Salt Stress Enhances Early Symbiotic Gene Expression in *Medicago truncatula* and Induces a Stress-Specific Set of Rhizobium-Responsive Genes

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Accepted 4 April 2021.

Salt stress is a major agricultural concern inhibiting not only plant growth but also the symbiotic association between legume roots and the soil bacteria rhizobia. This symbiotic association is initiated by a molecular dialogue between the two partners, leading to the activation of a signaling cascade in the legume host and, ultimately, the formation of nitrogen-fixing root nodules. Here, we show that a moderate salt stress increases the responsiveness of early symbiotic genes in *Medicago truncatula* to its symbiotic partner, *Sinorhizobium meliloti* while, conversely, inoculation with *S. meliloti* counteracts salt-regulated gene expression, restoring one-third to control levels. Our analysis of *early nodulin 11* (*ENOD11*) shows that salt-induced expression is dynamic, Nod-factor dependent, and requires the ionic but not the osmotic component of salt. We demonstrate that salt stimulation of rhizobium-induced gene expression requires *NSP2*, which functions as a node to integrate the abiotic and biotic signals. In addition, our work reveals that inoculation with *S. meliloti* succinoglycan mutants also hyperinduces *ENOD11* expression in the presence or absence of salt, suggesting a possible link between rhizobial exopolysaccharide and the plant response to salt stress. Finally, we identify an accessory set of genes that are induced by rhizobium only under conditions of salt stress and have not been previously identified as being nodulation-related genes. Our data suggest that interplay of core nodulation genes with different

accessory sets, specific for different abiotic conditions, functions to establish the symbiosis. Together, our findings reveal a complex and dynamic interaction between plant, microbe, and environment.

Keywords: ENOD11, exopolysaccharides, *exoS*, *exoY*, hyperinduction, infection, *Medicago truncatula*, nitrogen-fixation, NPL, NSP2, priming, RNA-seq, root nodules, salt stress, *Sinorhizobium meliloti*, succinoglycan, symbiosis

Legumes are integral to sustainable agriculture because of their high nitrogen content, the product of a mutualistic symbiosis that they form with a group of soil bacteria, collectively known as rhizobium (Graham 2003). The rhizobium–legume symbiosis involves a complex developmental process including both the creation of a new root organ, the nodule, and infection of the nodule by rhizobium, successfully coordinated in space and time (Ferguson et al. 2010; Oldroyd 2013). Within the nodule, differentiated rhizobia reduce atmospheric dinitrogen into ammonium in exchange for carbon from the host (Ferguson et al. 2010). However, the symbiotic interaction between rhizobia and their legume hosts is sensitive to environmental factors, such as extreme temperature, pH, soil nitrogen, drought, and salinity (Murray et al. 2017; Singleton and Bohloul 1984; Zahran 1999).

As our climate changes, rising temperatures lead to increased soil dryness, resulting in the concentration of salts, becoming an accelerating agricultural concern (HanumanthaRao et al. 2016; Sherwood and Fu 2014; St.Clair and Lynch 2010). Salt accumulation is exacerbated by irrigation, and the amount of cropland experiencing salinization is growing at 10% per year; by 2050, half of all agricultural land is predicted to be salt stressed (Nachshon 2018). Even moderate salt stress, not sufficient to inhibit growth, can have severe effects on nodulation, negatively regulating multiple aspects of the rhizobium–legume symbiosis (Borucki and Sujkowska 2008; Singleton and Bohloul 1984; Soussi et al. 1998; Tu 1981).

Establishment of the rhizobium–legume symbiosis is orchestrated by a series of signal exchanges between the two partners (Oldroyd 2013). Nodulation is initiated by the synthesis and

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Funding: Support was provided by Gene Ontology, the National Institute of General Medical Sciences (grants GM093628 and P20GM103449), and Hatch (grant VT-H02104); and Kyoto Encyclopedia of Genes and Genomes pathway analyses were supported by the Vermont Biomedical Research Network (VBRN) Bioinformatics Core.

*The e-Xtra logo stands for “electronic extra” and indicates that supplementary materials and a supplementary file are published online.

The author(s) declare no conflict of interest.

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secretion of rhizobial lipochitooligosaccharide signal molecules, known as Nod factors (NF), which signal the plant root, inducing changes in cytoskeletal organization and patterns of cell growth, as well as inducing major changes in gene expression (Roy et al. 2020). NF perception at the host plasma membrane by LysM receptor-like kinases initiates a signaling cascade that triggers nuclear calcium spiking, decoded by the nuclear calcium-calmodulin kinase, encoded by *Doesn't Make Infections 3* (*DMI3*) (Zipfel and Oldroyd 2017). *DMI3* signaling induces a transcriptional cascade, mediating changes needed for rhizobial infection, as well as activating cytokinin-dependent signaling in the underlying cortical cells (Catoira 2000; Marsh et al. 2007; Middleton et al. 2007; Mitra et al. 2004a; Murray et al. 2007; Oldroyd and Long 2003; Reid et al. 2017; Rival et al. 2012; Tirichine et al. 2007). These transcription factors act in concert to spatiotemporally modulate the expression of *Early Nodulin 11* (*ENOD11*), one of the earliest markers of rhizobium-legume symbiosis (Cerri et al. 2012; Journet et al. 2001; Vernié et al. 2015). *ENOD11* encodes a cell wall glycoprotein associated with the early stages of infection (Cerri et al. 2012; Journet et al. 2001; Vernié et al. 2015). The *ENOD11* promoter contains the distal NF-box, to which the transcription factor ERF required for nodulation 1 (ERN1) and the closely related ERN2 bind, activating its expression in patches of epidermal cells (Andriankaja et al. 2007; Cerri et al. 2012). There is a subsequent, rhizobial infection-associated upregulation of *ENOD11* mediated by a complex formed by the transcription factors nodulation signaling pathways 1 and 2 (NSP1 and NSP2), which binds a proximal region of the promoter, activating *ENOD11* expression independent of ERN1 (Cerri et al. 2012). As symbiosis proceeds, another transcription factor, nodule inception (NIN), competes with ERN1 for binding to the NF-box in the *ENOD11* promoter, repressing *ENOD11* expression in younger portions of the root system in order to maintain a developmentally sustainable number of nodules (Marsh et al. 2007; Vernié et al. 2015).

Rhizobial infection requires NF signaling, which induces changes within the root and root hair cells, thus preparing them for this process. Infection also requires production of bacterial exopolysaccharide (EPS) (Gage 2004; Jones et al. 2007). Together, these two molecules orchestrate the successful infection of the host root by rhizobia. Within minutes of NF exposure, root hair cells reorganize their cytoskeleton, part of a process of reorienting root hair growth to form tight curls entrapping the signaling rhizobia (Esseling et al. 2003; Jones et al. 2007). The plant rapidly modifies the cell wall of the developing infection chamber, secreting *ENOD11* only to this portion of the root hair cell wall (Fournier et al. 2015). Although NF signaling continues to play a role in planta, exit from this infection chamber and successful invasion of the host requires production of EPS (Jones et al. 2007). The predominant EPS produced by *Sinorhizobium meliloti* is succinoglycan (EPSI). Succinoglycan synthesis, export, and polymerization are controlled by the *exo* gene products (Jones et al. 2007). *S. meliloti* *exoY* mutants are unable to synthesize succinoglycan, and remain trapped in the root-hair-enclosed infection chamber (Cheng and Walker 1998). In contrast, root hair curls containing succinoglycan-synthesizing *S. meliloti* form an infection thread (IT), a tubular, transcellular apoplastic compartment, which initiates from the infection chamber, passing through the root hair cell and extending through underlying cortical cells; it is through this structure that the bacteria gain entry into the root. Localized cell wall degradation and inward growth of both the host cell wall and plasma membrane are needed for both initiation and extension of the IT (Fournier et al. 2008; Gage 2002, 2004). NF signaling induces expression of the host protein nodule pectate lyase (NPL), a cell-wall-degrading enzyme, which has been implicated in the process of infection, thus indicating a continuing role for NF signaling in the infection process (Xie et al. 2012). In *Lotus*

japonicus, NF induces expression of the LysM receptor kinase, EPR3, which binds the *Mesorhizobium loti* EPS octasaccharide and is required for progression of the IT through the root hair and into the root cortex and nodule primordium (Kawaharada et al. 2015, 2017). Thus, infection is initiated by NF signaling but entry into the root requires EPS.

Massive transcriptional reprogramming accompanies the initial response of plants to the symbiotic signals NF and succinoglycan as well as abiotic stresses such as salinity (Breakspear et al. 2014; Damiani et al. 2016a; Jones et al. 2008; Kreps et al. 2002; Larrainzar et al. 2015; Li et al. 2011; Mitra et al. 2004b; Shaar-Moshe et al. 2017; van Zeijl et al. 2015b). Perception of these biotic and abiotic signals result in transcriptional cascades that alter the expression pattern of thousands of genes and trigger hormone signaling to coordinate the plant response. Salt stress responses are mediated primarily by abscisic acid (ABA) and ethylene but gibberellins (GAs) and cytokinins also play an important role (Ku et al. 2018; Šimura et al. 2018; M. Zhang et al. 2016). All of these hormones also function in early root responses to NF and in subsequent organogenesis (Ferguson and Mathesius 2014). In particular, ABA and ethylene modulate the calcium spiking that functions upstream of the transcriptional cascade in the NF response, thus having the ability to block the response just after NF perception. Subsequently, ABA inhibits cytokinin induction of gene expression and strongly reduces nodule number (Ding et al. 2008; Oldroyd et al. 2001). Altering the signaling of any one of these hormones has profound effects on the transcriptome, thus leading to waves of transcriptional changes in response to these abiotic and biotic signals (Brenner and Schmülling 2012; Larrainzar et al. 2015; Luo et al. 2018; Okamoto et al. 2010; Xie et al. 2016).

