GRAS Proteins Form a DNA Binding Complex to Induce Gene Expression during Nodulation Signaling in *Medicago truncatula*  

Sibylle Hirsch,a,1 Jiyoung Kim,a,1 Alfonso Muñoz,a,b,1 Anne B. Heckmann,c,d J. Allan Downie,c and Giles E.D. Oldroyd,a,2

a Department of Disease and Stress Biology, John Innes Centre, Norwich NR4 7UH, United Kingdom  
b Centro Nacional de Biotecnologia, Darwin 3, 28049 Madrid, Spain  
c Department of Molecular Microbiology, John Innes Centre, Norwich NR4 7UH, United Kingdom  
d Department of Molecular Biology, University of Aarhus, 8000 Aarhus C, Denmark

The symbiotic association of legumes with rhizobia involves bacterially derived Nod factor, which is sufficient to activate the formation of nodules on the roots of the host plant. Perception of Nod factor by root hair cells induces calcium oscillations that are a component of the Nod factor signal transduction pathway. Perception of the calcium oscillations is a function of a calcium- and calmodulin-dependent protein kinase, and this activates nodulation gene expression via two GRAS domain transcriptional regulators, Nodulation Signaling Pathway1 (NSP1) and NSP2, and an ERF transcription factor required for nodulation. Here, we show that NSP1 and NSP2 form a complex that is associated with the promoters of early nodulin genes. We show that NSP1 binds directly to ENOD promoters through the novel cis-element AATTT. While NSP1 shows direct binding to the *ENOD11* promoter in vitro, this association in vivo requires NSP2. The NSP1-NSP2 association with the *ENOD11* promoter is enhanced following Nod factor elicitation. Mutations in the domain of NSP2 responsible for its interaction with NSP1 highlight the significance of the NSP1-NSP2 heteropolymer for nodulation signaling. Our work reveals direct binding of a GRAS protein complex to DNA and highlights the importance of the NSP1-NSP2 complex for efficient nodulation in the model legume *Medicago truncatula*.

INTRODUCTION

Legumes enter symbiotic interactions with rhizobial bacteria that help the plant in the acquisition of nitrogen. The interaction is newly established in every plant and involves recognition of rhizobially made Nod factor signal (Denarie et al., 1996; Downie and Walker, 1999). Perception of Nod factor leads to the induction of a unique organ, the root nodule that accommodates the bacteria and provides suitable conditions for nitrogen fixation. Nod factor perception by legume root hair cells leads to oscillations in calcium levels, termed calcium spiking (Ehrhardt et al., 1996). Genetic and pharmacological studies indicate that calcium spiking plays a pivotal role in the Nod factor signal transduction pathway that activates transcriptional changes in response to the symbiotic signal (Wais et al., 2000; Walker et al., 2000; Charron et al., 2004; Miwa et al., 2006).

Genetic dissection of nodulation in *Medicago truncatula* and *Lotus japonicus* revealed a signaling pathway necessary for Nod factor signal transduction (Oldroyd and Downie, 2004, 2006). A suite of receptor-like kinases are necessary for Nod factor perception (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). Following this perception presumably at the plasma membrane, all remaining components of the Nod factor signaling pathway are associated with the nucleus: a number of components at the core of the nuclear pore (Kanamori et al., 2006; Saito et al., 2007), as well as two cation channels (An et al., 2004; Imaizumi-Anraku et al., 2005), at least one of which is located on the nuclear membrane (Riely et al., 2007), are necessary for the Nod factor induction of calcium spiking. The calcium response itself is strongly associated with the nucleus (Ehrhardt et al., 1996). Downstream of the calcium response is a nuclear-localized calcium- and calmodulin-dependent protein kinase (CCaMK) (Levy et al., 2004; Mitra et al., 2004; Kalo et al., 2005; Smit et al., 2005; Gleason et al., 2006; Tirichine et al., 2006), three nuclear-associated transcriptional regulators, two GRAS family proteins, Nodulation Signaling Pathway1 (NSP1) and NSP2 (Kalo et al., 2005; Smit et al., 2005; Heckmann et al., 2006), and an ERF transcription factor, ERN1 (Middleton et al., 2007). CCaMK is regulated by autoinhibition that is relieved upon calmodulin binding. The removal of the autoinhibition domain leads to constitutive calcium-independent kinase activity in vitro and spontaneous nodule formation in vivo (Gleason et al., 2006).

In *M. truncatula*, several genes have been identified that are rapidly induced upon Nod factor application. The best studied of these early nodulins (ENODs) is *ENOD11*; however, a number of the genetic components identified as being essential for
nodulation are themselves induced following Nod factor elicitation. The autoactivated CCaMK induces ENOD11 expression, and this requires NSP1, NSP2, and ERN1 (Gleason et al., 2006; Tirichine et al., 2006; Middleton et al., 2007), indicating that the calcium signal is transduced by CCaMK through these transcriptional regulators to activate gene expression. ERN1 and two close homologs, ERN2 and ERN3, have been shown to directly activate transcriptional activators in nodulogenesis (Andriankaja et al., 2007). While ERN1 and ERN2 function as transcriptional activators, ERN3 represses ERN1/ERN2-dependent transcriptional activation of ENOD11 (Andriankaja et al., 2007). Nod factor induction of ENOD11 is confined to a zone of the root epidermis (Journet et al., 1999; Marsh et al., 2007).

