TECHNICAL ADVANCE

Virus-Induced Gene Silencing (VIGS) as a Reverse Genetic Tool to Study Development of Symbiotic Root Nodules

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Submitted 23 October 2007. Accepted 13 February 2008.

Virus-induced gene silencing (VIGS) can provide a shortcut to plants with altered expression of specific genes. Here, we report that VIGS of the *Nodule inception gene* (*Nin*) can alter the nodulation phenotype and *Nin* gene expression in *Pisum sativum*. *PsNin* was chosen as target because of the distinct non-nodulating phenotype of *nin* mutants in *P. sativum*, *Lotus japonicus*, and *Medicago truncatula*. The vector based on *Pea early browning virus* (PEBV) was engineered to carry one of three nonoverlapping fragments (*PsNinA*, *PsNinB*, and *PsNinC*) derived from the *PsNin* cDNA. Vector inoculation was mediated by agroinfiltration and, 2 weeks later, a *Rhizobium leguminosarum* bv. *viciae* culture was added in order to induce root nodulation. At this time point, it was estimated that systemic silencing was established because leaves of reference plants inoculated with PEBV carrying a fragment of *Phytoene desaturase* displayed photo bleaching. Three weeks after *Rhizobium* spp. application, plants inoculated with a control vector nodulated normally, whereas nodulation was almost eliminated in plants inoculated with a vector carrying *PsNinA* and *PsNinC*. For plants inoculated with a vector carrying *PsNinB*, nodulation was reduced by at least 45%. Down-regulation of *PsNin* transcripts in plants inoculated with vectors carrying *PsNin* cDNA fragments was confirmed and these plants displayed a relative increase in the root/shoot ratio, as expected if nitrogen fixation had been impaired.

Additional keywords: *sym35*, symbiosis, *Tobravirus*

The number of vectors for virus-induced gene silencing (VIGS) and their applications to study gene function continues to expand (Burch-Smith et al. 2004). Studies of pathogen-activated defense pathways are especially well represented (Bhattarai et al. 2007; Liu et al. 2005; Rowland et al. 2005).

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

However, application to studies of microbial symbioses with rhizobia or arbuscular mycorrhizal fungi is still lacking. Studies of symbiosis with nitrogen-fixating bacteria are limited to legume species that form nodules in response to *Rhizobium* spp. colonization (Spaink 2000). VIGS vectors have been developed for *Pisum sativum* (Constantin et al. 2004) and *Glycine max* (Zhang and Ghabrial 2006), whereas there are still no reports of efficient VIGS vectors for the main model legumes *Lotus japonicus* and *Medicago truncatula*. Earlier, *P. sativum* was used to characterize mutants affecting nodulation (Duc and Messenger 1989); however, studies in *P. sativum* have been limited because of low transformation efficiency (Krejci et al. 2007) and a genome estimated to be 10 times larger than the genomes of *L. japonicus* and *M. truncatula* (Bennett and Leitch 2004).

We have shown previously that the vector based on *Pea early browning virus* (PEBV, genus *Tobravirus*) can induce a phenotype in roots, indicating that VIGS was functional in roots and that plants infected by the PEBV vector carrying a neutral fragment nodulated normally (Constantin et al. 2004). The virus vector based on *Tobacco rattle virus* (TRV, genus *Tobravirus*) also induces silencing in roots (Bhattarai et al. 2007; Valentine et al. 2004) but is not known to infect any legume species systemically (Robinson 2003). The PEBV vector consists of two binary plasmids, pCAPE1 and pCAPE2, containing cDNA of PEBV RNA1 and RNA2, respectively, placed under control of the *Cauliflower mosaic virus* 35S promoter and nopaline synthase terminator (Constantin et al. 2004). The cDNA of PEBV RNA2 is modified to allow insertion of heterologous sequences replacing the genes required for nematode transmission (MacFarlane and Popovich 2000). Establishment of recombinant virus infection in the test plants is accomplished by agroinfiltration (Constantin et al. 2004), and this allows inoculation of many plants with high efficiency at a low cost.

