Systemic Regulation of Soybean Nodulation by Acidic Growth Conditions\textsuperscript{1}[OA]

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Mechanisms inhibiting legume nodulation by low soil pH, although highly prevalent and economically significant, are poorly understood. We addressed this in soybean (\textit{Glycine max}) using a combination of physiological and genetic approaches. Split-root and grafting studies using an autoregulation-of-nodulation-deficient mutant line, altered in the autoregulation-of-nodulation receptor kinase GmNARK, determined that a systemic, shoot-controlled, and GmNARK-dependent mechanism was critical for facilitating the inhibitory effect. Acid inhibition was independent of aluminum ion concentration and occurred early in nodule development, between 12 and 96 h post inoculation with \textit{Bradyrhizobium japonicum}. Biological effects were confirmed by measuring transcript numbers of known early nodulation genes. Transcripts decreased on both sides of split-root systems, where only one side was subjected to low-pH conditions. Our findings enhance the present understanding of the innate mechanisms regulating legume nodulation control under acidic conditions, which could benefit future attempts in agriculture to improve nodule development and biological nitrogen fixation in acid-stressed soils.

Most legume plants are able to facilitate biological nitrogen fixation through a symbiosis with soil bacteria, commonly called “rhizobia.” This relationship results in the formation of novel root organs, called nodules, which are critical for establishing an environment suitable for symbiotic nitrogen fixation (for review, see Ferguson et al., 2010; Desbrosses and Stougard, 2011; Ferguson, 2013). Nodule development is stringently controlled by internal plant mechanisms (for review, see Reid et al., 2011a) and can also be significantly affected by external factors, including nitrate and ethylene (Ferguson and Mathiesius 2003; Gresshoff et al., 2009).

One external factor that can diminish nodulation is low soil pH. It is estimated that about 30\% of the world’s land surface is acidic (pH < 5.5), including an extensive 40\% of arable land (Von Uexküll and Mutert, 1995). Low soil pH reduces nutrient availability, increases Al\textsuperscript{3+} toxicity, and is generally detrimental to crop yields. It has been estimated that Al\textsuperscript{3+} toxicity represents the greatest constraint on plant productivity in 67\% of the world’s acidic soil regions (Eswaran et al., 1997). These poor growth conditions lead to reductions in root development and nodulation and compromise nutrient transport (Horst, 1983, 1987; Marschner, 1991). This results in yield losses of more than 50\% in grain crops, such as wheat (\textit{Triticum aestivum}) and barley (\textit{Hordeum vulgare}), and in many legume crops, including common bean (\textit{Phaseolus vulgaris}), lentil (\textit{Lens culinaris}), and pea (\textit{Pisum sativum}; Mahler and McDole, 1987).

Low soil pH reduces the nodule numbers of legumes such as common bean, lentil, and pea by more than 90\% and nodule dry weight by more than 50\% (Lie, 1969; Mohebbi and Mahler, 1989; Vargas and Graham, 1989; Alva et al., 1990; Evans et al., 1990). However, certain legume species, such as some \textit{Lupinus} spp. and \textit{Mimosa} spp. found in the highly acidic Brazilian Cerrado and Caatinga biomes, can exhibit acid-tolerant nodulation (dos Reis et al., 2010; Sprent, 2009). Both determinate- and indeterminate-forming nodule types are affected by low pH, suggesting a common target. The loss of nodulation and nitrogen fixation caused by low soil pH is reflected by the fact that 75\% of the 3.6 million tons of nitrogen fertilizer used worldwide each year is applied in major soybean (\textit{Glycine max}) production regions, where acidic soils are prevalent (Fig. 1; http://www.fertilizer.org/). Attempts to combat this significant problem are now being enhanced by establishing a more detailed understanding of the molecular mechanisms regulated by soil acidity.

The reduced nodulation observed in acidic soil is not solely attributed to hindered plant development, as low pH also negatively affects rhizobia growth, persistence, and function. This is primarily caused by increased proton concentration and a resulting increased metal ion solubility in the growth substrate, which causes intracellular pH instability and inhibits cell function (Bhagwat and Apte, 1989; Graham et al., 1994).

