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Article Addendum

Legumes like more IAA

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The improvement of the effectiveness and survival of rhizobia in the rhizosphere of legume host plants is a common practice in agricultural legume production. We have recently reported that the overexpression of IAA in *S. meliloti* 1021 played a positive role in the adaptation to various stress conditions both in free-living bacteria and in nodulated plants. We show here that IAA triggers the coordinate enhancement of various cellular defense systems and that IAA-treated bacteria are more resistant to desiccation. In addition, Medicago plants nodulated by the IAA-overproducing strain RD64 (*Mt*-RD64), restore auxin/cytokinin balance by increasing the transcription of cytokinin signaling genes. Finally, we bring here that RD64 is less competitive in comparison to the wild type strain under normal conditions, but it works better under salt stress conditions.

Role of IAA in Defense Systems

The intracellular content of trehalose, a stress protector osmolyte, was previously analyzed. A higher trehalose accumulation both in IAA-treated and RD64 cells was observed as compared to control cells.[1] To investigate the general protection systems activated under stress conditions, the production of polymeric structures such as LPS, EPS and biofilm was here evaluated.[2] RD64 cells contained higher levels of LPS molecules and produced higher amount of biofilm as compared to 1021 cells (Table 1). Quantitative analysis of EPSs, the predominant components of biofilms, revealed that these cells released into the medium more EPS than the control strain (Table 1). The presence of more LPS molecules in the outer membrane of RD64 cells might help these cells to more adequately mask the innate plant defense against the symbiont bacteria[3] and to overcome stressful environmental conditions,[2] whereas the increased formation of biofilm might enhance rhizobium survival under free-living conditions.[4] In addition, the higher amount of EPS released by RD64 cells might improve their initial attachment to Medicago roots.[5] We think that the increased levels of trehalose,[1] LPS, EPS and biofilm are correlated to the enhanced resistance against stress conditions observed for RD64 cells.[1]

Table 1 **Yield of lipopolysaccharides (LPS), exopolysaccharides (EPS) and biofilm from *S. meliloti* cells**

	LPS (mg/g cells)	EPS (mg/g cells)	Biofilm (OD_{570})[a]
Control	33 ± 4	700 ± 100	0.032 ± 0.001
IAA-treated	46 ± 5	700 ± 98	0.030 ± 0.002
RD64	50 ± 3	900 ± 100	0.040 ± 0.003

The values reported in the Table are the averages ± standard deviation of at least five independent biological experiments. [a]Crystal violet staining quantification.

IAA addition to *E. coli* cells induced the expression of the chaperone protein DnaK (homolog of Hsp70).[2] This chaperone, by itself or with other molecular chaperone systems as HtpG (homolog of Hsp90), allows bacterial cells to survive conditions that cause protein unfolding.[6] To investigate whether IAA could trigger a similar effect in *S. meliloti,* the expression levels of *dnak* and *htpG* genes were measured by RT-PCR analysis. The expression level of *dnak* gene was slightly inhibited in both IAA-treated (0.80 ± 0.09, n = 4) and RD64 (0.60 ± 0.07, n = 4) cells. By contrast, *htpG* gene was strongly induced in both IAA-treated (31 ± 5, n = 4) and RD64 (11 ± 1, n = 4) cells. Considering different results,[7,8] we speculate that HtpG might contribute to the ability of both IAA-treated and RD64 cells to survive better at low temperatures.[1]

Desiccation and Competition Studies

Reduced viability with drought has been reported for different rhizobia including *S. meliloti*.[9,10] The effect of long-term storage under desiccation conditions on *S. meliloti* cells was evaluated by using a fast drying, a desiccation treatment that inhibits more effectively the rhizobia survival.[11] A decrease in viable cells was observed for both control and RD64 cells. The negative effect of fast drying was efficiently countered by adding IAA to a least concentration of 0.05 mM; indeed, even after 14 days of desiccation-treatment, the number of viable cells counted for IAA-treated cells (556 ± 46, n = 5)

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were higher than that measured for control cells (378 ± 30, n = 5).[12] This result suggests that high doses of exogenous IAA may promote cell survival by inducing stress-protective systems.

