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Nodulation and Growth of a Supernodulating Soybean Mutant SS2-2 Symbiotically Associated with *Bradyrhizobium japonicum*

Puji Lestari[1], Kyujung Van[2], Moon Young Kim[2], and Suk-Ha Lee[2]
[1]Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Jl. Tentara Pelajar No. 3A, Bogor 16111
[2]Seoul National University, Seoul, 151-742, Korea

ABSTRAK

Nodulasi dan Pertumbuhan Mutan Kedelai Penghasil Nodulsuper SS2-2 dalam Asosiasi Simbiotik dengan *Bradyrhizobium japonicum*. *Puji Lestari, Kyujung Van, Moon Young Kim, dan Suk-Ha Lee.* Mutan kedelai penghasil nodulsuper menunjukkan kelemahan dalam kontrol autoregulasi pada nodulasi dan perbedaan fenotip dibandingkan dengan tipe liarnya. Studi untuk mengevaluasi karakter pertumbuhan dan nodulasi dari kedelai penghasil nodulsuper dalam asosiasinya dengan *Bradyrhizobium japonicum* dilakukan dalam penelitian ini. Tiga genotip kedelai, yaitu mutan kedelai penghasil nodulsuper SS2-2, tipe liarnya Sinpaldalkong 2 dan kedelai kontrol Jangyeobkong diinokulasi dengan *B. japonicum* USDA 110, kemudian ditumbuhkan di rumah kaca dalam kondisi terkontrol. Karakter nodulasi, fiksasi nitrogen (Acetylene Reduction Activity/ARA), pertumbuhan tanaman, dan hasil biji ditentukan untuk mengevaluasi asosiasi simbiotik antara *B. japonicum* dan kedelai nodulsuper. Kedelai yang diinokulasi dengan *B. japonicum* menunjukkan peningkatan jumlah dan berat kering nodul serta berat kering total tanaman dibandingkan dengan tanpa inokulasi. Tanaman SS2-2 yang diinokulasi menunjukkan jumlah nodul sekitar 20 kali lipat lebih tinggi daripada tipe liarnya. Inokulasi *B. japonicum* ternyata juga meningkatkan fiksasi nitrogen seiring dengan perkembangan nodul. Tanaman S2-2 lebih pendek dan menghasilkan fiksasi nitrogen (ARA) lebih tinggi, tetapi spesifik ARA dan hasil biji lebih rendah dibandingkan dengan tipe liarnya. Berdasarkan hasil evaluasi terhadap nodulasi dan pertumbuhannya, interaksi *Rhizobium* dan kedelai penghasil nodulsuper SS2-2 mempunyai respon asosiasi simbiotik lebih rendah dibandingkan kedelai penghasil nodul normal (kedelai yang tidak mendapat perlakuan mutasi).

Kata kunci: *Bradyrhizobium japonicum*, fiksasi nitrogen, fenotip, kedelai nodulsuper.

INTRODUCTION

A symbiosis between the soil bacteria and leguminous plants is characterized by a specific multi step signal exchange (Israel *et al.* 1986). The nodulation of soybean plants is a developmentally complex process requiring interaction between *Bradyrhizobium* and the host plants (Sutton 1983). Successful development of nitrogen-fixing nodules, including supernodulating soybean requires on-going communication between the plant host and the endosymbiotic *Rhizobium* (Green and Emerich 1999).

In soybean, nodulation mutants have been isolated either from normal soybean population or by chemical or physical mutagenesis (Carroll *et al*. 1985a; Gremaud and Harper 1989). Three groups of scientists have independently isolated supernodulating or hypernodulating soybean mutants from cultivars Bragg (Carroll *et al.* 1985a), Williams (Gremaud and Harper 1989), and Enrei (Akao and Kouchi 1992). All these mutants were capable of producing several-fold more nodules than their parent lines in the presence of nitrate. These supernodulating soybean mutants may not only be unique biological materials for a study on host plant factors, which could be involved in the process of nodulation, but also be a useful germplasm in a breeding program on improvement of nodulation and nitrogen fixation (Lee *et al*. 1997).