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Symbiotic potency under low-pH conditions varies among rhizobia species. *Bradyrhizobium* spp. are generally more acid tolerant than *Rhizobium* spp., but the cause for this remains unclear (Brockwell et al., 1991; Sadowsky et al., 1998). Moreover, certain strains that are less potent under neutral conditions can outcompete other strains under low-pH conditions (Triplett and Sadowsky, 1992; Hungria and Stacey, 1997).

The expression of rhizobia nodulation genes (Richardson et al., 1988) and the production of Nod factor were also reduced in low-pH conditions (McKay and Djordjevic, 1993; Morón et al., 2005). This disrupts the signal exchange between the plant and bacterial partners (Hungria and Stacey, 1997), leading to a reduction of root hair deformation and root hair curling (Truchet et al., 1991; Miransari et al., 2006). Exogenous application of Nod factor and genistein can partially recover this reduction (Truchet et al., 1991; Miransari et al., 2006; Miransari and Smith, 2007). However, such treatments do not fully restore nodulation levels and are agriculturally impractical.

The attachment of rhizobia to legume root hairs requires important Ca$^{2+}$-dependent adhesions (Smit et al., 1992). However, low-pH conditions limit the availability of Ca$^{2+}$ in soils, which is suggested to impair rhizobia attachment, infection, and infection thread formation (Caetano-Anollés et al., 1989). Low soil pH also significantly reduces processes occurring downstream of or in parallel to root hair infection, including initial cell division events and primordia establishment (Lie, 1969).

Legume plants possess an internal regulatory mechanism called the autoregulation of nodulation (AON; Caetano-Anollés and Gresshoff, 1991; Reid et al., 2011b). In AON, the onset of nodule primordia formation triggers the production of CLE peptides in the root (Okamoto et al., 2009; Mortier et al., 2010; Lim et al., 2011; Reid et al., 2011a). These hormone-like peptides (GmRICs in soybean) are assumed to travel to the shoot, where they putatively interact with the Nodulation Autoregulation Receptor Kinase (GmNARK in soybean and its orthologs MmSUNN, LjHAR1, and PsSYM29 in other species; Krusell et al., 2002; Nishimura et al., 2002; Searle et al., 2003; Schnabel et al., 2005). This leads to the production of a shoot-derived inhibitor compound that is predicted to be transported down to the root, where it suppresses further nodulation events (Lin et al., 2010, 2011; Reid et al., 2012).

Interestingly, nitrate, a strong inhibitor of nodule formation, also triggers the production of a CLE peptide in soybean, called GmNIC1 (Reid et al., 2011a). However, this CLE acts locally through GmNARK in the root to inhibit nodule formation, as opposed to acting systemically through the shoot (Reid et al., 2011a).

Here, we present evidence that low-pH conditions trigger a systemic, shoot-controlled, and NARK-dependent mechanism that negatively regulates the early stages of nodule development in soybean. The involvement of AON and GmNARK was previously suggested when Alva et al. (1988) reported the insensitivity of soybean supermodulating mutants to pH-
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controlled hydroponic systems. This systemic component offers a new perspective on the acid regulation of nodulation and indicates that low-pH conditions could affect both the early nodulation signaling pathway (Indrasumunar et al., 2011) and the AON pathway (Alva et al., 1988) of legumes.

**Figure 2.** Soybean nodulation and development following pH 6.8 to 4.0 (A–D) and pH 4.0 to 2.0 (E–G) acid treatment. Acidic pH controls the nodulation of soybean plants (n = 10) treated with solutions of pH 6.8 to 4.0. A, Shoot dry weight (DW). B, Root dry weight. C, Number of nodules per plant. D, Number of nodules per unit root dry weight. E, Shoot dry weight. F, Root dry weight. G, Number of nodules per plant. H, Number of nodules per unit root dry weight. Error bars indicate SE. Different letters above the bars represent statistically significant differences (Student’s t test; P ≤ 0.05).
RESULTS

