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Auxins upregulate *nif* and *fix* genes

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In a recent publication we analyzed the global effects triggered by IAA overproduction in *S. meliloti* RD64 under free-living conditions by comparing the gene expression pattern of wild type 1021 with that of RD64 and 1021 treated with IAA and other four chemically or functionally related molecules. Among the genes differentially expressed in RD64 and IAA-treated 1021 cells we found two genes of *pho* operon, *phoT* and *phoC*. Based on this finding we examined the mechanisms for mineral P solubilization in RD64 and the potential ability of this strain to improve Medicago growth under P-starved conditions. Here, we further analyze the expression profiles obtained in microarray analysis and evaluate the specificity and the extent of overlap between all treatments. Venn diagrams indicated that IAA- and 2,4-D-regulated genes were closely related. Furthermore, most differentially expressed genes from pSymA were induced in 1021 cells treated with 2,4-D, ICA, IND and Trp as compared to the untreated 1021 cells. RT-PCR analysis was employed to analyze the differential expression patterns of nitrogen fixation genes under free-living and symbiotic conditions. Under symbiotic condition, the relative expression levels of *nif* and *fix* genes were significantly induced in *Mt*- RD64 plants and in *Mt*-1021 plants treated with IAA and 2,4-D whereas they were unchanged or repressed in *Mt*-1021 plants treated with the other selected compounds when compared to the untreated *Mt*-1021 plants.

We have previously shown that IAA triggered the upregulation of a central backbone of metabolism such as TCA cycle and the accumulation of PHB granules in free-living Rhizobium.[1] Under symbiotic conditions increased acetylene reduction and plant or seed dry weight production were observed for plants nodulated by IAA-overproducing strains.[1,2] More recently we showed that IAA led to an improvement of stress responses both in free-living and symbiotic conditions. It is known that plants develop a plethora of physiological, developmental and biochemical changes to deal with environmental stress conditions.[3-6] These changes require the activation of biochemical pathways that probably act additively and synergistically and depend largely on efficient nitrogen fixation in the root nodules, a sensitive target for abiotic stresses.[7,8] In this addendum, we comment our recent published data and report that *Mt*-RD64 plants exhibited enhanced expression of nitrogen fixation genes. The treatment of *Mt*-1021 plants with exogenous IAA led to similar upregulation. We speculate that this positive alteration might be of agronomic advantage: it could improve the adaptation of these plants to stressful environments as we have found for the salt-stress and P-starvation.[9,14]

Changes in Gene Expression Patterns Under Free-Living Condition

Microarray analysis was previously performed to compare the transcript profile of wild-type *S. meliloti* 1021 with that of RD64 and 1021 treated with IAA and other four chemically or functionally related molecules such as indole (Ind), tryptophan (Trp), indole-3-carboxylic acid (ICA) and 2,4-dichlorophenoxyacetic acid (2,4-D).[9] In order to derive useful biological