Rhizobium-legume symbiosis is particularly sensitive to salinity compared with the individual partners involved in this process. Salt concentrations that are too low to limit photosynthesis can inhibit nodulation of chickpea (Soussi et al. 1998), while free-living *S. meliloti* (strain Rm1021) is tolerant to up to 400 mM NaCl (Domínguez-Ferreras et al. 2006). Salt stress interferes with rhizobial infection, inhibiting root hair curling, rhizobial invasion, and release of rhizobia into nodule cells, producing fewer, less efficient nodules (Borucki and Sujkowska 2008; Cordovilla et al. 1994; Singleton and Bohlool 1984; Tu 1981; Zahran and Sprent 1986). Because of the central role of biological nitrogen fixation in sustainable agriculture and the increasing salinization of agricultural land, it is important to understand the mechanism of salt stress interference with symbiotic legume root nodule initiation and establishment.

The model legume *Medicago truncatula* is a Mediterranean species and displays a range of salt tolerance across natural populations (Soumaya et al. 2011). The genotype Jemalong A17 (used in this work) is widely used as a system for nodulation studies and is moderately tolerant to salt (de Lorenzo et al. 2007; Zahaf et al. 2012). Several genes in *M. truncatula* function in response to salt stress (Ariel et al. 2010; de Lorenzo et al. 2009; Gruber et al. 2009; Li et al. 2009, 2011; Merchan et al. 2003, 2007). *NAC969* is involved in both salt stress tolerance and nodule senescence, and its downregulation is associated with increased salt tolerance but aberrant nodules (de Zélicourt et al. 2012). Salinity is one of the key drivers of local adaptation in *M. truncatula* (Guerrero et al. 2018). The identification of specific genetic changes in *M. truncatula* associated with local adaptation to salt and to climatic variation in temperature and precipitation, as well as the identification of selective sweeps targeting nodulation genes, indicates the importance of both the rhizobial symbiosis and the salt response to legume survival and fitness (Bonhomme et al. 2015; Friesen et al. 2014; Yoder et al. 2014). Thus, we chose to examine the link between moderate salt stress and early symbiotic signaling. The focus on mild to moderate stress, as opposed to severe stress,

means that the stress responses are studied within a context of plant growth, not arrest, and has been proposed as an approach that may reveal new cellular mechanisms (Verslues 2017).

Here, we show that a moderate salt stress not sufficient to affect plant growth severely inhibits nodulation and rhizobial colonization yet stimulates the expression of several early symbiotic genes, increasing their responsiveness to NF. This hyperinduction is mediated by *NSP2*, and requires the ionic but not the osmotic component of salt. Inoculation with rhizobial succinoglycan mutants also increases the responsiveness of *ENOD11* expression to NF. In contrast, rhizobium inoculation antagonizes the effect of salt stress on the expression of over 100 genes. Finally, we report a set of genes induced by rhizobia only in the presence of salt stress, suggesting the existence of an accessory set of genes specific for nodulation under abiotic stress. Thus, our work reveals a complex interaction between salt stress and symbiosis, in which the abiotic environment influences the biotic interaction, with *NSP2* acting as a node to integrate environmental conditions and symbiotic signaling.

RESULTS

A moderate salt stress strongly inhibits nodulation.

In order to study the effect of salinity on nodulation, we established growth conditions in which *M. truncatula* seedlings were grown in a defined medium with sufficient water throughout the course of our experiments and were exposed to a concentration of salinity that strongly inhibited nodulation but did not impact overall root or shoot growth, and at which Rm1021 growth kinetics were unaffected (Miller-Williams et al. 2006) (Fig. 1). We found an 89% reduction in the number of nodules under a constant exposure to 100 mM NaCl; these nodules were globular, mostly white, sometimes brown, in contrast to well-developed pink nodules observed in the control condition. (Fig. 1A and B). Very few of these nodules showed expression of *nifH*, which encodes one of the subunits of nitrogenase, indicating that these are mostly non-functional nodules (Chakraborty 2018). In contrast, this salt treatment had a mild effect on overall growth of the plant during the timeframe of our experiment, with no significant change in primary root length, shoot biomass, or chlorophyll *a* content (Fig. 1C to E), although we did observe a significant decrease in chlorophyll *b* content (Fig. 1E). Thus, under our growth conditions, 100 mM NaCl acts as a moderate salt stress, which severely affects nodulation but has only a modest effect on root and shoot growth.

General response of *M. truncatula* root transcriptome to salinity and rhizobium.

Because we observed a strong reduction in the number of nodules under salt stress, we hypothesized that it would interfere with nodulation at a relatively early step. Given that significant transcriptional changes occur in the host root during the first day after inoculation with rhizobia (Breakspear et al. 2014; Larrainzar et al. 2015; Mitra et al. 2004b), we profiled the transcriptomes of salt-stressed and control primary roots with or without rhizobial inoculation at 1 day postinoculation (dpi). We hoped to identify both changes to expression of known nodulation-associated genes as well as the identification of genes that are associated with nodulation under stress conditions and thus might be missed in standard transcriptome analysis.

Principal component analysis revealed that the majority of variation (40%) was associated with salt, likely due to the longer salt treatment (5 days) as opposed to rhizobium treatment (1 day), and also that one replicate did not cluster with other samples from the same treatment group (Fig. 2A). To ensure that inclusion of this outlier did not bias our results, we analyzed the RNA sequencing (RNA-seq) data both with and without this sample (both datasets are provided in Supplementary File S1). This comparison revealed

that the majority of differentially expressed genes (DEGs) were shared between these analyses and, hence, we focused on these DEGs (for comparison, see Supplementary Figure S1 and Supplementary File S1). The strongest transcriptional response we observed was to salt stress, with 641 DEGs identified in samples containing a salt treatment (+S), either with (+R) or without (−R) rhizobial inoculation (Fig. 2B; Supplementary File S1). As expected, this set included upregulation of several genes associated with salt stress and ABA signaling, including genes encoding ABA 8'-hydroxylases, dehydrins, responsive to dehydration 22 (RD22), late embryogenesis abundant (LEA) proteins, and a dehydration-responsive element-binding protein (Medtr1g101600) (Supplementary File S1). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis further supported the strong effect of salt stress on the transcriptome, with the maximum number of significantly enriched pathways observed in its presence (Fig. 2C).

In contrast, we saw only 36 genes that were differentially expressed after rhizobium inoculation (Fig. 2B). This is fewer than reported in other studies and may be due to our growth conditions or the fact that the whole root was used instead of just the responsive zone or specific tissues or cell types. Most of the rhizobia-induced genes we identified were previously shown to be involved in nodulation, and include *ENOD11*, *ERN1*, *NIN*, *MtNPL*, *nuclear factor YA-1 (NF-YA1)*, *LOB domain protein 16 (LBD16)*, *vapyrin (VPY)*, *rhizobium-directed polar growth (RPG)*, *vestitone reductase cluster2 (VRC2)*, *β-carotene isomerase DWARF27 (D27)*, and a glycine-rich peptide (Tables 1 and 2; Supplementary File S1) (Arrighi et al. 2008; Breakspear et al.

Fig. 1. Moderate salt stress inhibits nodulation but not root or shoot growth. Effect of 100 mM NaCl on **A** and **B**, nodulation; **C**, primary root length; **D**, shoot dry weight; or **E**, chlorophyll (chl) content. Nodules visually categorized at 14 days postinoculation based on stages of development as immature (white), mature (pink), senescing or stressed (yellow), or severely senescing or stressed (brown) (A); color-coding of nodules matches the graph in B. Primary root length from $n = 14$, shoot dry weight from $n = 21$, and chlorophyll content from $n = 14$ seedlings per treatment per experiment. Data plotted as least square means (LSM) $\pm$ standard error of the mean from three independent experiments. Significance tested by pairwise comparison of LSM ($\alpha = 0.05$) and significant differences shown by asterisks (* and *** denote $P < 0.05$ and 0.0005, respectively). Asterisks in B reflect a significant difference in the total number of nodules (all colors) between treatments. Scale bar = 0.1 mm.

Fig. 2. Transcriptome profiling of roots reveals transcriptional regulation by salt and rhizobium. **A**, Principal component analysis (PCA) of patterns of differentially expressed genes (DEGs) from roots grown with or without 100 mM NaCl with either rhizobium or mock inoculation. Roots were harvested at 1 day postinoculation from three biological replicates, with 21 seedlings per treatment per replicate; each point represents one biological replicate. **B**, Venn diagram representing DEGs under salt, rhizobium, or a combination of these two treatments, in comparison with the untreated condition (control). **C**, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis for the effect of individual treatments compared with the control. MAPK = mitogen-activated protein kinase. Significantly overrepresented KEGG pathways are in the same group. Enrichment P value ≤ 0.05 , DEG input = fold change ≤ 11.51 , and false discovery rate = 0.05. The one pathway common to all treatments is the “Carotenoid biosynthesis” pathway.

2014; Cerri et al. 2012; Jardinaud et al. 2016; Journet et al. 2001; Laporte et al. 2014; Marsh et al. 2007; Middleton et al. 2007; Murray 2011; Schiessl et al. 2019; Soyano et al. 2019; van Zeijl et al. 2015b; Vernié et al. 2015; Wang et al. 2016; Xie et al. 2012).