The super- and hypernodulating soybean mutants formed considerably more nodules than their wild type, and an extensive region of the roots was covered with nodules (Akao and Kouchi 1992). Several soybean mutants generated from cv. Bragg (Carroll *et al.* 1985a) were described as supernodulators and nitrate tolerant symbiotic (*nts*) mutants. These mutants displayed N-fixation and produced nodule 10 times more than their wild type (Carroll *et al.* 1985b). Evaluation of the supernodulating soybean mutants in the field have been reported in previous studies, involving mutants of soybean cultivars Bragg and Williams (Herridge and Rose 1994). It was revealed that intermediate supernodulators could grow as vigorously as their parent cultivars. However, the yield of extreme supernodulators (*nts*382, *nts*1007, mutants of Bragg) and intermediate supernodulators (*nts*1116) have been consistently low (Song *et al.* 1995). Supernodulating soybean mutants also showed higher activities in the N fertilized than commercial cultivars Bragg, Centaur, and Manark (Song *et al.* 1995). It has been suggested that these were due to other mutations unrelated to nodulation (Mathews *et al.* 1989). In series of split-root and grafting experiment, the autoregulatory response was found to be systemic (Ollsson *et al*. 1989), requiring the action of the shoot (Delves *et al.* 1986).

The yield of supernodulating mutant SS2-2, generated from Sinpaldalkong 2 that was mutagenized by EMS (Lee *et al.* 1997), was not changed significantly due to nitrogen supply. Furthermore, non-inoculated plants of mutants, SS2-2 and *nts*382 showed greater nodules number and nodule mass, as well as greater ARA than their wild types, regardless of the level of exogenous nitrogen supply (Ha *et al.* 1999). Investigation of nitrogen fixation and agronomical traits of SS2-2 in association with *Rhizobium* has not been reported. This paper therefore, reported a study on a supernodulating soybean SS2-2 that was compared with its wild type in growth and nodulation in response to *Bradyrhizobium japonicum*.

MATERIALS AND METHODS

Plant Materials and Growth Condition for Bacteria

B. japonicum USDA 110 (obtained from the USDA *Rhizobium* culture collection, Belstsville, Md., USA) was used as inoculum in this study. Bacterial cells were grown in yeast extract mannitol/YEM medium which consists of K_2HPO_4 0.05%, $MgSO_4$ $7H_2O$ 0.02%, NaCl 0.01%, mannitol 1%, and yeast extract 0.04% with a pH adjusted to 6.8 (Vincent 1970). The bacterial cultures were incubated in a shaking incubator at 200 rpm, 30°C, for 6 days. In this study, three soybean genotypes, a supernodulating mutant SS2-2, its wild type Sinpaldalkong 2, and a control genotype Jangyeobkong were used for investigation.

Characterization of Nodulation and Growth

Surface sterilized soybean seeds were sown in plastic pots filled with 1 : 1 sand and soil mixture, then inoculated with *B. japonicum* USDA 110 (10^8 CFU/ml). A 3 x 2 factorial treatment combinations of three soybean genotypes and two inoculation levels (inoculated and non-inoculated treatment) were laid out in a completely randomized design with 3 replications. The plants were kept in a greenhouse and nitrogen-free Jensen's reagent (Fang and Hirsch 1998) was applied twice a week. The dry weight of each plant part (shoot, root, and nodule), nodule number on the root system, and acetylene reduction activity (ARA) representing the nitrogen fixation were measured at 28 and 47 days after planting (DAP) respectively, while plant height and yield were measured around R3 stage (82 DAP).

Analysis of Acetylene Reduction Activity (ARA)

Acetylene reduction activity was measured on removed root from the whole plant which was placed in a 1 liter jar, and sealed with a lid containing a serological stopper. Using a 50-cc syringe, a 50-cc aliquot of air was removed from the jar, and the same amount of C_2H_2 was then injected into the sample jar. The root system was allowed to remain in the jar with C_2H_2 for 30 min, after a 10-cc aliquot was withdrawn from the jar, and the aliquot was injected into a 10-cc vacutainer tube. From this 10-cc tube, 0.5-cc aliquot was later drawn for gas chromatography/GC (Model DS 6200) analysis of C_2H_2 (Denison *et al*. 1983). ARA was determined by measurement of C_2H_2 reduction activity per plant, while specific ARA based on the C_2H_2 reduction activity per nodule dry weight.