The GRAS family of putative transcriptional regulators is found throughout the plant kingdom, and these proteins have diverse roles in plant development, including root development, axillary shoot development, and maintenance of the shoot apical meristem (Bolle, 2004). GRAS proteins showed conserved residues in the C terminus but contain a variable N terminus with homopolymeric stretches of certain amino acids. It has recently been shown that two GRAS proteins that regulate root growth, SCARECROW (SCR) and SHORTROOT (SHR), interact with each other (Cui et al., 2007), while a class of GRAS proteins involved in regulating plant growth, the DELLA proteins, interact with a transcription factor involved in phytochrome signaling (de Lucas et al., 2008; Feng et al., 2008).

Although NSP1 and NSP2 both encode GRAS family members, they are not very similar (20.1% identity and 32.9% similarity), suggesting that they have different functions in the Nod factor signaling pathway. Here, we show that NSP1 is a DNA binding protein that binds to the promoter of the Nod factor-inducible genes ENOD11, ERN1, and NIN. The in vivo association between NSP1 and the ENOD11 promoter is enhanced by Nod factor elicitation. NSP1 and NSP2 form homopolymers and heteropolymers, and the binding of NSP1 to the ENOD11 promoter requires the action of NSP2. A point mutation in the leucine heptad repeat (LHRI) domain of NSP2, which is necessary for the heteropolymerization, reveals a function for heteropolymerization in nodule signaling. Our data reveal that NSP1 and NSP2 form a complex at the DNA to induce specific gene expression changes essential for the root nodule symbiosis.

RESULTS

NSP1 and NSP2 Form Heterodimers in *Saccharomyces cerevisiae*

The similarity in the *nsp1* and *nsp2* mutant phenotypes (Catoira et al., 2000; Oldroyd and Long, 2003) and the fact that they both encode GRAS domain proteins suggested the possibility that NSP1 and NSP2 may form a complex. To test this hypothesis, we assessed interactions between NSP1 and NSP2 in the Gal4 yeast two-hybrid system. Fusions of NSP1 or NSP2 to the Gal4 DNA binding domain caused autoactivation of the Gal4 system, indicating a function for NSP1 and NSP2 as transcriptional regulators (Figures 1A and 1C). Autoactivation is a function of the N-terminal domains of both NSP1 and NSP2, as has been previously reported for a GRAS protein from lily (Lilium longiflorum; Morohashi et al., 2003), since removal of the N-terminal domains of NSP1 (NSP1 156-554) and NSP2 (NSP2 113-508) abolished the autoactivation in *S. cerevisiae*, and the N-terminal domains alone (NSP1 1-155 and NSP2 1-112) were sufficient for autoactivation (Figures 1A and 1C). However, the autoactivation of NSP2 by the full-length protein is also regulated by the VHIID domain as a specific deletion of the VHIID domain (NSP2 1208-276) abolishes autoactivation (Figure 1C), and a point mutant in this motif (NSP2E232K) also inhibits autoactivation (Figure 1A). NSP2E232K was identified in the *nsp2*-3 mutant of *M. truncatula*, a weak *nsp2* mutant allele showing limited nodule formation in contrast with the null allele *nsp2*-2 that shows no nodule formation (Kalo et al., 2005).

The fact that *nsp2*-3 showed defects in nodule formation and this mutation abolishes autoactivation in *S. cerevisiae* indicates that the transcriptional activity by NSP2 is relevant for its function in the plant. *S. cerevisiae* containing NSP2E232K fused to the Gal4 DNA binding domain and NSP1 fused to the Gal4 activation domain showed increased reporter gene expression (Figure 1A), indicating an interaction between NSP1 and NSP2. The autoactivation of NSP1 and NSP2 limited this yeast assay, but by quantifying β-galactosidase activity, we found a significant enhancement in *S. cerevisiae* containing NSP1 and wild-type NSP2 compared with the autoactivation by NSP1 or NSP2 alone (Figure 1B).

Assays of β-galactosidase indicated that NSP1 and NSP2 interact when NSP1 or NSP2 was fused to the Gal4 DNA binding or activation domains (Figure 1B).