Table 1. Rhizobium-responsive genes that are significantly upregulated by salt stress^a

Gene	Fold change	Description
<i>Medtr3g415670</i>	8.37281988	Transmembrane protein, putative (<i>ENOD11</i>)
<i>Medtr3g086320</i>	7.52697871	Pectate lyase family protein (<i>MtNPL</i>)
<i>Medtr3g112460</i>	6.76060631	Nitrate transporter1/peptide transporter family
<i>Medtr8g012795</i>	6.69379079	Defensin-like
<i>Medtr7g074730</i>	6.21891491	Dihydroflavonol 4-reductase-like protein (<i>VRC2</i>)
<i>Medtr8g040940</i>	5.94651732	Hypothetical protein
<i>Medtr5g045470</i>	5.93956617	Serine protease inhibitor 1 (<i>SPI1</i>)
<i>Medtr4g081190</i>	5.47579623	ABC transporter B family protein
<i>Medtr5g011960</i>	4.28478737	Lipid transfer protein
<i>Medtr4g117000</i>	4.26927542	Glycoside hydrolase family 18 protein (<i>MtCHITb</i>)
<i>Medtr5g019460</i>	4.15736244	Geranylgeranyl pyrophosphate synthase (<i>GGPS1</i>)
<i>Medtr8g022270</i>	4.08146484	ABC transporter B family-like protein
<i>Medtr1g090807</i>	3.829778	Myosin heavy chain-like protein, putative (<i>MtRPG</i>)
<i>Medtr4g088510</i>	3.80251003	Hypothetical protein
<i>Medtr6g027840</i>	3.73624957	Ankyrin repeat RF-like protein, putative (<i>MtVAPYRIN</i>)
<i>Medtr3g031270</i>	3.39915861	Wound-induced-like protein
<i>Medtr4g076255</i>	3.26161212	Glucan endo-1,3- β -glucosidase
<i>Medtr3g087730</i>	3.21429686	Sulfate/bicarbonate/oxalate exchanger and transporter (<i>sat-1</i>)
<i>Medtr2g069245</i>	2.54506008	Nodule-specific glycine rich peptide
<i>Medtr3g436390</i>	2.03117086	Flavonoid hydroxylase

^a Genes listed by descending order of fold change with salt. Fold change calculated between salt-supplemented rhizobium-inoculated samples (+R+S) and rhizobium-inoculated samples (+R-S) excluding the outlier sample. *Medtr3g436390* and *Medtr4g076255* are repressed by rhizobium inoculation in the absence of salt. All other genes are induced by inoculation in the absence of salt stress. In addition to the listed genes, *ERN1* was observed to be hyperinduced by salt using quantitative reverse-transcription PCR (Supplementary Fig. S8). Top *Arabidopsis* protein BLAST results are provided in Supplementary Table S1.

Table 2. Rhizobium-responsive genes that are not affected by salt stress^a

Gene	Description
<i>Medtr0011s0280</i>	Putative oxidoreductase
<i>Medtr1g056530</i>	CCAAT-binding transcription factor (<i>NF-YA1</i>)
<i>Medtr1g471050</i>	Putative β -carotene isomerase (<i>D27</i>)
<i>Medtr2g019750</i>	AP2 domain class transcription factor
<i>Medtr3g081140</i>	Cysteine-rich TM module stress tolerance protein
<i>Medtr3g103260</i>	Hypothetical protein
<i>Medtr5g025850</i>	Leucine-rich repeat receptor-like kinase family protein
<i>Medtr5g075400</i>	Hypothetical protein
<i>Medtr5g099060</i>	Nodule inception protein (<i>NIN</i>)
<i>Medtr6g007160</i>	Transmembrane protein, putative
<i>Medtr6g007690</i>	Serine-threonine kinase
<i>Medtr6g056070</i>	Putative salicylate carboxymethyltransferase
<i>Medtr7g085810</i>	AP2 domain class transcription factor (<i>ERN1</i>)
<i>Medtr7g096530</i>	Lateral organ boundaries domain TF (<i>LBD16</i>)

^a All genes listed had no significant change in expression between salt-supplemented rhizobium-inoculated samples (+R+S) and rhizobium-inoculated samples (+R-S) in the core set of genes. The expression of *NIN* (+R+S versus +R-S) was further tested with quantitative reverse-transcription PCR with similar results (Supplementary Fig. S8). Top *Arabidopsis* protein BLAST results are provided in Supplementary Table S2.

We also observed induction of a gene encoding the chitinase *MtCHITb*, which can be converted into a NF hydrolase (L.-Y. Zhang et al. 2016).

Complex interplay between salinity and rhizobium in their regulation of gene expression.

To better understand the interplay between salinity and rhizobium in regulating gene expression, we analyzed rhizobium-responsive genes that are influenced by salinity and salt-responsive genes that are influenced by rhizobium. We found that 32 of the 36 rhizobium-regulated genes identified in our rhizobium in the absence of salt (+R-S) treatment were also regulated by rhizobium in the presence of salt (+R+S treatment). Of these, the expression of 20 was significantly different in the +R+S treatment relative to +R-S, indicating that the level of expression had changed (Fig. 2B; Supplementary File S1). Surprisingly, rather than inhibiting expression, as we predicted from salt's inhibition of nodulation, a moderate salt stress instead stimulated expression of all 20 genes (Table 1). This stimulation of expression included two genes that had been repressed by inoculation (*Medtr3g436390*, encoding a flavonoid hydroxylase, and *Medtr4g076255*, encoding a glucan endo-1,3- β -glucosidase), thus returning them to control levels. The remaining salt-enhanced rhizobium-induced genes were previously found to be upregulated by NF as early as 3 h postapplication (Damiani et al. 2016a; Jardinaud et al. 2016; van Zeijl et al. 2015b). Rhizobium-responsive genes that were unaffected by salt stress included the transcription factors *NIN*, *ERN1*, *NF-YA1*, *LBD16*, and *D27* (Table 2). *ENOD11* showed the highest hyperinduction by salt, closely followed by *MtNPL*, suggesting that salt stress particularly enhances expression of early rhizobium-induced genes (Table 1). We subsequently showed by quantitative reverse-transcription (qRT)-PCR that *ERN1* was significantly induced by salt but *NIN* was not (Supplementary Fig. S8).

In contrast to the enhancement of rhizobium-responsive genes by salt, we observed the opposite effect of rhizobium treatment on salt-regulated genes. We found that, of the 327 genes differentially expressed by salt in the absence of rhizobium (-R+S), 119 of them were no longer significantly different from control levels in the combined treatment (+R+S), indicating that the rhizobium treatment antagonized the effect of salt treatment on one-third of the genes. The majority (70%) of these 119 DEGs were upregulated by salt, indicating that rhizobium inoculation suppressed their induction (Supplementary File S1). This category included genes associated with salt stress and ABA signaling, encoding a LEA (*Medtr7g093140*), a dehydrin (*Medtr7g086340*), and an ABA 8'-hydroxylase (*Medtr1g019410*), as well as several encoding putative transcription factors, leucine-rich repeat (LRR) receptor-like kinase family proteins, proteins associated with lipid metabolism, and chlorophyll-binding proteins. A KEGG pathway enrichment analysis of the salt-regulated genes whose induction or repression is reversed by rhizobium inoculation indicates overrepresentation of genes involved in β -alanine metabolism, plant hormone signal transduction, photosynthesis, α -linolenic acid metabolism, porphyrin and chlorophyll metabolism, metabolic pathways, cutin, suberin and wax biosynthesis, and photosynthesis-antenna proteins (Fig. 2C), indicating the broad impact of rhizobium on salt stress responses in the plant root. Notably, this set also included *NSP2*, which was repressed by salt alone, a result that we confirmed with qRT-PCR (Supplementary Fig. S8).

Salt-rhizobium cotreatment strongly upregulates several symbiotic genes.

Given the strong stimulatory effect of salt on rhizobium-responsive genes, we next focused our attention on the set of DEGs observed exclusively in the combined presence of salt and rhizobia. We observed 314 DEGs in this set, which is the largest number of DEGs observed exclusively with any treatment in

our study (Supplementary File S1); of these, 216 (65.6%) were upregulated. Interestingly, 147 were previously identified as being responsive to purified NF or rhizobial inoculation in other studies (Damiani et al. 2016a; Jardinaud et al. 2016; van Zeijl et al. 2015b). The fact that we detected these DEGs only in the combined presence of salt and rhizobium further corroborates a strong positive regulation of salt stress on these rhizobium-responsive genes, such that the expression levels could cross the threshold of detection in our study. Interestingly, only a few of the repressed genes in this set have been shown to be induced during nodulation (21 of 147), indicating that, at this time point, the main effect of salt stress is not to repress nodulation-associated genes but, rather, to stimulate rhizobium-induced gene expression. We did not observe a strong correlation between expression patterns of genes in this set and those observed with a 2-day drought treatment on mature nodules, highlighting the complex interaction between development, environment, and symbiosis (Sańko-Sawcenko et al. 2019) (Supplementary File S1). Gene ontology (GO) term and KEGG pathway enrichment analyses of the genes differentially expressed only in the +R+S dataset highlighted overrepresentation of a diverse set of processes (Fig. 2C; Supplementary Figs. S2 and S3).

Several of the nodulation-associated genes in this set that were upregulated in the rhizobium and salt cotreatment only were previously known to play a role in early stages in nodulation. In particular, we identified genes encoding several LysM receptor-like kinases (LYK), one of which, LYK-related 3 (LYR3), is one of the putative NF receptors (Fliegmann et al. 2016), and another, LYK10 (Maillet et al. 2020), which plays a role in infection. We detected additional genes encoding kinases in this set, including the tyrosine kinase SPK1, whose expression is upregulated by both NF and hydrogen peroxide (Andrio et al. 2013; Jardinaud et al. 2016) and is involved in nodule formation, and the LRR receptor kinase *Medtr6g036780*, which is one of the most highly induced genes in this set, upregulated approximately 150-fold (Supplementary File S1).

We detected upregulation of other genes associated with early events in the rhizobium-legume interaction in this set, including the gene encoding the transcription factor NSP1, which binds the *ENOD11* promoter (Catoira 2000; Cerri et al. 2012; Hirsch et al. 2009; Smit et al. 2005), genes associated with rhizobial infection, such as *ENOD12* (Journet et al. 1994), which is closely related to *ENOD11*, *cystathionine-β-dynthase-like domain-containing protein 1 (CBS1)* (Sinharoy et al. 2016), *seven in absentia (SINA)* (Den Herder et al. 2008), and a gene encoding the putative rhicadhesin receptor (*Medtr2g031270*). The rhicadhesin receptor and *CBS1* were among the most strongly upregulated genes, exhibiting over 150- and 200-fold induction, respectively, compared with the control. In the set of genes including all three replicates, we further detected *annexin 1 (MtAnn1)*, which is thought to be involved in mitotic reactivation of cortical cells prior to infection (de Carvalho Niebel et al. 1998), and *CLAVATA3/embryo-surrounding region 13 (CLE13)* involved in autoregulation of nodulation; the expression of the latter is also detected in cortical cells undergoing mitotic reactivation during nodule organogenesis (Laffont et al. 2018; Mortier et al. 2010, 2012) (Supplementary File S1).

