating the inducible activation of *nodF*. More interesting, in those strains with increased copies of px_2 displayed about 26% higher expression level of *nodF* than that in the control strain. The effect is relatively slight, however it was specific because *nodD* expression in all strains tested was not effected. In another control assay, we replaced pKT230px_2(±) with pIJ1518 to perform the similar *in vivo* transcription assay. As known, pIJ1518 was constructed by cloning 1.7 Kb *Bcl*I fragment of pRL1JI into pKT230, containing the intact *nodD* gene expressed from vector promoter (Fig. 1), therefore, *nodD* is overexpressed in those *Rhizobium* stains that harbor pIJ1518. After pMP220F (*nodF-lacZ*) or pMP220D (*nodD-lacZ*) was mobilized separately into *Rhizobium* strain 8401 (pRL1JI) which harbored pIJ1518 or the empty vector pKT230, the β-galactosidase activity was monitored. The pattern, reflecting the effect on *nodF* or *nodD* expression by the *nodD* overexpression, was different from that by the px_2 overexpression. As shown in Fig. 3, when compared with

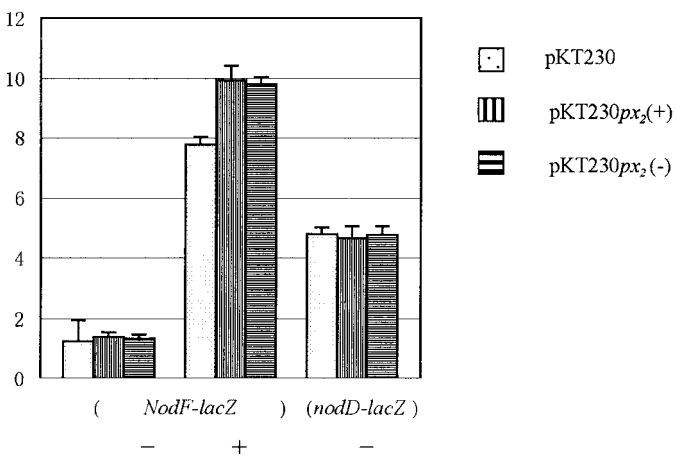

FIG. 2. Effect of px_2 overexpression on the expression of *nodF* or *nodD* gene. Cultures of *Rhizobium* stain 8401 (pRL1JI, pMP220F), 8401 (pRL1JI, pMP220D), harboring pKT230, pKT230px_2(+) or pKT230px_2(−), were grown separately in minimal medium at 28°C. β-galactosidase activities were assayed as described under Materials and Methods. All values were the means of three separate experiments and the error bars showed the standard deviations. +, 100 nM Naringenin was added; −, no Naringenin was added.

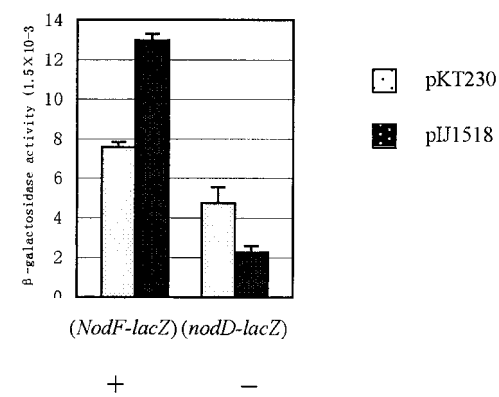

FIG. 3. Effect of *nodD* overexpression on the expression of *nodF* or *nodD* gene. Cultures of *Rhizobium* strain 8401 (pRL1JI, pMP220F), 8401 (pRL1JI, pMP220D) harboring pKT230 or pIJ1518, were grown separately in minimal medium at 28°C. β-galactosidase activities were assayed in the same way as above. +, 100 nM Naringenin was added; −, no Naringenin was added.