Acidic Growth Conditions Restrict Soybean Nodulation

To establish plant fitness and nodulation success under acidic conditions, soybean plants were grown over a range of pH values (pH 6.8–4.0 treatment; from here on, all pH values signify the acidity of the supplied nutrient solution, not the rhizospheric pH). Total nodule number was highest at pH 6.8 (near-neutral control) and decreased as the pH decreased (Fig. 2). The inhibition of nodulation remained similar from pH 5.5 to 4.0, possibly owing to the buffering/ion-exchange effect of vermiculite. Shoot and root dry weight remained constant throughout the pH range tested, demonstrating that the decrease in nodule number was not simply a reflection of decreased plant growth (Fig. 2). Indeed, when nodule numbers were scored on a per unit root dry weight basis, they still decreased as the pH decreased (Fig. 2). In contrast, plants supplied with either pH 3.0 or 2.0 were significantly compromised in root, shoot, and nodule growth (Fig. 2). Based on these findings, pH 4.0 treatment was deemed the most suitable to investigate the effects of acidity on nodulation using our growth conditions, as it did not hamper overall plant fitness.

Acid Inhibition of Nodulation Occurs Early during Nodule Ontogeny

To determine the developmental stage of nodule ontogeny that is affected by acidic growth conditions, plants were grown in either permissive (pH 6.8) or...
restrictive (pH 4.0) nodulation conditions and shifted to the opposite pH condition at 12, 24, 48, or 96 h post inoculation (hpi). Control plants were maintained at the same pH throughout the experiment. pH shifts at 96 hpi or earlier from permissive to restrictive conditions resulted in nodulation being inhibited (Fig. 3). However, nodulation remained unhindered when the shift to restrictive pH conditions occurred after 96 h (Fig. 3). This shows that inhibition occurs early in nodule development, within the first 96 hpi, and that after this time, nodulation had advanced to an insensitive stage. In contrast, nodulation did not recover in plants shifted from restrictive to permissive conditions at any of the time points tested, even for plants shifted as early as 12 hpi (Fig. 3).

The Inhibitory Action of Low pH on the Nodulation of Plants Grown in Vermiculite Is Independent of Al³⁺ Toxicity

As Al³⁺ toxicity is a primary factor causing diminished plant growth and yields in acid soils (Eswaran et al., 1997), the role of acid-induced Al³⁺ toxicity on soybean nodulation was investigated. Acid treatments (pH 6.8, 5.0, and 4.0) were supplemented with 0, 1, 10, and 100 μM AlCl₃. Nodule number per plant, and per mg root dry weight, decreased as pH decreased and as Al³⁺ concentration increased (Fig. 4). However, Al³⁺ inhibition of nodulation was independent of pH (Fig. 4), and the extent of inhibition of acid- and Al³⁺-treated plants was no greater than that observed in acid-treated Al³⁺-free plants (Fig. 4). This demonstrates that Al³⁺ has no added inhibitory effect on the nodulation of acid-stressed soybean plants using our experimental conditions.

Acid Inhibition of Nodulation Acts Systemically through the Shoot

To determine whether acid inhibition of nodulation functions locally or systemically, soybean split-root plants were used. A similar number of nodules were observed on both sides of a split-root system when both sides were treated with the same pH (either pH 6.8 or 4.0; Fig. 5). As expected, plants having both sides treated with pH 4.0 produced significantly fewer nodules than those having both sides treated with pH 6.8.

Figure 5. Systemic effects on soybean nodulation in response to low-pH stress using split-root systems. A, Number of nodules per split-root system (n = 8) subjected to various pH treatments on either rootstock (pH/pH). Error bars indicate s.e. Different letters above the bars represent statistically significant differences (Student’s t test; P ≤ 0.05). DW, Dry weight. B, The split-root apparatus involving a cut 15-mL Falcon tube spanning the two individual pots.

Figure 6. Effect of acidic conditions on supernodulation. Values show the number of nodules per plant of wild-type cv Williams 82 and its supernodulating GmNARK mutant, nod4 (V370D), following treatment with pH 6.8 or 4.0. n = 8. Error bars indicate s.e. Different letters above the bars represent statistically significant differences (Student’s t test; P ≤ 0.05). DW, Dry weight.
When one side of the split-root system was treated with pH 6.8 and the other with pH 4.0, both sides exhibited a similar number of nodules (Fig. 5), reminiscent of the inhibited treatment. This indicated that low pH inhibited nodulation locally as well as systemically through a mechanism that functions from root to shoot to root, similar to that caused by rhizobia-induced AON (Delves et al., 1986).