GRAS proteins contain two leucine heptad repeat domains (LHRI and LHRII) and three domains, VHIID, PFYRE, and SAW, that have residues conserved across all GRAS proteins (Bolle, 2004; Kalo et al., 2005). By generating both N- and C-terminal deletions of NSP1 and NSP2, we could identify the domains of these proteins that were responsible for the interaction. Most N- and C-terminal deletions of NSP1 and NSP2 abolished its interaction with NSP2 (Figure 1C; see Supplemental Table 1 online). Indeed, the only deleted version of NSP1 that still showed an interaction with NSP2 was an N-terminal deletion that removes the first 155 amino acids (NSP1 156-554). All the NSP1 deletions were stable in *S. cerevisiae*, as shown by antibody staining (see Supplemental Figure 1A online). This work suggests that more than one region of NSP1 is required for its interaction with NSP2. By contrast, C-terminal deletions of NSP2 (NSP2 1-276 and NSP2 1-180) had no effect on the interaction with NSP1, whereas N-terminal deletions completely abolished the interaction (NSP2 208-508 and NSP2 287-508) (Figure 1C; see Supplemental Table 1 online). Immunostaining showed that these NSP2 deletions were also stable in *S. cerevisiae* (see Supplemental Figure 1B online). From these studies we deduced that either the N-terminal region or the LHRI domain of NSP2 may be responsible for its interaction with NSP1. The LHRI domain of NSP2 (NSP2 113-180) was sufficient to interact with NSP1, and specific deletion of the LHRI domain (NSP2 113-508) abolished the interaction with NSP1 (Figure 1C; see Supplemental Table 1 online). This indicates that the LHRI domain of NSP2 is necessary for its interaction with NSP1.
and sufficient for the interaction with NSP1. However, two NSP2 constructs (NSP2 113-508 and NSP2Δ208-276) do not interact with NSP1, but contain the LHRI domain. At this stage, we cannot explain this inconsistency, but it seems likely that these two constructs are inappropriately folded to allow LHRI activity. These studies suggest that the LHRI domain of NSP2 interacts with multiple domains of the NSP1 protein.

NSP1 and NSP2 Form Homopolymers and Heteropolymers in Plant Cells

To validate the NSP1 and NSP2 interaction we had observed in S. cerevisiae, we assessed the interaction of these proteins inside plant cells. We tested the interaction of NSP1 and NSP2 in leaves of Nicotiana benthamiana using bimolecular fluorescence.
complementation (BiFC) and coimmunoprecipitation. We generated different fusions of the yellow fluorescent protein (YFP) domains to NSP1 and NSP2 and transiently expressed these proteins in *N. benthamiana* leaves using *Agrobacterium tumefaciens* infiltrations. Clear indications for an in vivo interaction between NSP1 and NSP2 were observed, with strong yellow fluorescence in the nuclei of *N. benthamiana* cells (Figure 2A). This nuclear localization of the NSP1 and NSP2 interaction in *N. benthamiana* is in agreement with earlier observations indicating a nuclear localization of NSP1 and NSP2 (Kalo et al., 2005; Smit et al., 2005; Murakami et al., 2006). Consistent with the analyses in *S. cerevisiae*, in planta BiFC experiments showed that the LHRI domain of NSP2 is sufficient for the interaction with NSP1 (Figure 2B), and removal of the LHRI domain abolishes the interaction with NSP1 (Figure 2C). The NSP2 construct lacking LHRI is stable in *N. benthamiana* (Figure 2C, inset).

Coimmunoprecipitation experiments also revealed an interaction between NSP1 and NSP2 in *N. benthamiana*. For these experiments, we generated constructs for the expression of NSP1 with a C-terminal triple HA tag and NSP2 with a C-terminal FLAG tag. These fusions are functional since they are able to complement *M. truncatula nsp1* or *nsp2* mutants, respectively (see Supplemental Table 2 online). Using the FLAG epitope, we immunoprecipitated NSP2 from leaf extracts and NSP1 coprecipitated as revealed by the presence of the HA epitope (Figure 2D). NSP1-HA was not precipitated from *N. benthamiana* leaves that lacked NSP2-FLAG (Figure 2D). Therefore, we conclude that NSP1 and NSP2 are able to interact in both *S. cerevisiae* and in plant cells.

To assess the possibility of self-interaction of NSP1 or NSP2, we coexpressed HA, green fluorescent protein (GFP), and FLAG-tagged NSP2 or HA and FLAG-tagged NSP1 in *N. benthamiana* leaves. All epitope fusions used in this study were able to complement their reciprocal *M. truncatula* mutant, indicating functionality. Using the FLAG epitope to isolate NSP1 or NSP2 from leaf extracts, we found the HA epitope coprecipitating in both cases (Figures 3A and 3B), indicating homopolymerization. This could be validated with BiFC for NSP1 (Figure 3C). We did not observe NSP2–NSP2 interaction using BiFC, but we could coprecipitate NSP2-GFP along with NSP2-FLAG using a FLAG epitope immunoprecipitation, confirming the previous result using NSP2-HA. This work indicates that NSP1 and NSP2 form homodimers or higher order homopolymers, as well as heterodimers or higher-order heteropolymers.