Cortical *NIN* acts through cytokinin and its receptor *cytokinin response 1 (CRE1)* to initiate nodule organogenesis (Vernié et al. 2015). We observed upregulation of several *NIN*-regulated genes with salt-rhizobium cotreatment, including *CBS1*, *NPL*, *RPG*, *ANN1*, and *LYR3* (Liu et al. 2019) (Supplementary File S1). Combined with the differential regulation of *CLE13* (upregulated) and the nitrate-responsive *CLE35* that regulates nodule number (downregulated) (Mens et al. 2020), our data suggest that, at this 1-dpi time point, salt hyperinduces genes associated with the cortical response pathway.

A previously unidentified set of genes associated with nodulation under salt stress.

Although nodulation is strongly inhibited by a moderate salt stress, nodulation does occur (Fig. 1). Transcriptome analysis of the early responses to rhizobium is usually carried out on plants grown in ideal growth conditions. A major goal of this study was to identify genes that might be required for nodulation under stress conditions, which might be missed under ideal conditions. Although we identified many genes known to function or be expressed during nodulation, 53.2% (167 of 314) of the genes exclusively induced in our +R+S set were not previously shown to be involved in nodulation. Some of these were among the most highly expressed genes.

We found a number of genes associated with detoxification. In particular, this set included 12 members of the cytochrome P450 superfamily, 4 of which were downregulated and 8 upregulated. Of those, three of the downregulated and two of the upregulated genes were not previously linked to early nodulation. Two glutathione S-transferase genes were upregulated, including one, *Medtr5g076900*, which was among one of the most highly induced genes (55-fold). We also identified 14 upregulated genes annotated as heavy metal transport/detoxification superfamily (MATE efflux family) proteins or other heavy metal-associated proteins, none of which had been previously associated with nodulation. Two of these genes, *Medtr3g434890* and *Medtr7g451200*, were upregulated approximately 80- and 100-fold, respectively (Supplementary File S1).

The transcriptional reprogramming triggered by the joint treatment of salt stress and rhizobium inoculation also included altered regulation of many genes with a likely role in signaling. Multiple F-box proteins, LRR receptor-like kinases, lectin receptor-type kinases, and others changed expression. In addition, many genes with homology to genes encoding disease resistance proteins of different classes—toll interleukin 1 receptor nucleotide binding site (NBS)-LRR; LRR; nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4; and coiled-coil-NBS-LRR—were either up- or downregulated, suggesting altered defense signaling.

Salt stress dynamically regulates symbiotic *ENOD11*.

Our transcriptome profiling data revealed that salt enhanced the expression of several symbiotic genes to various degrees. Of all the rhizobium-induced genes whose expression was further stimulated by salt, *ENOD11* showed the strongest hyperinduction (Table 1). Given this strong hyperinduction, we chose *ENOD11* for further analysis. To better understand how salt stress regulates *ENOD11*, we analyzed its expression at 2, 3, and 5 dpi, in addition to 1 dpi, the time point at which the transcriptome profiling was done. In validation of our RNA-seq findings, we observed a strong hyperinduction (10.2-fold with qRT-PCR), as well as an expanded zone of *pENOD11:uidA* expression by salt at 1 dpi (Fig. 3; Supplementary Fig. S4). At subsequent time points (2 and 3 dpi), the difference in transcript levels between the salt-treated and control seedlings was not significant but, at 5 dpi, we found salt to significantly inhibit *ENOD11* expression (2.8-fold reduction) (Fig. 3). Consistent with RNA-seq, we could not detect *ENOD11* transcript in uninoculated roots in the presence or absence of salt stress, nor did we observe any β-glucuronidase (GUS) staining in uninoculated *pENOD11:uidA* roots, indicating that salt stress alone cannot induce *ENOD11* (Fig. 4).

Plant response to salt stress is predominantly mediated by the phytohormones ABA and ethylene (Duan et al. 2013). Ethylene is an important mediator of salt stress in *Arabidopsis thaliana* (Cao et al. 2008; Chen et al. 2012) and a powerful inhibitor of early NF signaling, altering the frequency of calcium spiking and reducing symbiotic *ENOD11* expression (Oldroyd et al. 2001). Due to its dual roles in salt stress and NF signaling, we investigated whether ethylene mediates the hyperinduction of

ENOD11 expression by salt stress, using the ethylene-insensitive *sickle* (*skl*) mutant (Penmetsa and Cook 1997; Penmetsa et al. 2008). We found that salt-induced *ENOD11* hyperinduction at 1 dpi was the same in wild-type and *skl* plants, indicating that

Fig. 3. Salt stress dynamically regulates symbiotic *early nodulin 11* (*ENOD11*) expression. **A**, *ENOD11* expression as measured by quantitative reverse-transcription (qRT)-PCR and **B**, the corresponding *pENO-D11:uidA* expression using histochemical assay in inoculated seedlings grown in the presence or absence of 100 mM NaCl; dpi = days postinoculation. Transcript levels showing the mean and the images representative of three independent experiments, with $n = 14$ (histochemical assay) or $n = 21$ (qRT-PCR) per treatment per experiment. Statistical analysis was done using a two-way analysis of variance followed by *t* test on the least square means ($\alpha = 0.05$). Columns not connected by the same letter are significantly different. Error bars represent standard error of the mean. Scale bar = 0.5 mm. An asterisk (*) indicates a developing nodule. Expression levels with the salt-alone treatment were below the limit of detection in qRT-PCR.

Fig. 4. Hyperinduction of symbiotic *early nodulin 11* (*ENOD11*) occurs in response to salt, not drought or osmotic stress. *pENO-D11:uidA* expression at 1 day postinoculation in seedlings subjected to salt stress (100 mM NaCl or KCl), the equivalent osmotic stress (200 mM mannitol), or drought (seedlings maintained in half the volume of nutrient medium). Representative images from three independent experiments ($n = 7$ per treatment per experiment). Scale bar = 1mm.

ethylene signaling is not required for salt-regulated enhancement of *ENOD11* expression (Supplementary Fig. S5).

Salt stress is known to increase ABA levels in roots (Jia 2002), and ABA addition inhibits *ENOD11* expression (Ding et al. 2008). We tested whether the dynamic expression profile of *ENOD11* under salt stress reflected changing ABA levels in our system. To this end, we quantified ABA in the same region of the root where we observed *ENOD11* hyperinduction (1 dpi) and inhibition (5 dpi) by salt. We did not observe any significant difference in ABA levels between samples at 1 dpi. At 5 dpi, however, we found that roots treated with the combined salt and rhizobium treatment showed a significant increase in ABA, indicating that the combination of salt stress and rhizobium inoculation causes a stronger increase in ABA accumulation than either treatment alone (Fig. 5), and suggesting that the decrease in *ENOD11* expression at this time point may be due to the increase in ABA levels.

Together, our results reveal a dynamic regulation of *ENOD11* by salt stress in the presence of rhizobium, causing an early upregulation but inhibiting expression at a later stage of symbiosis, the latter response correlating with increased ABA accumulation.

Early hyperinduction of symbiotic *ENOD11* is due to the ionic effect of salt.

Salt imparts a two-fold stress: (i) osmotic, in which a sudden encounter with an excessive number of molecules leads to dehydration of plant cells via exosmosis, and (ii) ionic, where, due to physicochemical similarities with the macronutrient K^+ , cations in the salts disrupt cellular processes such as enzyme activity and protein translation (Tester 2003). To test whether *ENOD11* hyperinduction in the presence of NaCl is the result of an ionic imbalance or a result of osmotic stress, we subjected seedlings to a sodic or nonsodic salt (100 mM NaCl and KCl, respectively), an equivalent osmotic stress (200 mM mannitol), or water restriction. Our results show that *ENOD11* hyperinduction was triggered by salt treatments (both sodic and nonsodic) but not by nonsalt osmotic stress or drought, and that this salt response is contingent on the presence of rhizobia (Fig. 4).

Fig. 5. Salt and rhizobium together cause an increase in root abscisic acid (ABA) concentration at 5 days postinoculation (dpi). ABA quantification was determined by enzyme-linked immunosorbent assay. FW = fresh weight. Data represent the average of three independent experiments, with $n = 7$ per treatment per experiment. Statistical analysis was done using a two-way analysis of variance followed by Tukey's honestly significant difference test on the least square means ($\alpha = 0.05$). Columns not connected by the same letter are significantly different. Error bars represent standard error of the mean.

***ENOD11* hyperinduction by salt is dependent on NF signaling and mimicked by rhizobial succinoglycan mutants.**

Because salt hyperinduces *ENOD11* only in the presence of rhizobium, we tested whether the hyperinduction requires NF signaling or a different rhizobial signal. We inoculated seedlings with the SL44 mutant containing a deletion of *nodA*, *nodB*, *nodC*, and part of *nodD* ($\Delta(nodD1-ABC)$) that is unable to make NF (Fisher et al. 1988). None of the control seedlings inoculated with $\Delta(nodD1-ABC)$ showed any *ENOD11* expression, consistent with published work (Mitra et al. 2004b), nor did the salt-treated seedlings, establishing the requirement of NF in salt-regulation of *ENOD11* induction (Fig. 6). Similarly, we found that *dmi3* mutants, which are defective in NF signaling, also showed no symbiotic *ENOD11* expression in control or salt stress conditions (Supplementary Fig. S6), indicating that salt requires NF signaling to activate *ENOD11* expression.

Rhizobial EPS are also required for development of the rhizobium–legume symbiosis, particularly for infection. In *S. meliloti*, succinoglycan is required for the initiation of the IT in the root hair (Cheng and Walker 1998). To test whether surface EPS plays a role in *ENOD11* hyperinduction, we inoculated plants with an *S. meliloti* *exoY* mutant both with and without salt stress. We observed a 16-fold higher expression of *ENOD11* inoculated with the *exoY* mutant compared with the wild-type under the control condition, consistent with the similar infection phenotypes of salt-stressed and *exoY*-inoculated plants (Fig. 7).