Acidic Growth Conditions Inhibit Nodulation via the NARK Receptor

To further examine the systemic effect of low pH conditions on legume nodulation, a supernodulation GmNARK mutant of soybean, nod4 (V370D; Reid et al., 2011a), was investigated. Previously, Alva et al. (1988) used hydroponics to demonstrate that the nodulation

Table 1. Genes and associated primers used for qRT-PCR studies

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession (Phytozome)</th>
<th>Forward Primer (5’→3’)</th>
<th>Reverse Primer (5’→3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GmATP synthase</td>
<td>Glyma20g25920</td>
<td>GCGATTCTTAAGCCAGGCTTT</td>
<td>ACACCCCTGAAATCTGCTGA</td>
<td>Hayashi et al. (2012)</td>
</tr>
<tr>
<td>GmCons6</td>
<td>Glyma12g05510</td>
<td>AGATAGGGAAATGGTCTAAGG</td>
<td>CTAATGGCAATTGCAGCTCTC</td>
<td>Libault et al. (2008)</td>
</tr>
<tr>
<td>GmENOD40b</td>
<td>Glyma02g04180</td>
<td>GAGTGGCCGAAGCAAGATAAC</td>
<td>CTACATGCCATAGACCCCCAATG</td>
<td>Kouchi and Hata (1993)</td>
</tr>
<tr>
<td>GmNIN-2b</td>
<td>Glyma14g00470</td>
<td>ACAGGGATGTGCTGCTGGA</td>
<td>CGGCAGCTCTGATGTTCTGGCTTGA</td>
<td>Hayashi et al. (2012)</td>
</tr>
<tr>
<td>GmRabA2</td>
<td>Glyma14g07040</td>
<td>CAAACGAAATGGCGCTGAA</td>
<td>AAGGCCTACAAAGCTGCTTCTC</td>
<td>Blanco et al. (2009)</td>
</tr>
<tr>
<td>GmTIR-NBS-LRR</td>
<td>Glyma12g03040</td>
<td>GAGTCTTTAAGGTTGAGAAGGA</td>
<td>ATCCGCTGAAATGCTGAAATCTGTA</td>
<td>Hayashi et al. (2012)</td>
</tr>
<tr>
<td>GmCytochrome</td>
<td>Glyma11g37110</td>
<td>TCACCCAGAAACGCGAAGAG</td>
<td>ATCCCAATGCGAAGCATCAAAC</td>
<td>Hayashi et al. (2012)</td>
</tr>
<tr>
<td>P450</td>
<td></td>
<td></td>
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<tr>
<td>GmRIC1</td>
<td>Glyma13g36830</td>
<td>CAAATGCACAATGGCGCTACTG</td>
<td>GCCATGGAATCTAAGCTGCTG</td>
<td>Reid et al. (2011a)</td>
</tr>
<tr>
<td>GmRIC2</td>
<td>Glyma06g43680</td>
<td>GCCAACATCCATATGGCTCCTC</td>
<td>ACCACACCCTTGAGGTAGT</td>
<td>Reid et al. (2011a)</td>
</tr>
<tr>
<td>GmNIC1</td>
<td>Glyma12g33660</td>
<td>GCCAAAGGTTGTCACGAGAA</td>
<td>GCAAAACTGTCCTCAAGAC</td>
<td>Reid et al. (2011a)</td>
</tr>
</tbody>
</table>
Figure 8. Expression of nodulation genes in direct response to acid treatment. Values show the relative transcript abundance of the nodulation genes, *GmRabA2*, *GmENOD40b*, *GmNIN-2b*, *GmTIR-NBS-LRR*, *GmRIC1*, *GmRIC2*, and *GmNIC1*, in the ZON of soybean plants treated with either pH 6.8 or 4.0 and harvested at various times following inoculation with *B. japonicum*. *n* = 10. Error bars indicate s.e. Different letters above the bars represent statistically significant differences (Student’s *t* test; *P* ≤ 0.05).
of another GmNARK mutant, nts382 (Q920*), was not affected by treatment with pH 4.0 compared with pH 6.0 treatment. Likewise, in our experiments, pH 4.0 treatment failed to reduce the nodule number of the allelic nod4 plants, whereas it significantly reduced the nodule number of wild-type plants compared with those treated with pH 6.8 (Fig. 6).