**Figure 2.** NSP1 and NSP2 Form Heteropolymers in Planta.

(A) to (C) *N. benthamiana* leaves were cotransformed with YFP<sup>+</sup>-NSP1 and YFP<sup>+</sup>-NSP2 (A), YFP<sup>+</sup>-NSP1 and YFP<sup>+</sup>-NSP2-LHRI (B), and YFP<sup>+</sup>-NSP1 and YFP<sup>+</sup>-NSP2ΔLHRI (C). Overlays of confocal YFP and bright-field images of epidermal *N. benthamiana* leaf cells are shown. (A) and (B) show the nuclear localization of the NSP1 and NSP2 interaction. Removal of the LHRI domain of NSP2 abolished the interaction with complementation (BiFC) and coimmunoprecipitation. We generated different fusions of the yellow fluorescent protein (YFP) domains to NSP1 and NSP2 and transiently expressed these proteins in *N. benthamiana* leaves using *Agrobacterium tumefaciens* infiltrations. Clear indications for an in vivo interaction between NSP1 and NSP2 were observed, with strong yellow fluorescence in the nuclei of *N. benthamiana* cells (Figure 2A). This nuclear localization of the NSP1 and NSP2 interaction in *N. benthamiana* is in agreement with earlier observations indicating a nuclear localization of NSP1 and NSP2 (Kalo et al., 2005; Smit et al., 2005; Murakami et al., 2006). Consistent with the analyses in *S. cerevisiae*, in planta BiFC experiments showed that the LHRI domain of NSP2 is sufficient for the interaction with NSP1 (Figure 2B), and removal of the LHRI domain abolishes the interaction with NSP1 (Figure 2C). The NSP2 construct lacking LHRI is stable in *N. benthamiana* (Figure 2C, inset).

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The NSP1–NSP2 Interaction Is Required for Nodule Formation

To investigate the function of the heteropolymerization between NSP1 and NSP2 in M. truncatula, we transformed nsp2-2 mutants, which are unable to form nodules, with the NSP2 deletion construct lacking the LHRI domain responsible for its interaction with NSP1. Five weeks after inoculation with Sinorhizobium meliloti, no nodules were detected on the nsp2-2 roots transformed with NSP2::LHR1 (Figure 4B), in contrast with nsp2-2 roots transformed with full-length NSP2 that showed extensive nodulation (Figure 4A). This result suggests that the interaction that occurs through the LHRI domain is required for NSP2 action during nodule formation.

Point mutations are likely to cause less deleterious effects on protein function than deletions. To further assess the relevance of the interaction between NSP1 and NSP2, we identified a point mutation in the LHRI domain of NSP2 that reduced the interaction with NSP1 by approximately threefold (Figure 4C). This mutation resulted in the amino acid substitution A168V. We assessed the ability of NSP2A168V to complement the nsp2-2 mutant. Nodule numbers were reduced by approximately threefold in transgenic nsp2-2 roots harboring NSP2A168V compared with roots transformed with wild-type NSP2 (Figure 4D). To test whether the few nodules that formed on the nsp2-2 mutant roots transformed with NSP2A168V were functional, the rate of nitrogenase activity within the nodules was measured per milligram of nodule tissue. Acetylene reduction was impaired in the nodules of the nsp2-2 mutant roots transformed with NSP2A168V compared with nodules on nsp2-2 transformed with wild-type NSP2 (Figure 4E). Taken together, these results reveal that in the absence of the NSP1–NSP2 interaction, there is no nodule formation, while when the interaction is reduced, there is a reduced level of nodulation. These correlations imply that the NSP1–NSP2 interaction is important for nodulation signaling in M. truncatula.

NSP1 Directly Associates with ENOD Promoters

GRAS proteins have recently been shown to be associated with the promoters of target genes in vivo (Cui et al., 2007), although no direct binding of GRAS proteins to DNA has been observed. ENOD11 is rapidly induced by Nod factor treatment in M. truncatula roots, and this requires both NSP1 and NSP2 (Journet et al., 2001; Boisson-Dernier et al., 2005; Middleton et al., 2007). As ENOD11 is a potential target for inducibility by NSP1 and NSP2, we assessed whether these GRAS proteins could directly bind to the ENOD11 promoter. Using purified NSP1 and NSP2 from Escherichia coli, we tested if these proteins could associate with a radiolabeled 1049-bp fragment containing the start codon at the 3' end of the ENOD11 promoter that shows Nod factor inducibility (Boisson-Dernier et al., 2005). We saw a clear band shift in the electrophoretic mobility shift analysis (EMSA) when the ENOD11 promoter was incubated with NSP1, but not with either NSP2 or any of the controls (Figure 5A). The binding of NSP1 to the ENOD11 promoter can be outcompeted by the addition of unlabeled ENOD11 promoter (Figure 5A). Considering the interaction we have observed between NSP1 and NSP2, we assessed whether incubation with both proteins would cause a supershift in this assay but saw no evidence for this. Our work indicates that NSP1, but not NSP2, can directly bind to the ENOD11 promoter.

NIN and ERN1 are both essential for nodulation and are induced following rhizobia or Nod factor treatment (Schauer...
et al., 1999; Marsh et al., 2007; Middleton et al., 2007). The induction of NIN and ERN1 by rhizobia requires both NSP1 and NSP2 (Figure 5D) (Murakami et al., 2006). We tested whether NSP1 could bind to the NIN and ERN1 promoters in vitro by EMSA. We observed that purified NSP1 associates with the −892 to −13 region of the NIN promoter and the −862 to −29 region of the ERN1 promoter (Figures 5B and 5C). This work indicates that NSP1 binds directly to multiple early nodulin promoters.