To assess whether PEBV-mediated VIGS can alter expression of genes involved in nodulation to an extent that gives rise to a phenotype affecting nodule formation, we chose to target the *Nodule inception gene* (*Nin*) first described in *L. japonicus* (Schauser et al. 1999). In *P. sativum*, *PsNin* was characterized in *sym35* mutants that have a non-nodulating phenotype similar to *L. japonicus nin* mutants (Borisov et al. 2003). Recently, two *MtNIN* mutant alleles were cloned and characterized in *M. truncatula* (Marsh et al. 2007). Nodules of *L. japonicus* are of the determinate type whereas *P. sativum* and *M. truncatula*

produce indeterminate nodules. In all three species, *nin* mutants are characterized by excessive root hair curling, inability to form infection threads, and, consequently, failure to form any nodules. This phenotype places the function of NIN early in the nodulation process; however, several lines of evidence indicate that NIN also functions at later steps in nodulation. *Nin* transcripts are expressed in developing and mature nodules of *L. japonicus* (Schauser et al. 1999) and in meristematic cells and cells of the infection zone in indeterminate nodules of *P. sativum* (Borisov et al. 2003). Recent studies suggest that NIN is required for bacterial entry and initiation of nodule meristems developing into root nodules (Marsh et al. 2007; Tirichine et al. 2007). The *L. japonicus Nin* gene also was implicated in nitrate regulation of nodulation (Barbulova et al. 2007) and experiments in *M. truncatula* showed expression of an ENOD11 promoter GUS fusion in a larger area of the root in *Mtnin* mutants, suggesting that NIN is involved in regulation of nodule number (Marsh et al. 2007). Here, we describe the effect of targeting *PsNin* expression by VIGS mediated by a PEBV vector carrying different fragments of *PsNin*.

RESULTS

Adaptation of the PEBV VIGS protocol to accommodate controlled symbiosis.

To target *PsNin* transcripts for silencing mediated by PEBV, the procedure described previously (Constantin et al. 2004) was modified in order to allow systemic spread of virus and initiation of silencing before *Rhizobium* spp. inoculation of the plants (Fig. 1A). Initially, the effect of PEBV infection on nodulation was determined. One set of 6 plants did not receive PEBV inoculum and another set of 10 plants were agroinoculated with pCAPE1/pCAPE2-Con, which contained a 524-bp insert from the plant virus *Bean yellow mosaic virus* (Constantin et al. 2004). Two weeks later, *Rhizobium leguminosarum* bv. *viciae* was applied and nodulation was scored after another 3 weeks. Plants that did not receive PEBV inoculum on average developed 50 nodules, with an average of 24 pink nodules. Plants that had been agroinoculated with pCAPE1/pCAPE2-Con on average developed 61 nodules, with an average of 30 pink nodules. This difference was not significant ($P > 0.4$), implying that PEBV infection did not cause major changes to the nodulation phenotype.

As a visible marker for the onset of gene silencing, *Phytoene desaturase* (*PDS*) transcripts were targeted for silencing in a set of reference plants that were agroinoculated with pCAPE1/pCAPE2-PDS (Constantin et al. 2004). As a control to monitor the effect of PEBV infection on the plants, a second set of reference plants was agroinoculated with pCAPE1/pCAPE2-Con. In plants agroinoculated with pCAPE1/pCAPE2-PDS, photo bleaching started to develop on leaves 12 to 14 days postinoculation. At that time point, an *R. leguminosarum* bv. *viciae* culture was initiated and applied a few days later. As a control for unintentional rhizobia infection, a third set of reference plants was not inoculated with *Rhizobium* spp. Three weeks post-*Rhizobium* spp. application, 30 to 150 mature pink nodules had developed on the roots of virus-uninoculated plants and plants that had been agroinoculated with pCAPE1/pCAPE2-Con, whereas plants that were not *Rhizobium* spp. inoculated did not develop nodules. At the same time point, photobleaching still was developing on plants agroinoculated with pCAPE1/pCAPE2-PDS. This protocol assured that *Rhizobium* spp. application was timed according to the establishment of VIGS in each experiment and root nodules developed to maturity along with ongoing VIGS, measured as continued bleaching of leaves on plants inoculated with pCAPE1/pCAPE2-PDS.

VIGS phenotype of constructs carrying fragments of *PsNin* cDNA.

In legumes, mutants with lesions in the *Nin* gene display a distinct phenotype that makes it a suitable target to assess the feasibility of VIGS as a tool for nodulation studies. Two nonoverlapping *PsNin* cDNA fragments corresponding to nucleotides 1,720 to 2,149 (*PsNinA*) and 2,212 to 2,647 (*PsNinB*) of AJ493066, respectively (Borisov et al. 2003), were cloned into the PEBV RNA2 vector, thereby creating the constructs pCAPE2-*PsNinA* (430 bp) and pCAPE2-*PsNinB* (436 bp), respectively (Fig. 1B). Following the protocol described above, the nodulation phenotype was assessed in two independent experiments where 20 to 30 *P. sativum* plants