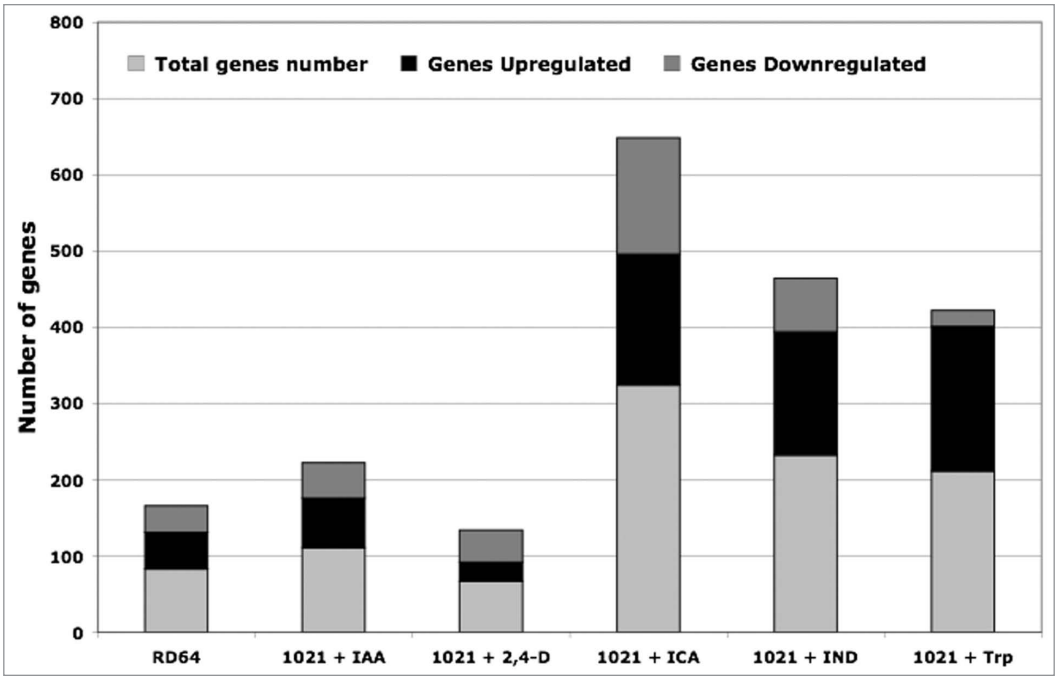

Figure 1. Number of genes significantly up or downregulated in RD64 and in 1021 cells treated for 3 hours with 0.5 mM Ind, Trp, ICA, 2,4-D or IAA than in untreated 1021 cells. Numbers shown are for genes where $p \leq 0.05$ and $M \geq 0.7$ (upregulated) or $M \leq 0.7$ (downregulated). The M value refers to the $\log_2$ of the ratio of intensities of each spots in the two channels. Microarray analysis is carried out as previously reported.[7]

knowledge from the datasets obtained in this analysis a more detailed classification of genes differentially expressed was performed.

The transcripts of 324, 232 and 211 genes were significantly up- or down-regulated after ICA-, IND- and Trp-treatment, respectively, whereas 112 and 67 genes were differentially expressed in 1021 cells treated with IAA and 2,4-D, respectively (**Fig. 1**). The number of genes regulated in RD64 cells (126) was closer to that found for the latter samples. This finding suggests that a common response mechanism is activated after IAA- and 2,4-D-treatment and that this mechanism differs from that induced by ICA-, IND- and Trp-treatment.

The evaluation of replicon location showed that the upregulated genes had a plasmid location for 1021 + 2,4-D, 1021 + ICA, 1021 + IND and 1021 + Trp (**Fig. 2A**), since more than 50% of all predicted protein-encoding genes were located on the megaplasmid pSymA, which is known to contain many genes specifically involved in symbiosis.[10] Indeed, among the genes significantly upregulated in these cells many *nif* and *fix* genes were found.[9] For 1021 + IAA and RD64 cells the upregulated genes were mainly mapped on chromosome (**Fig. 2A**). On the other hand, the majority of downregulated genes were located on the chromosome for all samples (**Fig. 2B**).