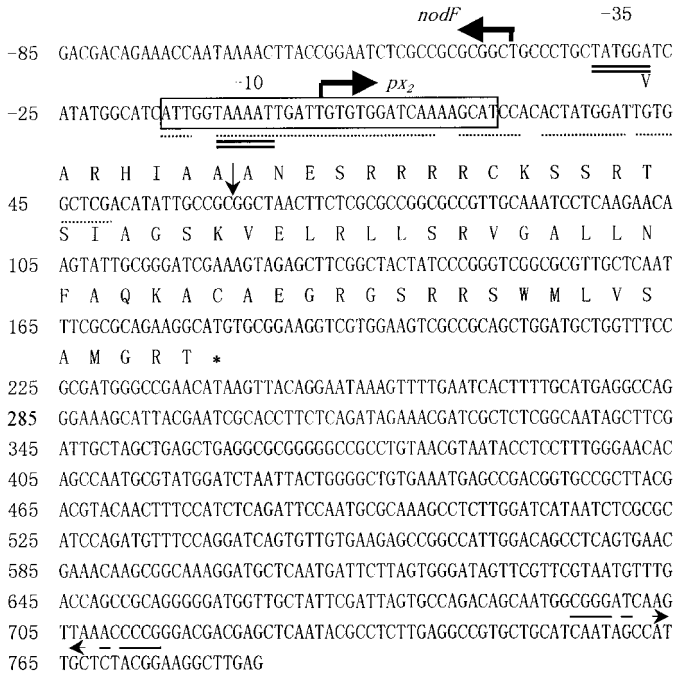

FIG. 4. nt sequence of px_2 and the deduced amino acids sequence for ORF_{66}. The numbers left to the nucleotides were relative to the TSP of px_2. The stop codon was shown by asterisk. The transcription directions, TSPs of *nodF* and px_2 were indicated. The double line under the sequence showed −10 and −35 promoter regions of px_2. The boxed regions are the highly conserved "*nod* box". The dotted line represents the protected region of Px that lied within the promoter region of px_2. The vertical arrow showed the restriction site of *Sac*II. Converging arrows show the potential transcriptional terminator, the sequence capable of forming secondary structure.

the control strain, in the strain containing pIJ1518 there was 1.7-fold enhancement on *nodF* expression, whereas, the β-galactosidase activity for the *nodD* had dropped by 50%, demonstrating the negatively autoregulatory expression feature of *nodD* (14, 28). It should be noted that the intact px_2 also remained on pIJ1518 (Fig. 1), therefore, although the *nodD* on the pIJ1518 was primarily responsible for such 1.7-fold increase in the *nodF* expression, the px_2 carried by the pIJ1518 also contributed to the total upregulating effect.

An Open Reading Frame of 66 Amino Acids in Length Was Revealed in px_2 Transcript

DNA sequence analysis of px_2 transcript revealed an open reading frame (ORF_{66}) consisting of 66 amino acids. Similar to some other genes identified in *Rhizobium*, this open reading frame used GTG (9, 10), 31 bp downstream of the transcription start point of px_2 (16), as the start codon (Fig. 4). Protein database searching found no homologous protein to this small polypeptide, indicating that if it is really the px_2 gene product, px_2 is a new gene.

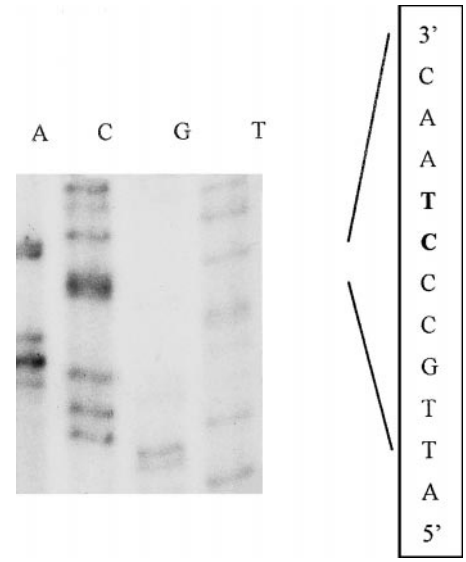

FIG. 5. Frame-shift mutation. The pUC19 px_2 (M) plasmid DNA was sequenced, and the mutated nucleotides are shown with boldface letters.

A Frame Shift Mutation Eliminated the Upregulating Effect of px_2 on nodF

Taking advantage of the *Sac*II restriction site located closed to the GTG codon of this ORF_{66}, a frame-shift mutation was constructed, where the wide type sequence of "**ATT GCC GCG GCT AAC**" in px_2 was switched into "**ATT GCC CTA AC**" sequence (Fig. 5). When the corresponding plasmid pKT230px_2(M+) containing the mutated px_2, together with pKT230px_2(+) and pKT230, were assayed under the same condition as above, in contrast, it was found that the specific upregulating effect was eliminated by this frame-shift mutation of ORF_{66} (Fig. 6). The results above provided