Hyperinduction of *ENOD11* by salt requires NF signaling in the presence of the wild-type, EPS-producing rhizobia (Fig. 6). To test whether *ENOD11* hyperinduction by the *exoY* mutant also requires activation by the NF signaling cascade, we created an *exoY* $\Delta(nodD1-ABC)$ double mutant (RFF1035), and found that, in the absence of NF signaling, *ENOD11* was not expressed in either the control or salt-stressed conditions whether or not the *exoY* mutation was present (Fig. 7), in line with previous findings that the role of succinoglycan in regulating the rhizobium–legume symbiosis follows NF perception (Dickstein et al. 1988; Yang et al. 1992).

ENOD11 hyperinduction with the *exoY* mutant could be due to either the absence of succinoglycan or to the shunting of succinoglycan precursors to a different pathway. To test whether the deregulated *ENOD11* expression is due to the absence of succinoglycan, we inoculated control or salt-stressed seedlings with the *exoS96* mutant. The *exoS* gene functions in a regulatory network that controls expression of the *exo* genes and likely controls other sets of genes as well. The *exoS96* mutants overproduce succinoglycan, and are also unable to infect plant roots (Cheng and Walker 1998). We observed a sixfold to sevenfold increase in *ENOD11* expression with the *exoS* mutant compared with the *exoY* mutant in both the control and salt-stressed roots, and this hyperinduced expression pattern was maintained upon coinoculation with a 1:1 ratio of *exoS* to *exoY*. As with *exoY*, there was no significant difference between *ENOD11* expression in the control and the salt-stressed conditions with either *exoS* or *exoY*/*exoS* coinoculation (Fig. 7). These results indicate that altered succinoglycan levels on infecting rhizobia are associated with *ENOD11* hyperinduction. The observation that the hyperinduction of *ENOD11* by *exoY* or *exoS* mutants and hyperinduction by salt stress is not additive suggests that succinoglycan and salt stress may function in a common pathway to regulate *ENOD11* gene expression.

***NSP2* regulates genes symbiotically hyperinduced by salinity.**

Given the requirement of NF to activate symbiotic *ENOD11* expression, salt must intersect with the pathway downstream of *DMI3*. Initially, *ENOD11* was shown to be directly regulated by

Fig. 6. Nod factor is required for early nodulin 11 (*ENOD11*) hyperinduction. *ENOD11* expression in control or salt-stressed roots inoculated with wild-type *Sinorhizobium meliloti* (Rm1021) or the Nod-factor-deficient SL44 ($\Delta(nodD1-ABC)$) strain at 1 day postinoculation. The graph represents relative expression of *ENOD11* based on quantitative reverse-transcription PCR, and representative images of *pENOD11::uidA* stained roots from plants grown in the different treatments are shown below. Relative expression was analyzed using a two-way analysis of variance followed by *t* test on the least square means ($\alpha = 0.05$) on two representative independent experiments of three, with $n = 7$ seedlings per treatment per experiment for each strain. The third experiment was excluded from the analysis because the consistent pattern of salt hyperinduction of *ENOD11* was not observed in the roots of seedlings inoculated with the wild-type rhizobia in this experiment. Images of *pENOD11::uidA*-stained roots are representative of three independent experiments, with $n = 7$ seedlings per treatment per experiment for each strain. An asterisk (*) indicates a significant difference between treatments within a genotype. Error bars represent standard error of the mean. Scale bar = 1 mm.

the transcription factors NIN, ERN1, and NSP1-NSP2, although, subsequently, *ENOD11* expression in root hairs was shown not to be dependent on *ERN1* or *NIN* (Cerri et al. 2012; Liu et al. 2019; Vernié et al. 2015). We studied *ENOD11* expression in the *nin*, *ern1*, and *nsp2* mutants to determine whether the hyperinduction requires the function of one or more of these genes (Fig. 8). Although the hyperinduction was maintained in the *nin-1* and *ern1-2* mutants (Fig. 8A and B), it was lost in the *nsp2-2* mutant (Fig. 8C), indicating that NSP2 function is required for *ENOD11* hyperinduction by salinity. Curiously, we noticed that *ENOD11* expression in the control condition in the *nsp2-2* mutant was not negligible, as previously reported (Marsh et al. 2007; Oldroyd and Long 2003) (Fig. 8C). As validation, we confirmed that the *nsp2-2* mutant used in our experiments contains a 0.5-kb deletion in *NSP2* (Kaló et al. 2005) (Supplementary Fig. S7). Thus, our work raises the possibility that NSP2 might play an inhibitory role in *ENOD11* expression that is revealed only under certain growth conditions. In line with previous findings, we further observed that *ENOD11* expression levels between the wild-type and the *nin-1* mutant in the absence of any salt stress are also comparable (Liu et al. 2019).

Genes with similar expression patterns are likely to be coregulated by a common factor. To determine whether *MtNPL* and *MtDEF*, genes whose expression was among those most strongly hyperinduced by salt, are regulated by NSP2 as well, we studied their expression in the *nsp2-2* mutant. Similar to *ENOD11*, the hyperinduction of *MtNPL* was abolished in the *nsp2-2* mutant, indicating that NSP2 regulates the hyperinduction of *MtNPL* (Fig. 8D). Although we observed a higher expression of *MtDEF* in wild-type roots in the presence of salinity compared with control roots, the difference was not statistically significant. Similarly, in the *nsp2-2* mutant, very little *MtDEF* expression was observed compared with the control but the difference was not significant

Fig. 7. Early nodulin 11 (*ENOD11*) hyperinduction by salt is mimicked by *exo* mutants. *ENOD11* transcript abundance in control or salt-stressed roots 1 day after inoculating with wild-type *Sinorhizobium meliloti* (Rm1021), the *exoY210* succinoglycan-defective mutant, the *exoS96* succinoglycan-overproducing mutant, coinoculation with *exoS:exoY* (1:1), or inoculation with RFF1035, which contains both the *exoY210* mutation and the deletion of the common *nod* region (*exoY Δ (nodD1-ABC)*). Statistical analysis was done using a two-way analysis of variance (ANOVA) followed by *t* test on the least square means ($\alpha = 0.05$) on two representative experiments of three, with $n = 7$ seedlings per treatment per biological replicate for each strain. The third experiment was excluded from the analysis because the consistent pattern of salt hyperinduction of *ENOD11* was not observed in the roots of seedlings inoculated with the wild-type rhizobia in this experiment (root tissue). An asterisk (*) indicates a significant difference between treatments within a genotype. *ENOD11* expression in control roots inoculated with all of the mutants was also significantly different from the wild-type rhizobia (two-way ANOVA followed by *t* test on the least square means, $\alpha = 0.05$). Error bars represent standard error of the mean.

Fig. 8. NSP2 regulates genes symbiotically hyperinduced by salinity. *ENOD11*, *MtNPL*, and *DEF* expression in roots inoculated with Rm1021 at 1 day postinoculation in the wild-type or mutant **A**, *nin-1*; **B**, *ern1-2*; or **C** to **E**, *nsp2-2* background in the presence or absence of 100 mM NaCl. Columns represent mean $\pm$ standard error of the mean. Statistical analysis done with a *t*-test within a genotype ($\alpha = 0.05$). An asterisk (*) indicates a significant difference. Results from three independent experiments with $n = 21$ seedlings total per treatment per genotype.

(Fig. 8E). Combined, our results are consistent with NSP2 as the mediator of salt hyperinduction of symbiotic genes.

Salt stress inhibits rhizobial infection in the epidermis.

Because *ENOD11* accumulates in the infection chamber in the root hair prior to the formation of an IT (Fournier et al. 2015), we tested whether *ENOD11* hyperinduction by salt correlates with an increased rhizobial infection in salt-stressed roots. To this end, we used the rhizobial strain carrying the *lacZ* gene under the constitutive *hemA* promoter to follow infection (Leong et al. 1985), comparing the number of infection events between control and salt-treated roots at 7 dpi. We chose this time point in order to capture progression of infection through the root hair, cortex, and nodule primordium. Contrary to our expectation, we found that the number of infection events that formed ITs in the root hair was significantly reduced under salt stress compared with the control (Fig. 9A), and was further reduced in the cortex (Fig. 9B). Few nodule primordia were made in salt-stressed plants (Fig. 9C) but the ones that were observed were infected with rhizobia (Fig. 9D). This observation indicates that salt interferes with the progression of infection in the root hair and cortex but suggests that, once rhizobia pass these stages, IT ramification in the nodule primordium can proceed.

Fig. 9. Moderate salt stress inhibits rhizobial infection in the epidermis and cortex. Progression of rhizobial infection in roots grown with or without 100 mM NaCl. Infection threads are monitored by inoculating with a *hemA::lacZ*-containing strain (Rm1021/pXLGD4) and staining for β -galactosidase activity at 7 days postinoculation. Graphs indicate number of infection events ending in **A**, root hairs and **B**, the cortex. Note that **C**, very few nodule primordia are formed in salt-grown roots but **D**, those that do form are infected (i.e., an average of 0.1 nodule primordia in 18 roots; all are infected). Representative images are shown below each graph. Data includes infection events where a clear end could be observed. Columns represent mean $\pm$ standard error of the mean. Statistical analysis done with a *t*-test ($\alpha = 0.05$). Asterisks indicate a significant difference (* and ** denote $P < 0.05$ and 0.005 , respectively). Results from three independent experiments with $n = 18$ to 19 seedlings total per treatment. Scale bar = $10\ \mu\text{m}$ for infection in root hair and cortex and $50\ \mu\text{m}$ for infection in nodule primordium.

DISCUSSION

The nitrogen-fixing symbiosis between legumes and rhizobia is more sensitive to salt than the growth of either partner on its own. Here, we examined the effect of salt stress on early symbiotic signaling and rhizobium infection. We found that moderate salt stress primes the response of a set of early nodulation genes, including *ENOD11*, to rhizobium, stimulating early symbiotic signaling. Conversely, rhizobium inoculation antagonizes approximately 37% of salt-responsive genes, returning gene expression to control levels. We showed that the increased responsiveness to rhizobium is an early response to salt stress, mediated by the NSP2 transcription factor, and that, by 5 dpi, salt represses expression, coincident with a rise in ABA levels. Curiously, inoculation with *exoY* or *exoS* mutants phenocopies salt hyperinduction of early symbiotic *ENOD11* expression, suggesting a link between EPS and salt stress. Finally, we identified a set of genes associated with nodulation only during salt stress, providing a window into the physiological changes needed to make nodulation work in an adverse environment. Together, our data reveal a complex interplay between the abiotic environment, the rhizobial endosymbiont, and its plant host.