To examine the specificity and the overlapping of the treatments examined we compared the expression profiles of 1021 + IAA and RD64 cells with those of 1021 + 2,4-D, 1021 + ICA, 1021+IND and 1021 + Trp cells by using Venn diagrams and classified the genes up or downregulated into groups (**Fig. 3**).[11] This analysis led to identification of 38 genes that were differentially expressed in both 1021 + IAA and RD64 cells (**Fig. 3D**). Moreover, 69% of 2,4-D-regulated genes were also regulated in these cells, thus indicating that similar response mechanisms were activated after treatment with these two auxin molecules (**Fig. 3D**). When similar comparisons were made with 1021+ICA (**Fig. 3C**), 1021 + IND (**Fig. 3B**) and 1021+Trp (**Fig. 3A**) we found that a high percentage of genes were differentially expressed solely in these cells. This result was particularly evident in 1021 + Trp cells for which only 1% of genes were also expressed in 1021 + IAA and RD64 cells (**Fig. 3A**). By contrast, the analysis of differences and cross-talk of gene expression among 1021 + ICA, 1021 + IND and 1021 + Trp cells revealed that the percentage of genes solely regulated in each treatments was very low, with 90% of genes expressed in 1021 + IND cells also expressed in 1021 + ICA and 1021 + Trp cells (**Fig. 3E**), suggesting a common regulatory mechanism involved in the response of 1021 cells to chemically related molecules such as ICA, IND and Trp. These results also indicated the existence of greater cross-talk between IAA- and 2,4-D-response processes than between IAA- and ICA-, IND- or Trp-response processes.

Quantitative Real-Time PCR Analysis Under Free-Living and Symbiotic Conditions

Microarrays results indicated that under free-living conditions the majority of genes differentially regulated in 1021 + 2,4-D, 1021 + ICA, 1021 + IND and 1021 + Trp were located on pSymA, which contains genes necessary for nodulation and nitrogen fixation. Therefore, RT-PCR analysis was employed to evaluate the relative expression levels of two *nif* genes (*nifA* and *nifK*) and two *fix* genes (*fixL* and *fixK2*) under free-living conditions and in symbiosis with Medicago plants.[12]