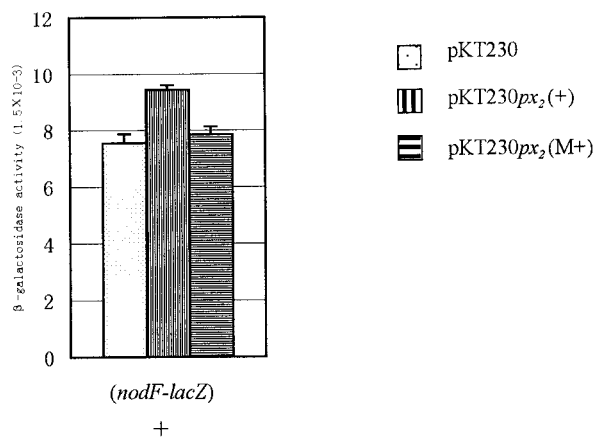

FIG. 6. Frame-shift mutation in ORF_{66} eliminated the upregulating effect on *nodF* expression. β-Galactosidase activities were monitored in the same way as above. +, 100 nM Naringenin was added.

strong evidence that px_2 was responsible for specifically upregulating the *nodF* expression. Since the ORF_{66} was the only open reading frame found within the px_2 transcript, this result also raised the big possibility that px_2 encodes this small polypeptide. However, it is still possible that the px_2 transcript itself may somehow act as a regulatory molecular due to that we have not yet obtained the px_2 protein product.

DISCUSSION

It was previously shown that *in vitro*, the transcription of px_2 was inhibited by Px protein, a newly identified protein participating in regulating the *nod* genes (15, 16). This result was consistent with the low expression level of px_2 *in vivo*, which was demonstrated evidently by *in vivo* transcription assay (data not shown). *nodF-lacZ* and px_2-*lacZ* fusion plasmids, pMP220F and pMP220px_2, in which *lacZ* gene in pMP220 was under the control of *nodF* and px_2 promoters, were constructed and transferred to the *Rhizobium* strain 8401 containing or lacking *nodD*. Distinct from the expression pattern of *nodF*, the promoter activity for px_2 was low and constant regardless of the inducer. Moreover, in the *Rhizobium* strain 8401 (pIJ1518), in which *nodD* was overexpressed, the promoter activity for px_2 was even lower than that in the *nodD*-absent *Rhizobium* strain 8401 (data not shown), suggesting that in addition to Px, NodD is also involved in repressing the px_2 expression. On the other hand, it has become apparent that, for *nod* genes, actually only rather modest induction could be tested *in vivo* (29–31), and a growing body of evidence suggested that high level of induction of *nod* genes inhibited nodulation of the host plants (29–32). Therefore, the observed positive effect of px_2 on *nodF* expression as well as the low expression level of px_2 *in vivo*, implies that the inhibition of px_2 expression by Px and NodD is of biological significance for avoiding the overexpression of px_2.

As for the relative slight (about 26%) upregulating effect of px_2 on *nodF*, the explanation may lie with two points as below. First, unlike those vectors that specified in overexpressing the interesting gene, pKT230 is not a type of high-copy vector. Second, since the DNA fragment F_1 that inserted into pKT230 contained the native promoter of px_2, while which was observed to be of low activity *in vivo*. So, such factors contributed to that the final amplification of px_2 expression is not significant. Anyway, an understanding of the mechanism by which px_2 affects *nodF* expression at the molecular level would require further investigation. However, as shown in Fig. 2, px_2 had no influence on *nodD* expression, indicating that the upregulating effect on *nodF* expression by px_2 overexpression was *nodD*-independent, which was different from other *nod* regulatory genes (30, 32, 33). One example is *dctB* in *R. leguminosarum*. Genetic analysis revealed that the *dctB* mutation caused the reduction in *nod* gene expression, in the meantime, a decrease in expression of regulatory gene *nodD* was also found in such mutant (33). Another example is the *nolR* in *R. meliloti*. It was found that NolR, *nolR* gene product, functions not only as a negative regulator of common *nod* genes but also the activator *nodD1* and *nodD2* genes (30, 32). Therefore, it was supposed that such genes exert their regulated role by first influencing the expression of *nodD*, the key regulatory *nod* gene.

In conclusion, our current study demonstrates an additional level of complexity governing the expression around the small region defining px_2 and *nodF* promoters that the expressions of px_2 and *nodF*, transcribed in the opposite directions, are highly coupled through the mediation of Px and NodD regulators.

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