In a competition assay, the relative ability of wild type and RD64 strains to occupy nodules formed on Medicago plants after co-inoculation was evaluated. We found that, when the plants were inoculated with equal numbers of control and RD64 cells, the majority of Medicago nodules were occupied by the wild type strain (Table 3). However, when ten times more RD64 cells were used, the number of nodules occupied by this strain increased as compared to the control. This increment was more evident after salt-stress: RD64 strain occupied about 60% of the nodules. Our results reinforce the suggestion that effective strains are not necessarily more competitive than ineffective strains.[13] To overcome this problem, massive inoculation with RD64 strain may help to dislodge the native strains. The plant growth promoting effects induced by the IAA-overproducing rhizobium on Medicago plants[14] was observed for other leguminous plants such as pea[15] and bean,[16] for which an increased dry weight production was already reported.

Hormone Remodeling

Mt-RD64 plants show an accumulation of IAA in nodules[1] that leads to an alteration in the concentration of this hormone in other plant tissues. Indeed, a decrease in the shoot and an increase in both the roots containing nodules and in nodules alone was observed for *Mt*-RD64 plants. The evaluation of the expression levels of the auxin responsive gene *GH3* confirmed these data. Here we found that the expression levels of Medicago genes encoding members of cytokinin signaling pathways were induced in the roots of *Mt*-RD64 plants (Table 2). These data suggest that the IAA-overproduction in the roots of these plants has a positive effect on cytokinin level. Our data reinforce the suggestion that the levels of auxin and cytokinin are intertwined[17] and that plants is able to attenuate the local hormone imbalance.[18] The upregulation of cytokinins signaling genes might trigger an equilibrate root nodule development.[19]

Conclusions

Engineered rhizobia strains able to overproduce IAA seem to be beneficial for plant growth and thus "provide a conceptual framework for rational human intervention for durable and broad-spectrum disease resistance in crops".[20] Indeed, despite the effectiveness of such kinds of rhizobia they have not (yet) been naturally selected. A possible explanation might be due to the low competitiveness of IAA-overproducing strains.

Table 2 **Real-time PCR analysis of cytokinin signaling genes in roots of medicago plants**

Gene	TIGR TC Accession number[a]	Relative level[b]
Mt CRE1	TC109250	1.5 ± 0.1
Mt HK2	TC102188	2.6 ± 0.3
Mt HK3	TC105228	1.8 ± 0.2
Mt HP1	TC106865	2.9 ± 0.6
Mt HP2	TC110295	3.0 ± 0.4
Mt RR1	TC95959	1.4 ± 0.2
Mt RR2	TC94164	1.9 ± 0.4
Mt RR3	TC96138	1.3 ± 0.1
Mt RR4	TC103991	2.2 ± 0.4
Mt RR5	TC95394	1.5 ± 0.2

The values reported in the Table are the averages ± standard deviation of four independent biological experiments. [a]The accession numbers are provided from "The Institute for Genomic Research" (TIGR) (http://www.tigr.org/tdb/e2k1/mta1/). [b]Relative gene expression levels from comparative CT method; $2^{-\Delta\Delta CT} > 1$, gene more highly expressed in RD64 cells; $2^{-\Delta\Delta CT} < 1$, gene more highly expressed in control cells.

Table 3 **Competitiveness assay between *S. meliloti* 1021 and RD64 strains**

Inoculum ratio (CFU ml^{-1}) 1021/RD64	Establishment ratio[a] (%) 1021/RD64	
	Control	Salt-stress[b]
1.0 x 10^4/1.0 x 10^4	90/10	83/17
1.0 x 10^5/1.0 x 10^4	100/0	93/7
1.0 x 10^4/1.0 x 10^5	63/37	41/59

[a]To determine strain establishment, bacteria were isolated from nodules and their identity verified by antibiotic resistance patterns (n = 15). [b]*M. truncatula* 2HA plants subjected for 3 days to salt stress by adding NaCl (final concentration 300 mM) to the growth medium.

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