We assessed which domains of NSP1 were responsible for the DNA binding. Most of the N- and C-terminal deleted derivatives of NSP1 still associated with the ENOD11 promoter (see Supplemental Figures 2A and 2B online), and the only derivatives that lost DNA binding capability were deletions that removed both LHRI and LHRII domains. The isolated LHRI and LHRII domains of NSP1 retarded the ENOD11 promoter fragment (see Supplemental Figure 2B online), indicating that NSP1 associates with the ENOD11 promoter through the LHRI domains.

We next attempted to define the NSP1 targeted cis-elements. Using random binding site selection to characterize the sequence binding specificity of NSP1, we identified several random oligonucleotides that bind NSP1; all contained the consensus motif AATTT (Figure 6A), indicating that this is likely to represent the NSP1 targeted cis-element. The ENOD11, NIN, and ERN1 promoters contain this AATTT motif. The domains of the ENOD11 promoter have been well characterized, and the −411 to −257 region has been shown to be responsible for Nod factor inducibility (Boisson-Dernier et al., 2005). This region contains two AATTT motifs (Figure 6B). When we specifically isolated these two motifs, embedded within a 27-bp region of the surrounding promoter sequence, we were able to see binding of NSP1 to both cis-elements (Figure 6C). The motifs were called NODULATION RESPONSIVE ELEMENT1 (NRE1)
and NRE2. Mutation of the AATTT motif to CCCCC in NRE1 (NRE1-C5) and NRE2 (NRE2-C5) greatly reduced the binding of NSP1 to these promoter fragments (Figure 6C). Deletion derivatives of NSP1 that contain either the LHRI or LHRII domains bound to NRE1 and NRE2, but not to the mutant forms NRE1-C5 or NRE2-C5 (see Supplemental Figure 2C online), indicating that the LHR domains are responsible for the specific recognition of the AATTT motif.

To test the relative importance of the AATTT cis-elements for Nod factor inducibility, we generated a fusion construct where a single copy of the 27-bp NRE2 region containing the AATTT motif of ENOD11 was fused to the minimal ENOD11 promoter, −257 to −1, that lacks Nod factor inducibility. In addition, we generated a construct where the AATTT motif of NRE2 was mutated to CCCCC. We tested the ability of these promoter fusions to drive the expression of β-glucuronidase (GUS) in M. truncatula roots. We found a small but significant induction of GUS following Nod factor treatment in the NRE2 fusion (Figure 6D). This induction is absent in an nsp1 mutant and is also absent in the mNRE2 fusion (Figure 6D). This work shows that NRE2 plays a role in Nod factor inducibility of the ENOD11 promoter, and this highlights the significance of NSP1 for Nod factor gene induction.

NSP1 and NSP2 Associate with the ENOD11 Promoter in Vivo

To test the NSP1 binding to the ENOD11 promoter in plants, we undertook chromatin immunoprecipitation (ChIP) to screen for the NSP1-ENOD11 promoter interaction in roots of M. truncatula. We generated antibodies against NSP1 and NSP2 to isolate the GRAS proteins from M. truncatula root extracts. These antibodies recognize their target proteins specifically and do not cross react with either NSP1 or NSP2 (see Supplemental Figure 3 online), indicating that the LHR domains are responsible for the specific recognition of the AATTT motif.

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**Figure 5.** NSP1 Associates with ENOD Promoters in Vitro.

(A) to (C) EMSA with NSP1 and NSP2 proteins using radiolabeled ENOD promoter probes. The promoter regions of ENOD11 (−1046 to +3) (A), NIN (−892 to −13) (B), and ERN1 (−862 to −29) (C) were used. The free ENOD promoter probe is present at the bottom of each image. The band shift, indicated with an arrow, revealed NSP1 association with the promoter of ENOD11 (A), NIN (B), and ERN1 (C) that is absent in the GST control and in NSP2. In (A), unlabeled ENOD11 fragment was used as competitor DNA. c10x and c50x, unlabeled competitor DNA in 10- and 50-fold excess, respectively.

(D) RT-PCR showing that the S. meliloti induction of NIN and ERN1 is absent in nsp1-1 and nsp2-2 mutants. Actin was amplified as a control. dpi, days post inoculation.
a mutual requirement of NSP1 and NSP2 for appropriate gene expression. However, the interactions we have observed between NSP1 and NSP2 suggest an interdependence of NSP1 and NSP2 for appropriate activity.