A moderate salt stress enhances the induction of a large number of early symbiotic genes.

As our climate warms, drying soil leads to increased concentration of salts, a problem exacerbated by irrigation (Pimentel et al. 2004; Sherwood and Fu 2014). As a result, many plants routinely experience moderate salt stress. Under a mild to moderate stress, growth still happens but the plant must alter its physiology to accommodate the stress, revealing the role of genes and pathways that function to alleviate its effects (Verslues 2017). We found that growing seedlings under moderate salt stress increases the expression of several early symbiotic genes induced by rhizobium. For some genes, this synergistic effect results in an induction of more than 100-fold, as was observed for *CBS1* and the LRR receptor kinase *Medtr6g036780*. We found that this hyperinduction is due to either the ionic component of salt, not the osmotic, or the increase in chloride ions. In addition, we showed that the stimulation of rhizobium-induced gene expression by salt is not due to salt-induced expression, because salt alone is unable to induce gene expression (Figs. 3 and 4; Supplementary File S1). Not all early nodulation genes show salt enhancement of rhizobium induction; the set of rhizobium-induced genes in our study whose expression was enhanced by salt, passing our twofold cutoff, included a number of genes involved in infection and early signaling (*RPG*, *VPY*, *CHIT5b*, *VRC2*, *NPL*, and *ENOD11*) (Table 1). Genes whose induction by rhizobium is not affected by salt include genes involved in an organogenesis initiation module (*NIN*, *NFYA-1*, and *LBD16*) (Table 2).

A number of rhizobium-induced genes that did not appear in our rhizobium-inoculated set (+R–S) were induced enough to exceed our cutoff threshold when salt was added (+R+S). This set included several genes that were CRE1-dependent, such as *CLE13*, *expansin A1* (*Medtr7g110990*), and *LYK10*, indicating that salt did not block the cytokinin signaling pathway but, rather, enhanced expression of some of the genes (Supplementary File S1) (van Zeijl et al. 2015b). GA also plays a major role in early NF signaling and rhizobium infection, and we found four genes encoding GA biosynthetic and catabolic enzymes differentially expressed in response to salt or to rhizobium plus salt. In particular, we observed a strong increase in expression of *Medtr3g088745*, encoding the biosynthetic enzyme GA 20-oxidase 5/6 (GA20ox5/GA20ox6), and of *Medtr4g074130*, encoding the degradative enzyme GA2-oxidase 13 (GA2ox13), exclusively in our +R+S dataset (Igielski and Kępczyńska 2017; Wang et al. 2020). Both of these genes are strongly induced

(14-fold for GA2ox13 and 42-50-fold for GA20ox5/GA20ox6) but did not pass the cutoff for differential expression in our +R–S dataset. The strong induction of these two GA enzymes mirrors the strong induction of both by NF seen in other studies (Damiani et al. 2016a; Jardinaud et al. 2016; van Zeijl et al. 2015b) and may reflect the complex role of GA during nodulation. This observation is consistent with the overall pattern we saw, in which moderate salt stress hyperinduces genes associated with early nodulation signaling.

The fact that 147 of the 314 genes differentially regulated by the combined treatment (+R+S) were previously identified as early rhizobium- or NF-induced in *M. truncatula* transcriptomes (Breakspear et al. 2014; Damiani et al. 2016a; Jardinaud et al. 2016; Larrainzar et al. 2015; van Zeijl et al. 2015b) indicates that this synergistic effect of a moderate salt stress on early symbiotic gene expression has a large impact, affecting expression of most of the early symbiotic genes in our dataset and including both cytokinin-dependent and -independent pathways.

Complex and dynamic interactions between the abiotic environment and the rhizobium–legume symbiosis.

The arbuscular mycorrhizal symbiosis is known to confer resistance to drought and salinity in its hosts (Evelin et al. 2019; Kivlin et al. 2013). Our results show that expression of 37% of the salt-regulated genes is restored to normal levels by rhizobium inoculation (Supplementary File S1). Thus, during the first day after inoculation, not only does salt influence the rhizobium-induced transcriptome but also the presence of rhizobia alters the salt-regulated transcriptome. Interestingly, genes known to be salt regulated that did not pass our threshold for differential gene expression in salt-treated roots were significantly repressed in roots treated with both salt and rhizobium. The transcription factor NAC969 regulates root architecture and nodulation and is known to be induced by 100 mM NaCl in roots (de Zelicourt et al. 2012). We found that NAC969 is downregulated in the presence of salt and rhizobium, as is the LEA protein Medtr8g022990 and the germin Medtr8g020650, providing a further example of the repression of the salt response by rhizobial inoculation.

NaCl in the root environment interferes with K homeostasis (Assaha et al. 2017; Wu et al. 2018); thus, potassium deprivation can mimic the effects of salt stress by NaCl. We observed only a modest 5.7% overlap between genes differentially regulated by salt stress and those detected under potassium deprivation (Supplementary File S1) by Garcia et al. (2017), likely due to differences in the age of the plants and growth conditions. However, we did find that several genes regulated by the combined +R+S treatment were also detected in older roots experiencing potassium deprivation; interestingly, the directionality of expression was opposite for all genes shared between the two studies. These genes included several oxidative stress-responsive genes and the *Medicago* homolog of the *Lotus* EPSI receptor, *EPR3* (*LYK10*). It is tempting to speculate that this action by rhizobia may ameliorate some of the negative effects of moderate salt stress.

In order to survive, a plant must continuously assess its environment and adjust its resource allocation in response. An immediate response to a new stress is replaced by a different set of responses when the stress persists, requiring different physiological solutions and substantially altering the transcriptome (Yang et al. 2014). In our experiment, at the time of inoculation, plants only experienced stress for 4 days, and that stress was only moderate, allowing root and shoot growth to proceed normally. We speculate that, at this early timepoint, short-term stress responses enhance the plant's efforts to maintain growth and development but that, after five additional days of stress, harsher measures are implemented, reducing allocation of resources to any nonessential processes. Under conditions of extended stress, inhibiting nodulation is likely beneficial, to allow conservation of resources for plant growth and

stress resistance. At this later time point, the combined effect of salt and rhizobia induce ABA accumulation to levels higher than either alone (Fig. 5). ABA is known to inhibit nodulation, altering calcium spiking and reducing symbiotic gene expression and rhizobium infection (Ding et al. 2008; Suzuki et al. 2004). At this stage, salt stress and rhizobia work additively, as opposed to the antagonistic effect of rhizobium on salt-regulated gene expression and the synergistic effect of salt and rhizobium we observed on early symbiotic gene expression. It will be interesting to explore the mechanism by which salt stress signaling and nodule development interact at this later stage in the symbiosis.

NSP2 as a node integrating environmental and symbiotic signals.

Plant response to external cues is coordinated through a suite of transcriptional regulators (Dietz et al. 2010). AP2/ERF, bZIP, NAC, and WRKY transcription factors function in communicating with environmental signals such as drought and salt stress, leading to an appropriate cellular response, and are often found at the point of integration in a signaling hub (Dietz et al. 2010; Goldack et al. 2011). Several transcription factors have been shown to participate in salt stress responses in *M. truncatula* roots (Ariel et al. 2010; Gruber et al. 2009; Merchan et al. 2003). The transcriptional regulation of *ENOD11* by salt made us explore salt regulation of its upstream regulators. The GRAS transcription factor NSP2 plays an important role in NF signaling and rhizobial infection, and transcriptionally regulates *ENOD11* (Cerri et al. 2012). In rice, the GRAS transcription factor OsGRAS23 is involved in drought and oxidative stress (Xu et al. 2015) and, in *M. truncatula*, several GRAS genes have been suggested to be involved in stress responses (Zhang et al. 2017). We found that salt stimulation of rhizobium-induced *ENOD11* and *NPL* expression requires NSP2 function but not the function of other NF signaling network transcription factors such as ERN1 and NIN (Fig. 8). In *L. japonicus*, *NPL* is downregulated in the *nin* and *nspl* mutants and NIN directly regulates *NPL* expression and, in *M. truncatula*, NIN-regulated *NPL* expression is required for rhizobial infection whereas *ENOD11* expression is independent of NIN function (Liu et al. 2019; Xie et al. 2012). Our study revealed that both *ENOD11* and *NPL* require NSP2 for hyperinduction, which places NSP2 as the integrator of salt stress and symbiosis.

Environmental stress triggers rapid cellular changes such as increases in calcium and reactive oxygen species (ROS), which function as secondary signals to regulate gene expression (Gilroy et al. 2016; Noctor et al. 2014). ROS play crucial roles in both salt stress and the rhizobium–legume symbiosis (Damiani et al. 2016b; Hossain and Dietz 2016). Concurrent with the role of ROS in salinity and symbiosis, several rhizobium-induced genes hyperinduced by salt stress have been shown to require ROS for induction (Andrio et al. 2013). *ENOD11*, the gene most strongly hyperinduced by salt in our study, is one of the three genes most strongly inhibited when ROS production is reduced; however, several other genes we identified as being hyperinduced by salt, such as *VPY*, *RPG*, *SP11*, *NPL*, and *VRC2*, also require ROS for induction (Andrio et al. 2013) (Supplementary File S1). Interestingly, some of the genes that were upregulated exclusively in our +R+S cotreatment but had previously been shown to be induced by rhizobium or NF also require ROS for induction. This includes some of the genes most strongly upregulated in the rhizobium and salt stress cotreatment: *CBS1*, an MtN21/EamA-like transporter family protein (Medtr3g012420), a caffeic acid O-methyltransferase (Medtr3g021440), *ENOD12*, and *SINA* (Andrio et al. 2013). The requirement of both ROS and NSP2 for the hyperinduction of both *NPL* and *ENOD11* raise the possibility that NSP2 may respond to an ROS signal downstream of salt.