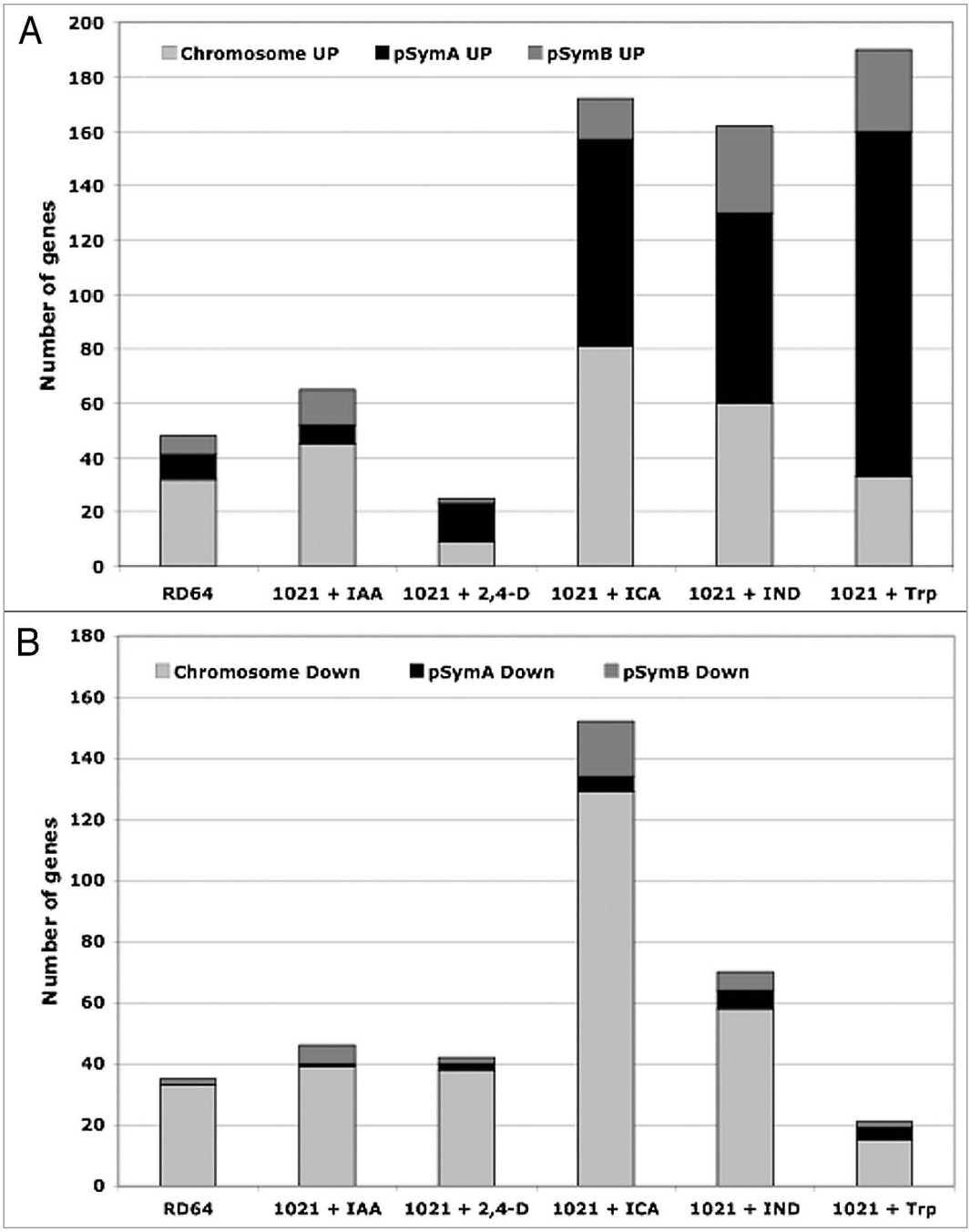

Figure 2. Replicon assignment of genes induced (A) or repressed (B) in RD64 and in 1021 cells treated for 3 hours with 0.5 mM Ind, Trp, ICA, 2,4-D or IAA than in untreated 1021 cells.

Under free-living conditions, the expression of *nifA, fixL* and *fixK2* genes was induced in 1021 + 2,4-D, 1021 + ICA, 1021 + IND and 1021 + Trp cells when compared to untreated 1021 cells (**Table 1**). The highest induction level was observed for *nifA* gene, the specific activator of nitrogen fixation genes.[13] These findings were consistent with the upregulation of genes located on pSymA and involved in nodulation and nitrogen fixation observed in microarray analysis.[7] For 1021 + IAA and RD64 cells *nifK* and *fixK2* genes were downregulated, whereas *nifA* and *fixL* genes were induced or unaffected.

During symbiosis the expression levels of these genes were completely different except for *Mt*-1021 plants treated with 2,4-D (**Table 2**). All the selected genes were upregulated in *Mt*-RD64 plants and in *Mt*-1021 plants treated with IAA and 2,4-D as compared to the untreated *Mt*-1021 plants. By contrast, for *Mt*-1021 plants treated with ICA, IND and Trp the expression of *nifA, nifK* and *fixL* genes was unaffected, whereas *fixK2* gene