The Mechanism of Systemic Inhibition of Nodulation Caused by Acidic Growth Conditions Is Both GmNARK Dependent and Shoot Controlled

To determine the role of the shoot’s genotype in acid-induced inhibition of nodulation, various genotype/graft/split-root combinations were tested. Split-root systems were established with both wild-type plants and near-isogenic GmNARK mutant plants (nod4). These plants were then grafted in reciprocal combinations, and their roots were treated with either pH 6.8 or 4.0.

Nodulation (measured as nodule number per mg dry weight per root portion) of wild-type/wild-type scion/stock grafted plants treated with pH 4.0 on both sides of their split-root system was significantly reduced compared with comparable plants treated with pH 6.8 (Fig. 7). Furthermore, nodulation was significantly reduced on the pH 6.8-treated side when the other side received pH 4.0 treatment (Fig. 7). The nodulation of

Figure 9. Expression of early nodulation genes in response to systemic acid treatment. Values show the relative transcript abundance of the early nodulation genes GmRabA2, GmENOD40b, GmNIN-2b, GmCytochrome P450, and GmRIC1 in the ZON of the split-root systems of soybean plants treated with either pH 6.8 or 4.0 and harvested 48 h following inoculation with B. japonicum. n = 10. Error bars indicate se. Different letters above the bars represent statistically significant differences (Student’s t test; P ≤ 0.05).
**Gmnark/Gmnark** scion/stock grafted plants remained similar regardless of pH treatment (Fig. 7).

When reciprocal grafts were produced using wild-type and Gmnark material, plants having a mutant scion exhibited a supersuppression phenotype, whereas those having a wild-type scion exhibited a wild-type nodulation phenotype (Fig. 7). Those having a wild-type scion formed significantly fewer nodules on both sides of their split-root system when one or both sides were treated with pH 4.0 (Fig. 7). In contrast, plants having a Gmnark scion were unaffected by acid treatment (Fig. 7). These findings clearly demonstrate that the systemic mechanism of acid inhibition on nodulation acts through the shoot and requires GmNARK.

**Low-pH Conditions Regulate Gene Expression Early in Nodule Development**

To determine the functional genomic stage of nodule development that is affected by acidic growth conditions, root tissue from acid-treated soybean plants (pH 6.8 or 4.0) was harvested 24, 96, and 336 hpi for expression analysis of early nodulation-related genes using quantitative reverse transcription real-time (qRT)-PCR. The tissue was harvested from the root region that is most susceptible to rhizobia infection (i.e. the zone of nodulation [ZON]; Bhuvaneswari et al., 1981; Hayashi et al., 2012). Focusing specifically on this zone has shown to be an effective way to measure early nodulation gene expression, as unwanted mRNA transcripts from remaining portions of the root system are removed that otherwise dilute the genes of interest (Hayashi et al., 2012).

Expression of all tested nodule development genes (Table I; GmENOD40b, GmNIN-2b, GmRabA2, and GmTIR-NBS-LRR) was down-regulated in pH 4.0-treated plants compared with pH 6.8+-treated control plants (Fig. 8). This suggests that the inhibition of nodule development by low-pH stress occurs very early in nodule ontogeny, perhaps even directly following Nod factor perception.

The expression of three CLE peptide-encoding genes was also investigated. GmRIC1 and GmRIC2 are up-regulated by *B. japonicum* inoculation and act through Gmnark in the shoot to inhibit nodule development (Reid et al., 2011a, 2011b). GmNIC1 is up-regulated by nitrogen and acts through Gmnark in the root as part of the nitrate regulation of nodulation pathway (Reid et al., 2011a). Because our results show that acid regulation of nodulation functions via Gmnark, we investigated whether low-pH conditions can also up-regulate the expression of these genes as a means of controlling nodulation. In fact, none of these genes were up-regulated in expression by acid treatment, and the expression of GmRIC1 and GmRIC2 actually decreased (Fig. 8), likely due to the decreased nodule primordia formation caused by the treatment (Lim et al., 2011).