Statistical Analysis

Parameters of growth and nodulation characters were measured as quantitative variations. The phenotypic data were analyzed using SAS procedure (Goodnight 1982), and LSD test was applied to evaluate the variance.

RESULTS AND DISCUSSION

Characterization of Nodulation and Growth

The supernodulating soybean mutant SS2-2 had smaller nodule size but greater nodule number than those of the wild type. The nodules on the root system of Sinpaldalkong 2 were clustered near the root crown (Figure 1). There was significant effect of soybean genotype but no significant difference of soybean-*Rhizobium* interaction effect on number and dry weight of nodule per plant. Inoculation of *B. japonicum* increased number and dry weight of nodule significantly at 28 and 47 DAP (Figure 2). Under given condition, 47-day-old SS2-2 plants had considerably higher both nodule number, about 20 times and nodule dry weight than the wild type and the control genotype.

Results of this study were consistent with the conclusion of Hansen *et al.* (1992) that the supernodulators were producing relatively more nodular tissue than normally-nodulating types (non-mutated plant) in the absence of soil nitrogen. Nodulation behaviour in SS2-2 was similar with that of a pea mutant (*Pisum sativum*) (Jacobsen and Feenstra 1984) and a lupine hypernodulating mutant (*Lupinus albus*) L-62 (Burity *et al.* 1999). It was shown that without nitrogen supply, the mutant presented better nodulation in association with *Rhizobium*. Addition-

Figure 1. Nodulation types of supernodulating mutant SS2-2, its wild type Sinpaldalkong 2, and control genotype Jangyeobkong inoculated with *B. japonicum* at 47 days after planting.

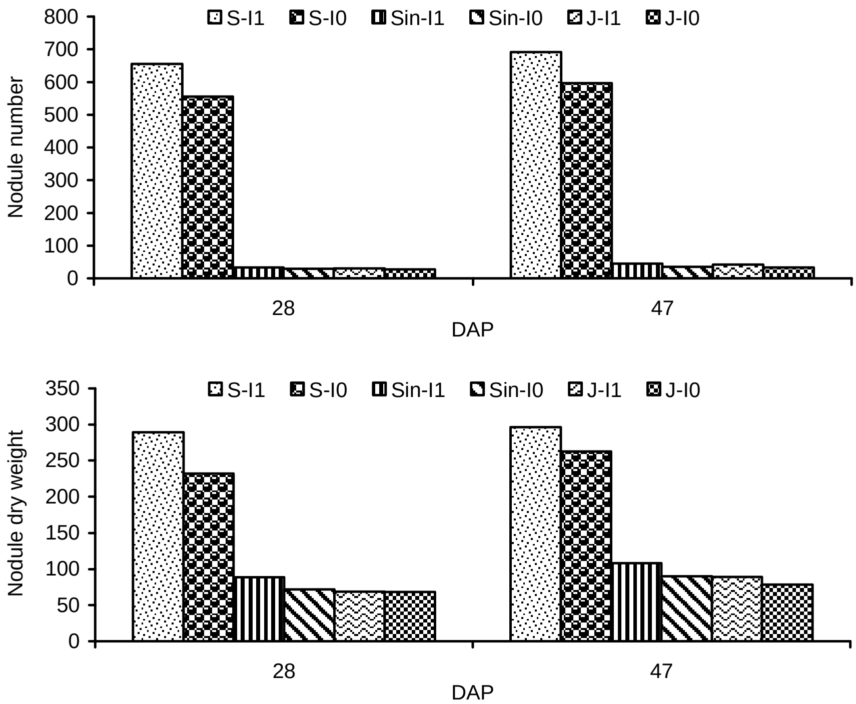

Figure 2. Number and dry weight of nodules in response to inoculation of *B. japonicum* in three soybean genotypes. S = SS2-2, Sin = Sinpaldalkong 2, J = Jangyeobkong, I1 = inoculated, I0 = non-inoculated treatment, DAP = days after planting.

ally, *B. japonicum* was effective to induce nodulation and increase nitrogen fixation (Israel *et al.* 1986).