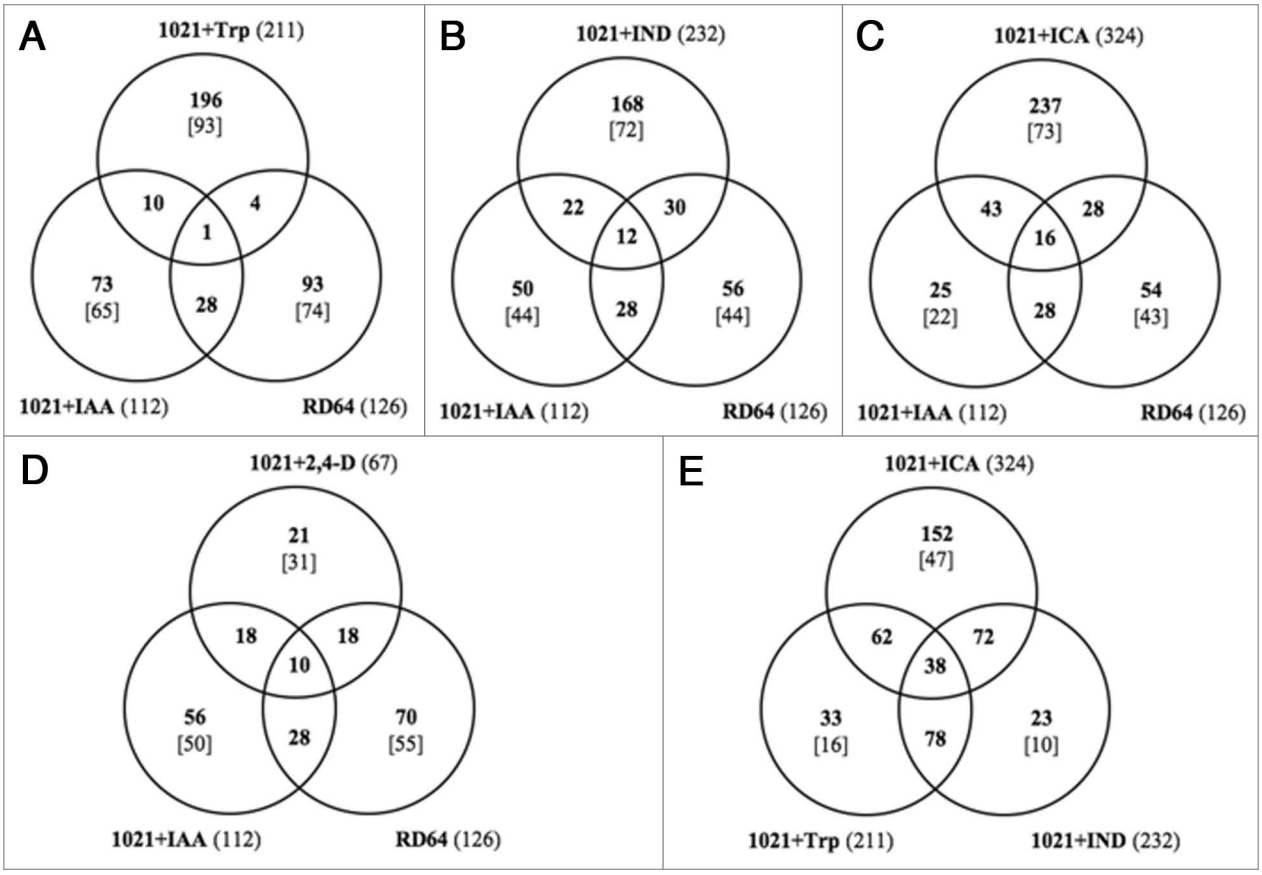

Figure 3. Venn diagram showing the classification of *S. meliloti* genes significantly up or down regulated in RD64 and in 1021 cells treated for three hours with 0.5 mM Ind, Trp, ICA, 2,4-D or IAA than in untreated 1021 cells on the basis of microarray analyses. Numbers in parentheses represent the total genes with $p \leq 0.05$ and $M \geq 0.7$ (upregulated) or $M \leq 0.7$ (downregulated). The M value description is as reported in **Figure 1**. In total, there were 112 IAA-regulated genes, 126 RD64-regulated genes, 67 2,4-D-regulated genes, 211 Trp-regulated genes, 232 IND-regulated genes and 324 ICA-regulated genes. Numbers in bracket represent the percentages of genes differentially expressed exclusively in each samples.

was downregulated as compared to the untreated *Mt*-1021 plants.

Final Remarks

Our previous investigations provided evidence that overexpression of IAA in *S. meliloti* 1021 played a positive role in the adaptation to different environmental conditions, including P starvation, both in free-living bacteria and in nodulated Medicago plants.[7,14] We also reported that plants nodulated by the IAA overproducing strain exhibited improved nitrogen-fixing ability, both under normal and salt stress conditions.[12] Here we suggest that this finding was connected to the upregulation of genes that control nitrogen fixation such as *nifA*, *fixL* and *fixK2* (**Table 2**). We also speculate that the effects observed under symbiotic conditions were specifically due to the hormonal activity of IAA, since the synthetic auxin 2,4-D led to similar effects that were not revealed in the case of structurally related molecules.