Fig. 1. Application of virus-induced gene silencing to nodulation experiments in *Pisum sativum*. **A**, Seed were germinated day zero and transferred to sterile clay granules connected by a wick to a reservoir of nutrient medium without N. Two weeks after germination, plants were agroinoculated with *Pea early browning virus* (PEBV) vector cDNA constructs. Reference plants were agroinoculated with a construct targeting *P. sativum* *Phytoene desaturase* to provide a visual marker for establishment of silencing. At 12 to 14 days after agroinoculation, reference plants displayed photo bleaching and *Rhizobium* culture was started and applied to test plants approximately 30 days after sowing. Three weeks later, approximately 50 days after sowing, nodulation was analyzed. **B**, PEBV vectors with expression cassettes of RNA1 (pCAPE1) and RNA2 (pCAPE2) inserted between right and left border (RB and LB) of a binary plasmid. Transcriptional control was exerted by a 35S promoter and a NOS terminator (T). pCAPE2-*Nin* vectors contained cDNA of PEBV RNA-2 with insertions of cDNA derived from *P. sativum* *Nodule inception* gene (*PsNin*). The origin of the inserted fragments is indicated below the box illustrating the complete mRNA sequence (AJ493066). The black bar marks the region encoding the conserved RWP-RK domain (Schauser et al. 2005).

were agroinoculated with pCAPE1 in combination with pCAPE2-PsNinA, pCAPE2-PsNinB, or pCAPE2-Con. Ten plants were inoculated with pCAPE1/pCAPE2-PDS in each experiment to time the application of *Rhizobium* culture according to initiation of photobleaching as described above. The total number of nodules produced per plant was counted 3 weeks after application of *Rhizobium* spp. The absolute number of nodules formed varied markedly between the two experiments but the relative differences between the groups of plants tested were similar (Fig. 2A). On roots of plants inoculated with pCAPE1/pCAPE2-Con, the average number of nodules

per plant was 122 in the first experiment and 44 in the second experiment. On roots of plants agroinoculated with pCAPE1/pCAPE2-PsNinA, an average of 0.7 nodules was formed per plant in the first experiment and 0 in the second. On roots of plants agroinoculated with pCAPE1/pCAPE2-PsNinB, nodulation was reduced 45% or more, with an average of 34 and 24 nodules in the first and second experiment, respectively (Fig. 2A). Statistical analysis confirmed that these differences were significant at $P < 0.0001$ for pCAPE1/pCAPE2-Con versus pCAPE1/pCAPE2-PsNinA and for pCAPE1/pCAPE2-Con versus pCAPE1/pCAPE2-PsNinB, and at $P < 0.0003$ for pCAPE1/pCAPE2-PsNinA versus pCAPE1/pCAPE2-PsNinB. Roots of all plants agroinoculated with pCAPE1/pCAPE2-Con developed both pink and white nodules whereas roots on >75% of plants agroinoculated with pCAPE1/pCAPE2-PsNinB developed both pink and white nodules, 4% developed only white nodules, and 20% developed no nodules. On roots of plants agroinoculated with pCAPE1/pCAPE2-PsNinA, nodules were seen on only 15% of the plants in the first experiment.

Pink nodules can fix N whereas white nodules have not developed bacteroids capable of N fixation (Ott et al. 2005). In three experiments including only plants agroinoculated with pCAPE1/pCAPE2-PsNinB and pCAPE1/pCAPE2-Con and experiment 1.1 and 1.2 (Fig. 2A), the ratio of pink/total nodules ranged from 0.15 to 0.44 on roots of plants agroinoculated with pCAPE1/pCAPE2-PsNinB, whereas this ratio ranged from 0.44 to 0.62 on roots of plants agroinoculated with pCAPE1/pCAPE2-Con (Supplementary Fig. S1). This suggested that agroinoculation with pCAPE1/pCAPE2-PsNinB caused a greater reduction in pink nodules than in the total number of nodules.

siRNA Scan to predict off-target silencing induced by PsNinA and PsNinB.