**The Regulation of Nodulation Genes by Low-pH Conditions Is Systemic**

To further examine the systemic function of acidity on nodulation, the expression of the early nodulation genes GmENOD40b, GmNIN-2b, GmRIC1, GmRabA2, and GmCytochrome P450 (Table I) was examined in the ZONs of split-root plants (Fig. 9). These genes were selected on the basis of their early nodulation response. Each side of the split-root plant was treated with either pH 6.8 or 4.0 and was inoculated 48 h later with either *B. japonicum* wild type (+; effective, control) or its nodC mutant (−; ineffective, unable to synthesize Nod factor). Three treatment combinations were investigated: 6.8+/6.8+, 6.8+/4.0+, and 6.8+/4.0−. The ZONs were harvested 48 hpi for gene expression analysis using qRT-PCR.

The transcript abundance of all genes tested was significantly reduced in the ZON of pH 4.0+-treated roots compared with pH 6.8+-treated roots (Fig. 9). They were also all significantly reduced in the ZON of pH 6.8+-treated roots where the alternate side of the split-root system received pH 4.0 treatment (Fig. 9). This reduction was irrespective of the strain of inoculum used on the pH 4.0-treated side. Collectively, these findings demonstrate that the systemic inhibition of nodulation is directly triggered by low pH and is not dependent on the induction of nodulation.

**DISCUSSION**

We present evidence from experimentation with soybean that low pH acts systemically through the receptor kinase GmNARK in the shoot to inhibit...
nodule development in the root. Acid inhibition of nodulation occurs early in nodule development, as significantly fewer nodules formed on plants exposed to low-pH treatment 0 to 96 hpi (Fig. 3). This is consistent with previous reports using pea (Lie, 1969) and soybean (D. Conlan, unpublished data; Honours, 1982). Once triggered, the inhibition is maintained, as nodulation failed to recover in plants transferred to favorable pH conditions, even as early as 12 hpi (Fig. 3). However, at later time points (after 96 hpi), no suppression was observed in plants transferred to low-pH conditions (Fig. 3). This stage coincides with the development of a defined nodule primordium, which may have gained developmental autonomy (Newcomb et al., 1979; Calvert et al., 1984). AON is also believed to act at this stage in soybean (Mathews et al., 1989). This indicates that the nodule primordia (meristems) had progressed to a stage where they were no longer inhibited by acid treatment.

Acid inhibition acts early in the signaling pathway of nodulation, as the transcript abundance of critical genes associated with nodule ontogeny was reduced under low-pH conditions (Fig. 8). This adds to the biological, genetic, and developmental findings described here that acidify acts at an initial stage of nodulation. Supporting this conclusion is the finding that ectopic overexpression of the Nod factor receptor component GmNFR1a, but not GmNFR1β or GmNFR5α or GmNFR5β (Indrasumunar et al., 2010, 2011), in transgenic soybean roots not only increased the nodule number per plant but also conferred the ability of modified roots to nodulate at wild-type levels in acid soil (pH 4.6).

Long-distance suppression of gene expression may have been directly caused by alterations of the GmNARK-facilitated pathway. Low-pH conditions can also negatively impact the growth of plants and rhizobia (Brockwell et al., 1991; Marschner, 1991; Smit et al., 1992; Hungria and Stacey, 1997; Morón et al., 2005) and can decrease the synthesis of legume flavonoids and rhizobia Nod factor (McKay and Djordjevic, 1993; Hungria and Stacey, 1997). These factors may have also contributed to the down-regulation of early nodulation gene expression observed here (Figs. 8 and 9). In parallel, the accumulation of ethylene, reactive oxygen species, and salicylic acid resulting from low-pH-induced oxidative stress (Borch et al., 1991; Velikova et al., 2000) may have also played a role.