This function of NSP2 as a node that responds to both NF signaling and environmental conditions is consistent with its role in

the regulation of the strigolactone synthesis gene, *D27*. NSP2 and NSP1 regulate *D27* expression via the NF pathway in high phosphate but via an NF-independent pathway in low phosphate, indicating that NSP2 can respond to a second input (low environmental phosphate levels) distinct from NF signaling (Liu et al. 2011; van Zeijl et al. 2015a). Studies in *Arabidopsis* find that low phosphate levels in the environment trigger an increase in ROS levels in the root tip, which likely also mediates transcriptional changes (Balzergrue et al. 2017; He et al. 2018; Shin et al. 2005). Together, these findings place NSP2 as an integrator of abiotic and biotic signals and suggest that NSP2 may respond to a common secondary signal such as ROS shared by different abiotic stress response pathways.

Interestingly, moderate salt stress represses *NSP2* expression in the root, and rhizobium reverses this, restoring *NSP2* to wild-type levels (Supplementary File S1). This ability of rhizobium to counteract the effect of salt stress suggests that rhizobium may modulate the plant salt response to benefit the symbiosis and indicates a more complex set of feedbacks between environment, plant, and microbe.

Salt stress at the host-symbiont interface.

Salt stress causes increased accumulation of amino acids, polyamines, and amino acid derivatives in plants (Alcázar et al. 2006; Trinchant et al. 2004; Verbruggen and Hermans 2008). We have previously observed increased accumulation of arginine in 14-dpi nodules from plants grown under moderate salt stress (Keller et al. 2018). In the present study, we observed downregulation of *Medtr3g114870*, annotated as ornithine decarboxylase (ODC), in salt-stressed roots (–R+S versus –R–S). Arginine and ornithine are substrates in the polyamine synthesis pathway, in which ODC converts ornithine to the polyamine putrescine, which can then be converted to other polyamines. In addition to protecting against salt stress in both plants and bacteria, polyamines in *S. meliloti* 1021 appear to regulate EPS production, which is required for infection of the plant root (Becerra-Rivera and Dunn 2019; Chen et al. 2019). The reduced ODC expression in salt-stressed roots could alter polyamine levels in the plant and, thus, rhizobial EPS production in the rhizosphere or IT, affecting infection of the host root. Interestingly, ODC *Medtr3g114870* is one of the salt-regulated genes whose repression is reversed by rhizobium inoculation, providing a specific example of rhizobia interacting with their plant host to modify the plant salt response to potentially benefit the symbiosis.

Although free-living Rm1021 can survive in the presence of salinity as high as 400 mM NaCl (Domínguez-Ferreras et al. 2006), salt stress might affect rhizobial physiology ex planta in a way that influences rhizobium–legume symbiosis. Salt stress induces *nod* genes independent of flavonoids in the halotolerant *Rhizobium tropici* CIAT899 (del Cerro et al. 2017; Guasch-Vidal et al. 2013; Pérez-Montaña et al. 2016). Furthermore, there is a strong overlap between genes upregulated by the flavonoid apigenin and by salinity in CIAT899 (Pérez-Montaña et al. 2016). In *S. meliloti*, osmotic stress promotes biofilm formation and the core *nodDIABC* genes are required to establish the three-dimensional architecture of biofilm (Fujishige et al. 2008; Rinaudi et al. 2006). In contrast, an inhibitory effect of salinity on *nod* gene expression has been suggested in *R. tibeticum*, which is alleviated by the flavonoids apigenin and hesperitin (Abd-Alla et al. 2014). It is possible that hyperinduction of early nodulation genes by 100 mM NaCl that we observed is due to increased *nod* gene expression in *S. meliloti*, similar to CIAT899, which leads to increased production of NF.

EPS on the rhizobial surface plays an important role in resilience in the face of environmental stress but also plays an essential role in infection of the plant host root (Cheng and Walker 1998; Janczarek 2011). Increased salt in the environment promotes the

production of EPS on the surface of *S. meliloti* and protects the bacterium against deleterious effects of ROS (Lehman and Long 2013). The *S. meliloti* *exoY* gene is required for synthesis of the EPSI molecule, succinoglycan, and *exoY*-defective mutants are able to initiate infection but are blocked in the root hair. In *L. japonicus*, EPS acts as a signal molecule, binding the EPR3 receptor, which distinguishes compatible and incompatible EPS molecules; however, the *M. truncatula* EPR3 homolog, *LYK10*, does not appear to have this function (Kawaharada et al. 2015; Maillet et al. 2020).

The relationship between salt, EPS, and *ENOD11* is curious. Reducing *exoY* function increases *ENOD11* expression, indicating that a functional *exoY* gene must normally limit *ENOD11* expression. Yet adding salt when inoculating with an *exoY* mutant does not further induce *ENOD11* expression, suggesting that *exoY* and salt function in the same pathway. If salt stimulates *ENOD11* induction and wild-type *exoY* inhibits it, the simplest model is that salt inhibits succinoglycan signaling. However, salt actually increases the production of both *exoY* and succinoglycan (Breedveld et al. 1990; Rüberg et al. 2003). Therefore, if salt inhibits succinoglycan signaling, it is not at the level of production, and must be downstream. Succinoglycan binds cations and EPS viscosity is thought to be affected by side chains and, probably, cation binding (Breedveld et al. 1990; Hawkins and Oresnik 2017; Sutherland 1997). Because 100 mM NaCl interferes with succinoglycan binding to the antimicrobial NCR247 peptide (Arnold et al. 2018), it could also potentially affect interactions of succinoglycan with other molecules during infection. Interestingly, *exoA* mutants also appear to enhance *ENOD11* induction (Mitra et al. 2004b), indicating that this may be a common effect of altering EPS structure or levels. It will be interesting to see whether the hyperinduction of *ENOD11* expression by both EPS defects and salt treatment reflect a shared pathway or are the result of distinct mechanisms.

Additionally, inoculation with the *exoY* mutant causes an increased expression of defense-related genes in the host (Jones et al. 2008). Our KEGG pathway enrichment analyses revealed an overrepresentation of the phenylpropanoid biosynthesis pathway, alluding to the possibility that salt-stressed plants employ genes involved in defense responses to inhibit allocation of limited resources into developing a nodule (Fig. 2).

A distinct set of accessory genes associated with nodulation under stress conditions.

The rhizobium–legume symbiosis is generally studied under ideal conditions in a controlled laboratory setting. These studies have led to the identification of a core set of plant nodulation genes that are robustly associated with nodule initiation and development and required for their function. However, natural conditions are far from ideal. Are there nodulation genes required under different environmental conditions that we miss when abiotic stress is eliminated from the environment? We designed this RNA-seq experiment to explicitly look for an accessory set of genes associated with nodulation under moderate salt stress, a common problem in agricultural settings. We found 314 genes that were differentially expressed exclusively in roots exposed to salt and rhizobium. Of those, 167 genes, several of which were upregulated, had never been identified in transcriptomes of nodules or inoculated roots. Could these genes be playing a negative role in symbiosis? Overexpressing one or more of these genes and studying nodulation phenotype in the absence of salt stress could address this question. Among the unique genes, we found a number of genes involved in detoxification or defense. We also found a number of genes involved in the proteasome and ubiquitination such as *SINA*, *PUB64*, several genes encoding F-box proteins, and other E3 ubiquitin ligases, indicating the importance of regulation at the level of protein accumulation. The U-box containing PUB proteins was induced in response to a number of environmental stresses in

M. truncatula (Song et al. 2017). With the new finding that DMI2 is stabilized after rhizobium inoculation (Pan et al. 2018), it is clear that protein stabilization and destruction plays an important role in regulation of nodulation.

The core set of nodulation genes is important but, most likely, the core set never functions alone but, instead, functions with different accessory gene sets used for different environmental conditions. Thus, in the natural environment, it is the interplay of those core genes with the accessory set that establishes the symbiosis. If so, the nodulation accessory set genes should be important for plant success in different environments. In fact, genes identified in genome-wide studies for genes with signatures of local adaptation (i.e., genetic changes in populations associated with specific climatic conditions or geographic regions) were also part of our set of genes differentially expressed exclusively when plants were inoculated with rhizobium in the presence of salt (Supplementary File S1). *Medtr5g019070* is of particular interest, because it is induced 40-fold when both salt and rhizobium are present and was strongly associated with two climate temperature variables often linked to drier climates (Yoder et al. 2014), suggesting a possible role in the interaction between abiotic and biotic signaling in the root. Additionally, a candidate gene identified by Friesen and colleagues (2014) in their search for genes with alleles that sort with saline soils included a fusaric acid resistance-like protein (*Medtr2g096670*), shown to be downregulated in response to NF (van Zeijl et al. 2015b), which we also find to be downregulated in our +R+S set of genes (Supplementary File S1).

A genome-wide study for candidate genes associated with different nodulation traits identified four genes from our +R+S accessory gene set, all associated with nodule occupancy or location of nodulation on the root system (Stanton-Geddes et al. 2013). Three of these genes were repressed in our dataset, including the putative GA transporter *MtNPF3.1* (*Medtr5g055000*) (Tal et al. 2016), an LEA gene (*Medtr8g022990*), and the chaperone *DNAJ 11*-like gene; none of these had been previously identified as being nodulation-related. In contrast, the LOB domain protein *Medtr5g075020* was induced more than twofold in our study and was upregulated as early as 3 or 4 h after NF treatment in others (Supplementary File S1). These genes are all linked to nodulation phenotypes via this genome-wide association study but only one (*Medtr5g075020*) had previously been identified as being regulated transcriptionally by NF or inoculation. Identifying them in our +R+S-only set of genes indicated that salt stress can increase the expression of genes known to be required later for nodulation but which may not usually be induced at early time points by rhizobium or at high levels.