It was not expected that agroinoculation with pCAPE1/pCAPE2-PsNinA and pCAPE1/pCAPE2-PsNinB would result in different nodulation phenotypes because the PsNin inserts were of equal size. The PsNinA fragment covered a region encoding the RWP-RK domain of NIN that is highly conserved in proteins encoded by the *Nin* gene family, whereas the PsNinB fragment covered a region with little or no conservation between proteins encoded by the *Nin* gene family (Schauser et al. 2005). Small interfering RNAs (siRNAs) derived from the trigger sequences may target transcripts of other genes (off-targets) with regions of identity or complementarity to the trigger sequences (Xu et al. 2006). It was shown that a 22-nucleotide (nt) sequence identity was sufficient to cause off-target silencing; however, genes with 21- to 22-nt identity but with one mismatch to the trigger sequence were not silenced. Therefore, it was possible that the different effect of PsNinA and PsNinB was due to off-target silencing. To predict off-targets of PsNinA and PsNinB, a sequence similarity search using the blastn program was conducted against the National Center for Biotechnology Information nucleotide collection and expressed sequence tag (EST) sequences from *P. sativum*. For PsNinA, the longest sequence of complete identity was 20 nt in the gene for chalcone synthase (X80007). For PsNinB, the longest sequence of complete identity was 17 nt in a β -galactosidase-like mRNA (DQ435678). This result probably reflected the limited sequence information available for *P. sativum*. Therefore, the corresponding *Nin* fragments from *M. truncatula* (MtNinA and MtNinB) and *L. japonicus* (LjNinA and LjNinB) were used to scan the *M. truncatula* and *L. japonicus* datasets for targets of predicted efficient siRNAs using siRNA Scan (Xu et al. 2006). Default settings were applied to search for genes with at least 21 nt identity or complementarity. With these settings, only MtNin and LjNin were

Fig. 2. Nodules developing on *Pisum sativum*. Columns display the average number of visible nodules per plant (left) and average number of pink nodules per plant (right). Bars represent 95% confidence intervals. **A**, Nodulation on roots of *P. sativum* plants agroinoculated with *Pea early browning virus* (PEBV) vectors carrying a fragment that was not expected to target any endogenous genes (Con), or fragments of *P. sativum* Nodule inception gene (*PsNin*) corresponding to nucleotides 1,720 to 2,149 (PsNinA of 430 bp) and nt 2,212 to 2,647 (PsNinB of 436 bp), respectively. The two experiments are presented separately because nodulation on control plants varied significantly between experiments **B**, Nodulation on roots of *P. sativum* plants agroinoculated with PEBV vectors carrying the fragments Con, PsNinA, PsNinB, a fragment corresponding to nucleotides 106 to 538 of *PsNin* cDNA (PsNinC of 433 bp), a 253-bp subfragment of PsNinA corresponding to nucleotides 1,897 to 2,149 (PsNinA253), or a 186-bp subfragment of PsNinA corresponding to nucleotides 1,964 to 2,149 (PsNinA186). Results of two experiments were combined because inoculation on control plants was not considered significant between experiments ($P > 0.05$).

identified as targets. An siRNA SCAN of PsNinA and PsNinB against the *Arabidopsis* dataset identified a sequence in TC255366 with 24-nt identity to PsNinA. Although these results did not predict serious off-target effects, off-target silencing cannot be ruled out. Therefore, to test whether off-target effects were responsible for the phenotype generated by PsNinA, the phenotype induced by a third fragment was analyzed because the chance that two different fragments target the same genes is low.

VIGS phenotype induced by a third fragment, PsNinC, and shorter fragments of PsNinA.

A third cDNA fragment with a size equal to PsNinA and PsNinB was cloned into pCAPE2, generating pCAPE2-PsNinC (433 bp). The PsNinC fragment covered nucleotides 106 to 538 of the *PsNin* cDNA, which encodes the N-terminus of PsNIN (Fig. 1B) that is not conserved in proteins encoded by other members of the *Nin* gene family (Schauser et al. 2005). Blastn of PsNinC against the *P. sativum* nucleotide collection and EST did not produce any hits, and siRNA Scans of the corresponding fragments in *L. japonicus* (LjNinC) and (MtNinC) identified only *LjNin* and *MtNin* as targets with at least 21 nt perfect match. In addition, to test whether the silencing phenotype induced in plants inoculated with pCAPE1/pCAPE2-PsNinA were dependent on the size of the fragment, two shorter cDNA fragments, corresponding to nucleotides 1,879 to 2,149 and 1,964 to 2,149, were cloned into the PEBV RNA2 vector, thereby creating the constructs pCAPE2-PsNinA253 (253 bp) and pCAPE2-PsNinA186 (186 bp), respectively (Fig. 1B). Because we had observed that the absolute number of nodules varied between experiments, PsNinA and PsNinB were included in two experiments that were set up to determine the nodulation phenotype induced upon agroinoculation with pCAPE1/pCAPE2-PsNinC, pCAPE1/pCAPE2-PsNinA253 and pCAPE1/pCAPE2-PsNinA186. In each experiment, nine plants were agroinoculated with each construct. Combined results (Fig. 2B) indicated that decreasing the size of the PsNinA fragment resulted in an increase in nodule formation. Statistical analysis confirmed that plants inoculated with pCAPE1/pCAPE2-PsNinA186 formed significantly more nodules ($P < 0.0004$) and more pink nodules ($P < 0.02$) than plants agroinoculated with pCAPE1/pCAPE2-PsNinA. Plants inoculated with pCAPE1/pCAPE2-PsNinA253 formed more nodules ($P < 0.05$) than plants inoculated with pCAPE1/pCAPE2-PsNinA but the difference in formation of pink nodules was not significant. Plants agroinoculated with pCAPE1/pCAPE2-PsNinC formed very few nodules and statistical analysis showed that there was no significant difference between the number of nodules ($P > 0.17$) or pink nodules ($P > 0.20$) formed on plants agroinoculated with pCAPE1/pCAPE2-PsNinA and plants agroinoculated with pCAPE1/pCAPE2-PsNinC. This result suggested that the nodulation phenotype seen upon agroinoculation of pCAPE1/pCAPE2-PsNinA and pCAPE1/pCAPE2-PsNinC was due to silencing of *PsNin*.