The fact that acid-induced inhibition of nodulation acts through GmNARK (Figs. 5 and 6) was somewhat expected, as preliminary findings had been reported by Alva et al. (1988). However, the specific involvement of GmNARK was unknown at the time, as it was not yet identified, and a systemic role was not yet established. This is especially important considering that nitrate acts through GmNARK in a root-localized fashion to suppress nodule development, and hence, a local mechanism is in place that could have been usurped by acid to exert nodulation control. Indeed, the debilitating effects of low pH on plant and rhizobia health (Brockwell et al., 1991; Marschner, 1991; Sadowsky et al., 1998) and on their symbiotic interactions (Smit et al., 1992; Hungria and Stacey, 1997; Morón et al., 2005) have long been considered local rhizosphere effects induced directly by H⁺ ions. This now appears to be only one component of pH-regulated nodulation control.

The systemic nature of pH inhibition of nodulation was rigorously tested here using combinations of plant and rhizobia mutants, coupled with split-root and grafting procedures, and phenotyping and transcript abundance analyses. The effects are ultimately the result of both abiotic stress responses across the plant-rhizosphere interface as well as synergistic interactions among numerous internal regulatory mechanisms (Fig. 10). Importantly, the existence of an active systemic inhibitory mechanism involving long-distance signaling that suppresses nodulation under low-pH conditions offers the potential for new targets and approaches to combat this prevalent and global agricultural problem.

MATERIALS AND METHODS

Plant Growth Conditions

Seeds of two soybean (Glycine max) wild-type cultivars, Bragg and Williams 82, and the supernodulation Gmnr mutant, nod4, were surface sterilized by full immersion in 70% ethanol for 30 s, followed by rinsing five times with sterile water. Unless otherwise stated, the seeds were sown in sterile grade-2 vermiculite in 1-L pots. Vermiculite was used as a substrate because it has a partial buffering capacity (McBride and Homenath, 1994), enabling acid treatments to be made without significantly harming the plant. Plants were watered with a modified Herridge nutrient solution every 2 d (Herridge, 1977): KH₂PO₄ 1 mM; K₂HPO₄ 1 mM; MgSO₄ 7H₂O 2 mM; KCl 1.5 mM; CaCl₂ 2H₂O 2.5 mM; Fe[II]-EDTA 34.8 mg L⁻¹; H₂BO₃ 2.86 mg L⁻¹; MnCl₂ 4H₂O 1.812 mg L⁻¹; ZnCl₂ 0.112 mg L⁻¹; CuCl₂ 2H₂O 0.052 mg L⁻¹; Na₂MoO₄ 2H₂O 0.024 mg L⁻¹; The pH of the solution was adjusted using 5 M HCl, and 300 mL was applied every 2 d via watering. Glasshouse growth conditions were controlled using a 16-h-day/8-h-night cycle at 28°C/23°C, respectively. Unless stated otherwise, all plants were grown for 18 d post inoculation, when they were then harvested for scoring. Where dry weights were recorded, the tissue was placed in an oven at 60°C for 4 d prior to weighing. In all cases, unless otherwise stated, n = 15 plants per pH treatment.

Bacteria Growth Conditions

Bradyrhizobium japonicum strains CB1809, USDA110, and its mutant, nodC (the latter kindly provided by G. Stacey), were grown in yeast mannitol broth (mannitol 2 g L⁻¹; yeast extract 0.4 g L⁻¹; K₂HPO₄ 0.5 g L⁻¹; MgSO₄ 7H₂O 0.2 g L⁻¹; NaCl 0.1 g L⁻¹; pH 7) for 48 h at 28°C. Cultures were diluted with water to a final concentration of optical density at 600 nm = 0.1 prior to inoculating plants. For acid-treated plants, the inoculum was first diluted to pH 4.0 using water adjusted with 5 M HCl.