There was no significant effect of soybean genotype on nodule dry weight at 28 and shoot dry weight at 47 DAP, but significant difference measured at 47 DAP on root dry weight (Table 1). *B. japonicum* induction produced healthier and more vigorous plants and stimulated growth of SS2-2, Sinpaldalkong 2 and Jangyeobkong better than in non-inoculated treatments. In this study, the dry weights of root and shoot were lower than those in infertile soil (clay/river sand/horticultural bed = 6 : 3 : 1) conducted by Ha and Lee (2001). Regardless of the genotype, SS2-2 growth was consistently lower than that of its wild type. According to shoot/root ratio, it was clear that SS2-2 had smaller roots and shoots with greater nodules. However, shoot growth of the wild type and the mutant were identical under symbiotic condition, while the root growths were still lagged off behind (Table 1). Small size and short root of SS2-2 were predicted as the reduction of plant growth that had also been observed in other supernodulating or hyper-nodulating mutants of soybean (Carroll *et al*. 1985a; Day *et al.* 1986) and other legumes (Sheng and Harper 1997). The short root phenotypes of other legume

Table 1. Genotypic differences of growth and nitrogen fixations in three soybean genotypes as affected by inoculation with *B. japonicum.*

Soybean genotype	28 DAP			47 DAP		
	Non-inoculated	Inoculated	Mean	Non-inoculated	Inoculated	Mean
			Root dry weight (mg/plant)			
SS2-2	351.7	378.7	365.2a	393.0	548.3	470.6b
Sinpaldalkong2	402.7	434.7	418.7a	433.0	740.0	586.5a
Jangyeobkong	380.7	434.7	407.7a	428.3	689.0	558.6ab
Mean	378.7a	416.0a		418.1b	659.1a	
			Shoot dry weight (mg/plant)			
SS2-2	1045.0	1508.0	1276.5a	1274.3	1712.0	1493.1a
Sinpaldalkong2	1241.0	1346.0	1293.5a	1485.0	1703.3	1594.1a
Jangyeobkong	1192.3	1325.0	1258.6a	1536.0	1731.7	1633.8a
Mean	1159.4b	1393.0a		1434.8b	1715.7a	
			Shoot/root ratio			
SS2-2	3.00	3.98	3.49a	3.20	3.10	3.15a
Sinpaldalkong2	3.08	3.10	3.09a	3.40	2.30	2.85a
Jangyeobkong	3.13	3.05	3.09a	3.60	2.51	3.05a
Mean	3.07a	3.38a		3.4a	2.64b	
			C_2H_2 reduction activity (mol h^{-1} $plant^{-1}$)			
SS2-2	3.62	4.94	4.28a	3.95	5.20	4.57a
Sinpaldalkong2	1.53	2.68	2.10b	1.62	4.00	2.81b
Jangyeobkong	1.23	1.45	1.34b	1.43	3.90	2.67c
Mean	2.13a	3.02a		2.33b	4.37a	
			Specific C_2H_2 reduction activity (mol h^{-1} nodule dry weight g^{-1})			
SS2-2	15.60	17.08	16.34a	15.17	17.60	16.38b
Sinpaldalkong2	21.34	30.21	25.77a	18.00	37.04	27.52a
Jangyeobkong	18.00	21.01	19.50a	18.26	43.82	31.04a
Mean	18.31a	22.77a		17.14b	32.82a	

Within categories, means (column or row) not followed by the same letter are significantly different at P 0.05 based on LSD.

mutants had also been documented, such as in a *Lotus japonicus* hypernodulating mutant *har1-1* (de Bruijn *et al.* 1998) and a pea hypernodulating mutant *nod3* (Jacobsen and Feenstra 1984). Thus, it was suggested that the short root length in the supernodulating mutant SS2-2 was likely to be due to a decrease or modification in root meristem activities (Wopereis *et al.* 2000), because of a defect of essential component of the regulatory mechanism responsible for maintaining the root growth.

B. japonicum increased the total dry weight (shoot, root, nodule) significantly compared to that of the control (Table 1). For this reason, the increase in total dry weights on rhizobia application in all soybean genotypes were more pronounced. Regardless of the *Bradyrhizobium* inoculation, during the plant growth period, the total dry weights of SS2-2 climbed to levels which were not significantly different from those of the wild type and control genotypes. Total plant dry weight in SS2-2 was not different from that of the normally-nodulating soybean. According to the root and shoot growth of the supernodulating mutant, it was suggested that the development of the soybean-*Bradyrhizobium* symbiosis was under the control of both shoot and root factors and these factors interact each other (Delves *et al.* 1986). Furthermore, rapid regulatory response in soybean was reproducible elicited by *Rhizobium japonicum* and autoregulatory control mechanism (Van Workum *et al.* 1998).