Interestingly, there was very little overlap, if any, between the genes identified in these different genome-wide studies. However, genes from each study overlapped with our putative abiotic accessory gene set, indicating that our approach of looking for abiotic-specific nodulation genes was successful in identifying a number of genes that appear to have been targets of local adaptation to different soil, saline, and climate conditions. These findings also demonstrate a strong link between environmental stress, soil conditions, and nodulation. Most of these genes identified in population-wide studies were found by virtue of their association with either stress or nodulation. Our study, which explicitly examined nodulation under salt stress conditions that permit relatively normal levels of growth, revealed a role for these genes in nodulation under stress conditions. This connection suggests that nodulation genes may be under selection in dry, saline, or hot areas, and that the process of nodulation can be adjusted for success under many different environmental conditions. The very small overlap between these genome-wide studies suggests that there are different sets of accessory genes for different conditions and, also, that there are many accessory genes yet to find.

Together, our data paint a picture in which salt stress primes the response of rhizobium-induced genes to NF, stimulating early symbiotic signaling under salt stress and inducing an accessory set of genes involved in detoxification, biotic stress, and signaling. The importance of these genes to plant success can be seen by their identification in screens for genes that have signatures of local adaptation. We showed that *NSP2* functions as a node to integrate NF and salt signals, coordinating symbiosis with environmental conditions. In addition, we found that rhizobium inoculation reverses much of the salt-responsive changes in gene expression, providing a possible mechanism for the observation that rhizobium inoculation can ameliorate effects of salt stress. Finally, our findings provide insight into the dynamic interplay between salt stress and the rhizobium-legume interaction and the molecular changes that occur in salt-stressed roots as they respond to rhizobium. Our findings lay the foundation for further analysis of the interaction between host, microbe, and environment at a mechanistic level.

MATERIALS AND METHODS

Plant growth conditions.

All experiments were performed using *M. truncatula* Jemalong A17. Seed were scarified in concentrated sulfuric acid for 15 min, surface sterilized with 30% bleach for 10 min, and imbibed for 4 to 6 h at room temperature. Imbibed seed were stratified at 4°C for 1 to 4 days or germinated immediately. Stratified seed were germinated overnight in the dark. Germinated seedlings were placed in CYG seed germination pouches, in 10 ml of buffered nodulation medium (Ehrhardt et al. 1992) supplemented with 1 mM KCl, referred to here as modified nodulation medium (MNM). We observed a significant increase in the number of nodules in pouch-grown seedlings in MNM. Seven seedlings were planted per pouch. The lower portions of the pouches were wrapped with aluminum foil to shade the roots. Well-spaced pouches were placed in a Styrofoam box, with a transparent lid, and the box was taped closed to prevent dehydration. Two water-saturated empty pouches were used to flank the remaining pouches, to prevent partial drying out of the pouches placed at the ends. The boxes were placed in MTR30 Conviron growth chambers, set at 20°C, 50% humidity, and a cycle of 16 h of light and 8 h of darkness, with an intensity of 100 $\mu\text{E m}^{-2} \text{s}^{-1}$. Media were replenished twice a week; to maintain salt concentration, excess medium was poured out and fresh medium added to the pouch, pipetting onto the paper. All experiments were performed using 7 to 21 roots per treatment per replicate. Unless otherwise noted, the data shown are the average of three biological replicates. The exact number of roots used is noted in the respective figure legends.

Rhizobial strains and inoculation.

All rhizobial strains used are in the *S. meliloti* (Rm1021) background (Meade et al. 1982) and are listed in Supplementary Table S3. Pelleted cells from overnight cultures grown in low-salt Luria-Bertani (LB) medium, containing half the amount (85.5 mM) of NaCl present in regular LB medium (Bertani 1951) and supplemented with appropriate antibiotics, were resuspended in 10 mM MgSO_4 , and the optical density at 600 nm was adjusted to 0.07. Each pouch was inoculated with 1 ml of the rhizobial suspension, or mock inoculated with the same volume of 10 mM MgSO_4 , added near the tip of the roots.

RNA extraction for transcriptome profiling and qRT-PCR.

Primary roots were frozen, ground in liquid nitrogen, and stored at -80°C. Total RNA was extracted from the ground tissue using the RNeasy Plant Mini kit (Qiagen), genomic DNA was removed using the TURBO DNA-free kit (Ambion), and the resulting RNA

was further cleaned up using the RNeasy MinElute Clean-up kit (Qiagen). RNA quality was assessed with an Agilent 2100 Bioanalyzer, and RNA samples with RNA Integrity Number (RIN) values higher than 8 were processed further.

RNA-seq.

Twelve dual-indexed TruSeq stranded RNA-seq libraries were created for the four treatments from three biological replicates. The libraries were combined into a single pool and sequenced in a single lane of a HiSeq 2500 (Illumina) High Output, 125-bp paired-end run. Approximately 220 million pass filter reads for the lane were generated. All expected barcodes were detected. The mean quality scores were above Q30 for all libraries. The libraries were gel size selected to have average inserts of approximately 200 bp.

FastQ (125-bp) paired-end reads were trimmed using Trimmomatic (v 0.33) enabled with the “-q” option: 3-bp sliding-window trimming from the 3′ end requiring minimum Q30. Quality control checks on raw sequence data for each sample were performed with FastQC. Read mapping was performed via Tophat2 (v2.0.13) using the *M. truncatula* reference genome (4.0v2).

Gene quantification was done via Cuffquant for fragments per kilobase of exon model per million reads mapped values and feature counts for raw read counts. DEGs were identified using the edgeR (negative binomial) feature in CLCGWB (Qiagen) using raw read counts. The generated list was filtered based on a minimum 2× absolute fold change and false discovery rate (FDR) corrected $P < 0.05$.

GO enrichment analysis.

GO annotation was downloaded from the *M. truncatula* Genome Database, which included 4,415 GO terms for 29,846 genes. DEGs were mapped to this dataset and 278 GO terms were obtained for the 450 genes, and their ontology (biological process, molecular function, and cellular component) distribution was summarized and drawn by R package 3.6.0 (R Core Team 2013). GO term enrichment analysis was performed with Fisher’s exact test and significant GO terms were reported.

KEGG pathway enrichment analysis.

Partek Genomics Suite (version 7, build 7.17.1222; Partek) was used to perform a KEGG pathway enrichment analysis for DEGs defined by a corrected P value (FDR) of 0.05 and a fold change of 1.51 from the following contrasts: 1 versus 3 (36 genes), 1 versus 2 (543 genes), and 1 versus 4 (1,020 genes). The enrichment analysis parameters were configured to use Fisher’s exact test and to only consider pathways containing more than two genes. The resulting enriched KEGG pathways for each gene set were defined based on an enrichment score of three or an enrichment P value < 0.05 .

qRT-PCR.

cDNA was synthesized from 1 µg of total RNA using iScript cDNA synthesis kit (Bio-Rad). For each round of cDNA synthesis, one reaction lacking reverse transcriptase was prepared as a control to test for genomic DNA contamination. qPCR was performed with SYBR green (Quanta Perfecta) in a StepOnePlus real-time PCR system (Applied Biosystems) using the delta-delta cycle threshold (CT) method and a standard cycle. All samples were run in duplicate. The *helicase* and *UBC9* genes were used as endogenous controls (Kakar et al. 2008). The geometric means of the expression of these two genes were used to normalize CT values of the target genes. All primers are listed in Supplementary Table S4.

Construction of the *exoY* Δ(*nodD1-ABC*) mutant.

S. meliloti strain SL44 (Fisher et al. 1988) was grown on tryptone yeast extract medium supplemented with streptomycin (500 µg/ml) and neomycin (50 µg/ml), and transduced with a N3 phage lysate made on *S. meliloti* strain Rm7210Sp (Lehman and Long 2013); transductants were selected on LB supplemented with streptomycin (500 µg/ml), neomycin (50 µg/ml), and spectinomycin (50 µg/ml).

Reporter gene expression and chlorophyll quantification.

M. truncatula plants stably transformed with *pENOD11:uidA* (Journet et al. 2001) were stained for GUS activity as described by Oldroyd et al. (2001), omitting the fixation step. Expression of *lacZ* was assessed as described by Boivin et al. (1990). Roots were observed under a Leica dissecting microscope (LeicaMZ8) for a wide field of vision or Olympus compound microscope (Olympus BX60) for a better spatial resolution. Chlorophyll quantification was performed as described by Lichtenthaler (1987).

ABA extraction and quantification.

ABA was extracted as described by Walker-Simmons (1987), with modifications from Liang et al. (2007). Briefly, ground and frozen tissue was suspended in extracting methanol (2.5 mM citric acid monohydrate and 0.5 mM 2,6-di-tert-butyl-4-methylphenol in absolute methanol) at a ratio of 1 ml of solution per 100 mg of tissue and nutated at 4°C in darkness for 36 h. Following centrifugation at $1,500 \times g$ at 4°C for 15 min, the cell debris pellet was discarded and the supernatant was adjusted to 70% methanol. Supernatant was then passed through a conditioned (absolute methanol followed by deionized water) C18 column (Sep-Pak light C18 cartridge; WAT023501), frozen briefly at –80°C, and evaporated to dryness using a SpeedVac. The residue was resuspended in tris-buffered saline and stored at –20°C. ABA was quantified using competitive enzyme-linked immunosorbent assay (PDK09347/0096; Agdia), following the manufacturer’s instructions. All reactions were done in duplicate.

AUTHOR-RECOMMENDED INTERNET RESOURCE

CYG seed germination pouches: <https://mega-international.com>

ACKNOWLEDGMENTS

We thank S. Long (Stanford University) for providing the rhizobial strains SL44 and Rm7210; M. Arnold and G. Walker (Massachusetts Institute of Technology) for providing the strain Rm7096; A. Becker (University of Minnesota Genomics Center) for generating the RNA-seq libraries; T. Delaney, S. Keller, J. Preston, and M. Tierney (University of Vermont) for their advice and generosity with the use of equipment; University of Vermont Advanced Genome Technologies Core for help with RNA quality assessment; J.-M. Ané (University of Wisconsin-Madison) for critical reading of the manuscript; A. Howard for statistical advice; and members of the Harris lab for suggestions and support.

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