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Table 1. Quantitative RT-PCR analysis of *nif* e *fix* genes in free-living *S. meliloti* cells

Sample	Relative level[a]			
	nifA	***nifK***	***fixL***	***fixK2***
RD64	2.6 ± 0.3	0.43 ± 0	2.3 ± 0.2	0.27 ± 0.03
1021 + IAA	2.3 ± 0.3	0.53 ± 0.06	0.93 ± 0.1	0.45 ± 0.07
1021 + 2,4-D	22.5 ± 0.6	0.81 ± 0.05	1.60 ± 0.1	5.7 ± 0.7
1021 + IND	25.6 ± 1.3	0.29 ± 0.3	1.10 ± 0.05	3.0 ± 0
1021 + Trp	8.3 ± 0.7	0.35 ± 0.03	1.4 ± 0.1	3.7 ± 0
1021 + ICA	37.0 ± 4.0	0.85 ± 0.07	2.1 ± 0.2	4.4 ± 0.2

[a]Relative gene expression levels from comparative C_T method. The relative expression level was >1 for genes more highly expressed in RD64 cells and in 1021 cells treated for 3 h with 0.5 mM Ind, Trp, ICA, 2,4-D or IAA than in untreated 1021 cells. The values reported in the Table are the means ± SD of at least three biological replicates conducted at different times. All averages differ significantly according to the Tukey's test ($p < 0.01$). Total RNA isolation, cDNA synthesis and quantitative PCR were performed as previously described.[9] Specific primer pairs, designed using the Primer3 software, are shown. *nifA*: 5'-CCT TGC AAG AGC ATT CCT TC-3' and 5'-TCT TTG ACC TGG CGA GAG TT-3'; *nifK*: 5'-GGA GGT CAT TGG TGA CGA CT-3' and 5'-TTG ATC GAG CCA GGG TTT AC-3'; *fixL*: 5'-AAA AGC GCA TCA TCG GTA TC-3' and 5'-TTT CGC CCA TCT CAT TTA GG-3'; *fixK2*: 5'-AAG CCA AAC CAC AGT CCA TC-3' and 5'-CAT CTG AAA GGA GGC GGT AG-3'.

Table 2. Quantitative RT-PCR analysis of *nif* e *fix* genes in *S. meliloti* cells during symbiosis

Sample	Relative level[a]			
	nifA	***nifK***	***fixL***	***fixK2***
Mt-RD64	1.9 ± 0.2	2.3 ± 0.2	3.2 ± 0	2.3 ± 0.3
Mt-1021 + IAA	2.1 ± 0.2	2.8 ± 0.4	3.0 ± 0.2	1.5 ± 0
Mt-1021 + 2,4-D	1.9 ± 0.3	2.0 ± 0.2	2.7 ± 0.1	1.6 ± 0.1
Mt-1021 + IND	1.2 ± 0.2	1.0 ± 0.3	1.2 ± 0.2	0.66 ± 0
Mt-1021 + Trp	1.2 ± 0.2	1.1 ± 0.2	0.93 ± 0.09	0.66 ± 0.06
Mt-1021 + ICA	0.85 ± 0.05	1.0 ± 0.2	1.6 ± 0.2	0.47 ± 0.05

[a]Relative gene expression levels from comparative C_T method. The relative expression level was >1 for genes more highly expressed in nodules of *Mt*-RD64 plants or in nodules of *Mt*-1021 plants treated for 4 hours with 0.5 mM Ind, Trp, ICA, 2,4-D or IAA than in untreated *Mt*-1021 plants. The values reported in the table are the means ± SD of at least three biological replicates conducted at different times. All averages differ significantly according to the Tukey's test ($p < 0.01$). Experimental procedures were as described in Table 1.

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