Nitrogen fixation was observed by determination of ARA per plant. Based on the nodule dry weight, specific ARA was obtained from the ARA. The more nodule dry weight of the plant, the higher specific ARA would be. Under symbiotical growth, there was significant difference on ARA at 28 and 47 DAP between mutant SS2-2 and its wild type (Table 1). SS2-2 gave a higher ARA than the wild type and control genotype, positively correlated with the nodule dry weight. *B. japonicum* caused the increase of ARA at 47 DAP, in part because of the nodule development. However, as the symbiosis progressed, the lowest specific ARA was observed on supernodulating soybean mutant. The specific ARA was important to be setermined to know the ability of nitrogen fixation on soybean plant. Moreover, leghaemoglobin content in the nodule would be able to be detected qualitatively.

Even though nitrogen fixation rate (ARA) of supernodulating mutant was higher than that of the wild type, specific ARA in nodules of the supernodulating plants was reduced. Such differences in the

ARA could be as results of differences in the ratio of bacteroidal in the nodules or differences in the nitrogenase activity per unit of bacteroid (Israel *et al.* 1986). Whereas, ARA attributable to these nodule would steadily declined at later stage of growth, not only because of nodule senescence but also because of the steady increase in nodule dry weight. Therefore, this apparent discrepancy might be also related to nutritional status of the host plants (Hattory and Johnson 1984), eventhough contribution of indigenous *Rhizobium* could be considered, however it was not strong enough to increase nitrogen fixation at the late stage of growth. Regardless of *Bradyrhizobium* inoculation, supernodulating soybeans showed less tolerant to effects of nitrogen (Song *et al*. 1995). Day *et al.* (1989) showed that lower specific ARA on supernodulating mutant that represented specific nitrogenase activity, was mainly due to the reduction in amount of symbiotic tissue which was characterized by smaller nodule cells, fewer bacteroids per peribacteroid membrane vesicle, and lower haem content per nodule. Consequently, although nodule emergence and subsequent nodule growth were specific parameters, ARA was not coordinately regulated with this nodule development in the mutant.

Regardless of the soybean genotypes, the lower soil fertility (non-inoculated) resulted in the lower plant height as well as pod and seed numbers per plant, and plant yield. There were significant effects of the soybean genotypes and the inoculation levels on the plant height, pod and seed number per plant, and plant yield (Table 2). It was clear that plant height of SS2-2 was shorter than the wild type under symbiotical growth. The mutants gave less plant yield than those of the wild types.

Low yield of SS2-2 was consistent with previous results of other supernodulating mutants (Maloney and Oplinger 1997). Apparently, under symbiotical growth, both SS2-2 and Sinpaldalkong 2 gave better yield than non-inoculated plants. The ability of the supernodulating mutant SS2-2 to develop more root nodules and to have greater nitrogen fixation did not result in increased grain yield compared with its normally-nodulating parent. This result was supported previous studies (Song *et al*. 1995), in which grain yield of the wild type soybean plant was significantly higher than that of supernodulating mutant. The greater yield in normally-nodulating soybean seemed to be associated with the photoassimilates. The supernodulating soybean mutant needed more leaf photoassimilates from the top for nodule development and maintenance. This caused the reduced yield of the supernodulating soybean mutant when compared to the wild types. Based on these results, SS2-2 might be able to maintain a fairly high total dry weight at low level of nitrogen even in the absence of exogenous nitrogen.

Table 2. Yield and yield components of three genotypes of soybean as affected by inoculation of *B. japonicum* at 82 DAP.