DISCUSSION

Much evidence exists for GRAS proteins functioning in transcriptional regulation: they are generally located in the nucleus (Bolle, 2004), they show association with promoters in vivo (Cui et al., 2007), and a GRAS protein has been shown to have an innate capability to activate RNA polymerase in both S. cerevisiae and in plants (Morohashi et al., 2003). However, transcription factor classification generally requires direct DNA association, and the emerging evidence suggests that GRAS proteins may function to regulate the action of other transcription factors (de Lucas et al., 2008; Feng et al., 2008). Here, we show that the GRAS protein NSP1 binds directly to DNA and does so in a complex with a second GRAS protein, NSP2. NSP1 can activate RNA polymerase in S. cerevisiae, and this transcriptional activity of GRAS proteins has been previously validated in plant cells using L. longiflorum SCL, a GRAS protein from lily (Morohashi et al., 2003). The association of NSP1 with the ENOD11 promoter through a novel cis-element is relevant for Nod factor induction of this promoter in M. truncatula. Our work shows the GRAS protein NSP1 functioning as a classic transcription factor, with direct binding to a promoter element. By contrast, we have no evidence

Figure 6. NSP1 Binds Specifically to an AATTT Motif Present in the ENOD11 Promoter.

(A) Identification of the NSP1 cis-element by random binding site selection (RBSS). NSP1 was found to bind to the indicated 15 oligonucleotides from a pool of 4^20 random-sequence oligonucleotides. All oligonucleotides bound to NSP1 contained the AATTT motif.

(B) The -411 to -257 region of the ENOD11 promoter contains two AATTT motifs (underlined). Regions around these motifs were synthesized and named NRE1 and NRE2.

(C) EMSA analysis showed that NSP1 binds to NRE1 and NRE2. Mutation of the AATTT motif to CCCCC in both NRE1 and NRE2 (indicated as NRE1-C5 and NRE2-C5) caused a dramatic reduction in NSP1 binding.

(D) A synthetic promoter was generated by fusing the minimal ENOD11 promoter lacking Nod factor inducibility to the NRE2 element and used to drive expression of GUS. This construct was transformed into plants and assessed for Nod factor inducibility, measured by a fluorimetric GUS assay. Nod factor inducibility was observed that was absent in nsp1-1 mutants and when using NRE2-C5. The asterisk indicates a significant difference to all other data points, measured with a t test at 95% confidence. NF, Nod factor. Values are means ± sd (n = 15 plants for each treatment).
The Nod factor signaling pathway is regulated by a number of components, including the putative transcriptional regulator NIN. We have previously shown that nin mutants show excess Nod factor–induced ENOD11 expression in epidermal cells (Marsh et al., 2007). ENOD11 expression is strongly induced following Nod factor treatment, analogous to the expression of early nodulin, such as ENOD11 (Schauser et al., 1999; Marsh et al., 2007). Here, we show that NSP1 binds directly to the NIN promoter and is necessary for NIN induction. This suggests that NSP1 is likely to induce a negative feedback loop through the induction of NIN that regulates the Nod factor signaling pathway.

Nod factor signaling uses oscillations in calcium as a secondary messenger that links Nod factor perception at the plasma membrane to gene expression changes in the nucleus. The observed enhancement of NSP1 and NSP2 binding to the ENOD11 promoter in vivo following Nod factor treatment indicates that some modification of these GRAS proteins occurs during Nod factor signaling. Considering that NSP1 and NSP2 are necessary for CCAmK-induced gene expression (Gleason et al., 2006), CCAmK is a likely candidate for such modification. It is possible that NSP1 or NSP2 are modified by CCAmK to facilitate their interaction or their association with DNA. Further investigations are necessary to define the role of calcium and CCAmK in the activation of NSP1–NSP2 interaction, DNA association, and specific gene induction. Our work reveals that a NSP1-NSP2 complex binds to the promoters of early nodulin genes, and this binding is in close proximity to ERN1, ERN2, and ERN3 binding sites. We propose that NSP1, NSP2, ERN1, ERN2, ERN3, and NIN all act in combination to regulate the expression of early nodulins with the appropriate spatial and temporal pattern. The promotion of NSP1-NSP2 association with ENOD...
promoters is likely to be an important target of Nod factor signal transduction.

METHODS

Plant Material and Bacterial Strains

Seeds of Medicago truncatula cv Jemalong A17, nsp1-1, and nsp2-2 were scarified with sand paper, surface-sterilized in 100% bleach, imbibed in sterile water, and plated on 1% deionized water agar plates. Seeds were left for 24 to 48 h at 4°C and germinated on inverted agar plates at room temperature for ~30 h. Seedlings were then transferred to modified buffered nodulation medium containing 0.1 μM 2-aminooxyvinylglycine (Sigma-Aldrich). For complementation tests, M. truncatula seedlings were transformed with Agrobacterium rhizogenes strain ARqua carrying the appropriate binary vector using standard protocols (Boisson-Dernier et al., 2001). One week after the transfer of the plants to growth pouches, roots were inoculated with Sinorhizobium meliloti 1021 pXLGD4 (~10^7 colony-forming units/mL). Nodule formation was scored 2 to 5 weeks after inoculation. The presence of the transformed gene was verified by DNA extraction and PCR. For transient expression, Nicotiana benthamiana plants were grown in greenhouses or growth chambers at 21°C and 16 h of light for 2 to 5 weeks, and leaves were infiltrated with Agrobacterium tumefaciens C58C1 pCH32.