Permissive versus Restrictive pH-Shift Experiments

Six-day-old cv Bragg plants (n = 15) were treated with either pH 6.8 or 4.0 solution every 2 d. When 8 d old, the plants were inoculated with B. japonicum strain CB1809. At different times after inoculation (12, 24, 48, or 96 hpi), a subset of plants from each pH treatment was flushed with 1 L of the opposite pH solution (i.e., a pH shift). The plants were subsequently watered every 2 d with the new pH solution. Treatments continued for 18 d until the plants were harvested for scoring.
pH and Aluminum Toxicity Studies

Aluminum solutions were made by dissolving AlCl₃ in sterile water. Six-day-old cv Bragg plants (n = 15) were subjected to a combination of pH (300 mL of pH 6.8, 5.0, or 4.0) and AlCl₃ (300 mL of 0, 1.1, or 100 μM) treatment. At 8 d old, the plants were inoculated with B. japonicum strain CB1809. Treatments were given every 2 d for 18 d until the plants were harvested for scoring.

Split-Root and Grafting Experiments

Surface-sterilized cv Bragg seeds were initially sown in 4-L pots. When seedlings were 3 d old, they were uprooted and cut 3 mm from the root tip with a sterile scalpel blade to encourage lateral root development. The cut seedlings were immediately replanted into modified 50-mL Falcon tubes that had been cut in cross-section to form 70-mm open-ended plastic tubes (Fig. 4). The tubes also had two longitudinal slots (30 × 10 mm) cut along their sides for the purpose of binding together two 1-L square pots (Fig. 4). The replanted seedlings were grown for a further 7 d to ensure the proliferation of their roots into each pot. Acid treatments commenced following this 7-d period, and inoculation with 100 mL of B. japonicum CB1809 culture occurred 2 d after that. Treatments were given every 2 d for 18 d until plants (n = 8) were harvested for scoring.

For reciprocal grafting experiments, grafts were made using cv Williams 82 and old cv plants having already-established split-root systems (initiated 7 d prior). Grafts were performed as described by Delves et al. (1987). Grafted plants were kept under clear plastic bags and watered as necessary to maintain high humidity, which encourages graft unions to form. After 7 d, the plastics bags were removed and the first acid treatments were administered. The plants were inoculated with 100 mL of B. japonicum CB1809 culture 2 d following the commencement of the acid treatments. The pH treatments were continued every 2 d for 14 d until the plants (n = 10) were harvested for scoring.

Root Tissue Collection for Gene Expression Analysis

Roots of treated plants (n = 10) were observed with a dissecting microscope to record the region between the first emerging root hairs and the fully mature root hairs. This region represents the ZON (Bhuvaneswari et al., 1981; Hayashi et al., 2012) and was harvested for gene expression studies.

RNA Extraction and cDNA Synthesis

All samples collected were immediately snap frozen in liquid nitrogen and stored at −80°C. RNA extraction was performed according to the manufacturer’s instructions using TRIzol reagent (Invitrogen). Genomic DNA was removed by DNase treatment of 1 μg of RNA using 1 unit of DNase (Fermentas) at 37°C for 40 min. cDNA was synthesized in 20-μL reactions using 0.5 μg of DNase-treated RNA, 5× first-strand buffer (Invitrogen), 1 μL of 10 μM oligo(dT)₁₂‐₁₈ primers, 0.5 mM deoxyribonucleotide triphosphates, 40 units of RNaseOUT (Invitrogen), and 100 units of SuperScript III reverse transcriptase (Invitrogen) at 50°C for 1 h. Resulting cDNA was verified using PCR with GmATP synthase primers (Table I).

qRT-PCR

qRT-PCR primers used in this study are listed in Table I. SYBR Green (Applied Biosystems) was used in reactions distributed onto 384-well plates using the Eppendorf epMotion 5075 Robotics System. qRT-PCR and analysis were run for 40 cycles on an ABI Prism 7900D Sequence Detection System (Applied Biosystems) with an annealing temperature of 60°C and duplicate technical replicates for each reaction. Thermal cycle conditions were as follows: initial 95°C for 10 min, then 40 cycles of 95°C for 15 s, 60°C for 1 min, and 95°C for 2 min (dissociation stage to verify primer specificity). LinRegPCR 7.5 software (Ramakers et al., 2003) was used to determine PCR efficiency for each reaction. GmATP synthase and GmCor1δ (Libault et al., 2008) were used as housekeeping genes.

Statistical Analysis

All values and sds were generated using group averages. Statistical differences among treatment groups were determined using Student’s t tests to generate P values. A threshold of P ≤ 0.05 was used to indicate a significant statistical difference between values.

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