Soybean genotype	Non-inoculated	Inoculated	Mean
Plant height (cm)			
SS2-2	34.0	38.3	36.1b
Sinpaldalkong 2	46.0	53.2	49.6a
Jangyeobkong	45.0	52.2	48.6a
Mean	41.7b	47.9a	
Pod number per plant			
SS2-2	46.0	48.0	47.0b
Sinpaldalkong 2	74.0	78.0	76.0a
Jangyeobkong	73.0	78.0	75.5a
Mean	64.3b	69.3a	
Seed number per plant			
SS2-2	67.0	77.0	72.0b
Sinpaldalkong 2	105.0	120.0	112.5a
Jangyeobkong	103.0	118.0	110.5a
Mean	91.7b	105.0a	
Plant seed yield (g)			
SS2-2	11.0	14.0	12.5b
Sinpaldalkong 2	18.0	24.0	21.0a
Jangyeobkong	17.0	23.2	20.1a
Mean	15.3b	21.7a	

Within categories, means (column or row) not followed by the same letter are significantly different at P 0.05 based on LSD.

Table 3. Responses of three soybean genotypes to *B. japonicum* USDA 110 induction in nitrogen-free condition at 82 days after planting.

Parameter	Ratio of inoculated/non-inoculated treatment		
	SS2-2	Sinpaldalkong 2	Jangyeobkong
Plant height	1.126	1.156	1.160
Pod number per plant	1.043	1.054	1.068
Seed number per plant	1.149	1.143	1.145
Plant seed yield	1.273	1.333	1.365

SS2-2-*Bradyrhizobium japonicum* Symbiosis

SS2-2 less responded to *B. japonicum* induction in terms of plant growth and plant yield than its wild type (Table 3). Eventhough SS2-2 performed an effective symbiosis as the normally-nodulating soybean, it showed less response to *B. japonicum* induction in terms of nodulation, nitrogen fixation and nodulation characters (Lestari *et al.* 2005). Accordingly, although SS2-2 is a supernodulator, it is not a constitutive nodulator, since it still requires an inducer (*R. japonicum*) to be present. It was to be likely that some mechanisms were undergone in the supernodulating mutant. The nodulation in the supernodulating mutant suggested a mutational alteration of the autoregulation system, indicating closed relationship between nitrogen fixation and autoregulation of nodule development (Delves *et al.* 1986). Supernodulating mutant might maintain its growth by biologically fixed nitrogen (Delves *et al.* 1986). Clearly, SS2-2 is a mutant in the autoregulation pathway and was less sensitive to regulation by external conditions. Hence, interaction of rhizobia and the supernodulating soybean mutant SS2-2 had less symbiotically associated response than normally-nodulating soybean.

Based on the character of nodulation in SS2-2 especially nitrogen fixation which was represented by ARA observed in this study, supernodulating mutant that is potential for reducing uptake of soil N, would be contribute to increase amounts of N-rich legume residues (Song *et al.* 1995). The low yield of soybean supernodulator SS2-2 and its small plant size, would make this mutant more attractive for planting with "high density population" to increase the yield. Hansen *et al.* (1992) reported that although the large number of nodules of supernodulators were to be some degree parasitic on the host, they were potentially useful as N contributors in the cropping systems (Day *et al.* 1989) and might represent a source of N for the succeeding crops (Song *et al*. 1995). These results suggested that a major advantage of the supernodulation trait was in crop rotation, particularly for the subsequent cereal crop. Further more, the supernodulating soybean mutant SS2-2 would be useful for an environmental-friendly agriculture.

CONCLUSION

Evaluation of symbiotic association between *B. japonicum* in a supernodulating soybean mutant SS2-2 compared with the wild type, Sinpaldalkong 2 and the control genotype, Jangyeobkong revealed distinct phenotypic characters among them. Soybean plant inoculated with *B. japonicum* increased number and dry weight of nodule significantly either at 28 or 47 DAP than non-inoculated plants. The inoculated SS2-2 plants produced nodules almost 20 times more than the wild type. An ARA was observed lower in normally-nodulating soybean than that of SS2-2, otherwise, specific ARA was observed the lowest on SS2-2. Inoculation with *B. japonicum* lead to the increase of ARA in 47 DAP, due in part to nodule development. SS2-2 had smaller plant and gave less yield, but revealed greater number of nodule and nitrogen fixation than its wild type, either grown symbiotically or non-symbiotically. Thus, SS2-2 less responded to *B. japonicum* induction in terms of nitrogen fixation, nodulation characters, growth and plant yield than its wild type. In another word, the interaction between *Rhizobium* and the supernodulating mutant SS2-2 was less symbiotically associated response than the normally-nodulating soybean. Clearly, SS2-2 was a mutant in the autoregulation pathway that was less sensitive to regulation by external conditions.

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