Plasmid Construction

The appropriate genes were cloned into the Gateway donor vector pDONR207 by BP recombination reactions using appropriate primers (see Supplemental Table 3 online), sequenced, and then recombined into the appropriate Gateway destination vectors by LR reactions (Invitrogen). The DNA binding and activation domain vectors, pGBK7 and pGADT7 (Clontech), had been previously made Gateway compatible by introducing the Gateway cassette B into the Smal/Xmal restriction site and called pDEST-GBK7 and pDEST-GADT7. To test the interaction in N. benthamiana using the BiFC approach, the genes were recombined into the Gateway destination vectors pGPTVII.Bar.YN-GW, pGPTVII.Bar.GW-YN, pGPTVII.Hyg.YC-GW, and pGPTVII.Hyg.GW-YC. For Escherichia coli expression, the following destination vectors were used: pDEST15, pDEST17, and pBAD-DEST49. For each experiment, N. benthamiana hairy root transformation, the C58C1 strain carrying the p19 silencing suppressor plasmid (Voinnet et al., 2003) were brought to an optical density (OD600) of 0.2 to 0.5 with 10 mM MgCl2 and 150 μM acetosyringone (Sigma-Aldrich). The strains were mixed and incubated at room temperature for ~2 h. The A. tumefaciens mixture was infiltrated into N. benthamiana leaves as described previously (Voinnet et al., 2003; Witte et al., 2004). The fluorescence was assayed 60 to 80 h after infiltration using a ×40 1.25 numerical aperture oil-immersion objective on an inverted Leica DM IRB confocal microscope. For monitoring YFP signals, an argon laser at 514-nm wavelength was used for excitation. Immunoblot analysis was used to validate the stability of the protein fusions in N. benthamiana with α-HA (HA tag fused to activation domain) or α-Myc (c-Myc tag fused to DNA binding domain) antibodies.

BiFC Analysis

A. tumefaciens strain C58C1 carrying the helper plasmid pCH32 was transformed with the BiFC constructs by electroporation. The strains of interest and the C58C1 strain carrying the p19 silencing suppressor plasmid (Voinnet et al., 2003) were brought to an optical density (OD600) of 0.2 to 0.5 with 10 mM MgCl2 and 150 μM acetosyringone (Sigma-Aldrich). The strains were mixed and incubated at room temperature for ~2 h. The A. tumefaciens mixture was infiltrated into N. benthamiana leaves as described previously (Voinnet et al., 2003; Witte et al., 2004). The fluorescence was assayed 60 to 80 h after infiltration using a ×40 1.25 numerical aperture oil-immersion objective on an inverted Leica DM IRB confocal microscope. For monitoring YFP signals, an argon laser at 514-nm wavelength was used for excitation. Immunoblot analysis was used to validate the stability of the protein fusions in N. benthamiana with α-GFP used to detect fusions to YFP or α-Nsp1/α-Nsp2 used to detect fusions to YFP.

EMSA

Recombinant Nsp1-His and GST-Nsp2 were produced in E. coli strains DH5α and BL21 and purified using the ProBond resin (Invitrogen) and glutathione-agarose (Novagen). The amount of purified proteins was estimated by the Bradford method using a protein assay kit (Bio-Rad). EMSAs were performed as described previously (Kim and Kim, 2006). The oligonucleotide probes were labeled with 32P-dATP using T4 nucleotide kinase (Invitrogen) or 32P-dTTP using Klenow fragment (Invitrogen). The binding reactions were performed in 20 μL binding buffer (25 mM HEPES-KOH, pH 8.0, 50 mM KCl, 1 mM DTT, 0.05% Triton X-100, and 15% glycerol). After 20 min of incubation at room temperature, the reactions were resolved by 6 to 8% native polyacrylamide gels with 0.5× TBE buffer.

ChiP

For each experiment, 5 g of root tissues from 10-d-old seedlings were used for ChiP with α-Nsp1 and α-Nsp2 antibodies. Nuclei were isolated as described previously (Delaney et al., 2006). Purified nuclei were fixed with 1% formaldehyde for 20 min immediately after extraction. The immunoprecipitation of purified chromatin was performed using a ChiP assay kit (Upstate) according to the manufacturer’s instructions. The presence of the ENOD11 promoter in the ChiP immunoprecipitate was tested by PCR analysis (see Supplemental Table 3 online).

RBSS

The RBSS assay was performed essentially as described previously (Wilson et al., 1993). Briefly, the selection was performed by mixing 100
ng of double-stranded random oligonucleotides, 20 bp in length (4^20 possible combinations) with 200 ng of NSP1-His fusion protein attached to 40 μL of ProBond resins (Invitrogen). The supernatant was removed, and the pellet was resuspended in 50 μL of water, boiled for 3 min, and centrifuged rapidly. The supernatant (5 μL) was used as the template for a PCR. The PCR product was purified from 2% agarose gel. The procedure was repeated 10 times. After the last PCR amplification step, the PCR products were ligated to a T/A cloning vector (Promega), and individual selected clones were sequenced.

Fluorimetric GUS Assay

Transgenic roots of the NRE2 element driving the expression of GUS were generated using A. rhizogenes. GUS activity was measured following 1 nM Nod factor or buffer alone treatment using a fluorimetric assay as described previously (Jefferson et al., 1987). The transgenic roots were ground in liquid nitrogen and homogenized in GUS extraction buffer for total protein extraction. Enzymatic reactions were performed using 1 μg of total protein extract with 4-methylumbelliferyl-D-glucuronide as substrate (Sigma-Aldrich), GUS activities were measured using a microtiter fluorimeter.

Coimmunoprecipitation

NSP1 and NSP2 fusions were transiently expressed in N. benthamiana and infiltrated leaves harvested 3 d after infiltration. Two grams of the corresponding N. benthamiana leaves were ground in liquid nitrogen and incubated in extraction buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 50 mM glutamate, 50 mM arginine, 0.1% Triton X-100, 5 mM DTT, 2% polyvinylpolypyrrolidone, and protease inhibitors [Sigma-Aldrich]) with end over end shaking for 20 min. After centrifugation at 2500g for 20 min, the extracts were ultracentrifuged at 100,000g for 30 min. The supernatant was collected and its protein concentration was measured by the Bradford method (Bio-Rad). Coimmunoprecipitation mixtures were made containing the same amounts of total protein in the same volume. These mixtures were precleared by incubation with 35 μL of IgG agarose beads for 1 h. After centrifugation, the supernatant was incubated with constant rotation for 1.5 h with 35 μL of α-FLAG agarose beads (Sigma-Aldrich), previously blocked to avoid unspecific binding with 1 mg/mL BSA in extraction buffer lacking polyvinylpolypyrrolidone for 2 h. Subsequently, the beads were collected by centrifugation, washed six times with 1 mL of the extraction buffer lacking polyvinylpolypyrrolidone, and eluted with 70 μL of 0.2 mg/mL FLAG-peptide (Sigma-Aldrich) at room temperature for 10 min. The eluted fractions and crude extracts were run in SDS-PAGE and subjected to immunoblots with the corresponding antibodies. For the immunoblots, the polyclonal α-FLAG (Sigma-Aldrich), the polyclonal α-GFP (Santa Cruz), and the high-affinity clone 3F-10 α-HA (Roche) antibodies were used with their respective horseradish peroxidase secondary antibodies. Enhanced chemiluminescent reagent (Amersham) was used to detect the proteins.

RT-PCR

Total RNA was extracted from uninoculated and S. meliloti–inoculated roots (2 and 4 d after inoculation) of 10-d-old seedlings. One microgram of total RNA was reverse transcribed and subjected to RT-PCR. The following PCR conditions were used: 30 cycles at 96°C for 30 s, 54°C for 30 s, and 72°C for 2 min, followed by 10 min of a final extension at 72°C.

Acetylene Reduction Assay

Ten to fifteen nodules were placed into a small sealed vial, and 5% volume acetylene was injected. The reaction was incubated for 1 h and the amount of produced ethylene was measured by gas chromatography using 1 mL of each sample. The fresh weight of the nodules was determined and the concentration of ethylene calculated in nanomoles per hour and milligram.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: NSP1, AJ972473 and AJ972478; NIN, FJ719774; NSP2, AJ832138; ERN1, EU038802; and ENOD11, AJ297721.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Stability of NSP1 and NSP2 Deletions.

Supplemental Figure 2. Defining the Domains of NSP1 Required for DNA Binding.

Supplemental Figure 3. Specificity of α-NSP1 and α-NSP2 Antibodies.

Supplemental Table 1. Quantification of NSP1/2 Autoactivation and Interaction.

Supplemental Table 2. Complementation Tests of the C-Terminal Tagged Versions of NSP1 and NSP2 Used in the Coimmunoprecipitation Experiments.

Supplemental Table 3. Primers Used for Plasmid Construction (GW, Gateway), ChiP-PCR, and RT-PCR Analysis.

ACKNOWLEDGMENTS

We thank Carole Thomas for help with yeast two-hybrid assays and Katia Marrocco and Thomas Kretsch for supplying the gateway-compatible plasmids necessary for BiFC. We also thank Verity Bonnell for constructing the point mutation in NSP2. This work was supported by the Biotechnology and Biological Science Research Council as grant BB/D521749/1, John Innes Foundation funding to S.H., a Marie Curie training fellowship to J.K., and a postdoctoral fellowship to A.M. from the Ministerio de Educación y Ciencia, Spain.

Received November 19, 2008; revised January 29, 2009; accepted February 11, 2009; published February 27, 2009.

REFERENCES


GRAS Proteins Form a DNA Binding Complex to Induce Gene Expression during Nodulation Signaling in Medicago truncatula
Sibylle Hirsch, Jiyoung Kim, Alfonso Muñoz, Anne B. Heckmann, J. Allan Downie and Giles E.D. Oldroyd

Plant Cell 2009;21:545-557; originally published online February 27, 2009;
DOI 10.1105/tpc.108.064501

This information is current as of July 9, 2017