Root/shoot ratio and growth of plants agroinoculated with constructs PsNinA and PsNinB.

To rule out the possibility that the reduction in nodulation was caused by a reduction in root size, the fresh weight of roots was measured 3 weeks after *Rhizobium* spp. application on all plants in the first experiment. There was no difference in the average fresh weight of roots of plants inoculated with pCAPE1/pCAPE2-PsNinA or pCAPE1/pCAPE2-PsNinB compared with plants agroinoculated with pCAPE1/pCAPE2-Con (Fig. 3). This indicated that reduction in the number of root nodules observed for plants agroinoculated with pCAPE1/

pCAPE2-PsNinA or pCAPE1/pCAPE2-PsNinB was not caused by unspecific impairment of root growth.

Inhibition of nodule formation can be expected to increase N-starvation in plants growing under limiting N conditions and, thereby, lead to an increased root/shoot ratio (Davidson 1969; Levin et al. 1989). To use the root/shoot ratio as an indicator for N-starvation, shoot fresh weights were measured and root/shoot ratios calculated (Fig. 3). Plants agroinoculated with pCAPE1/pCAPE2-Con or a second control vector, pCAPE1/pCAPE2-Con*, containing a 400-bp fragment of the GUS gene, both had an average root/shoot ratio of 1.5, whereas plants agroinoculated with pCAPE1/pCAPE2-PsNinA or pCAPE1/pCAPE2-PsNinB had a significantly higher average root/shoot ratio of 2.8 (Fig. 3). Phenotypically, shoot growth was reduced in plants agroinoculated with either pCAPE1/pCAPE2-PsNinA or pCAPE1/pCAPE2-PsNinB compared with plants agroinoculated with either pCAPE1/pCAPE2-Con or pCAPE2Con* (Supplementary Fig. S2). Shoots of plants agroinoculated with pCAPE1/pCAPE2-PsNinA maintained two green fully developed leaves that were more light green due to N-starvation than shoots with three fully developed leaves seen on plants agroinoculated with pCAPE1/pCAPE2-PsNinB. Application of nutrient medium containing KNO₃ resulted in recovery of shoot growth. In general, growth on nutrient medium without N stressed the plants and the primary shoot died. Even plants agroinoculated with control construct did not receive any nitrogen supplement until pink nodules had developed 5 to 6 weeks after germination. Taken together, the significantly altered root/shoot ratios of plants agroinoculated with pCAPE1/pCAPE2-PsNinA or pCAPE1/pCAPE2-PsNinB and the phenotypes described above were consistent with an inhibition of N-fixation as would be expected by interference with nodule development.

In wild-type *P. sativum* and other nodule-forming plants, application of symbiotic bacteria results in root hair curling and formation of infection threads whereas *nin* mutants are characterized by excessive root hair curling and inability to form infection threads (Borisov et al. 2003; Marsh et al. 2007; Schauser et al. 1999). Root hairs of plants agroinoculated with pCAPE1/pCAPE2-PsNinA or pCAPE1/pCAPE2-Con were

Fig. 3. Fresh weight of roots and shoots and root/shoot ratios. Roots and shoots of plants agroinoculated with pCAPE1/pCAPE2-Con (Con), pCAPE1/pCAPE2-Con* (Con*), pCAPE1/pCAPE2-PsNinA (PsNinA), and pCAPE1/pCAPE2-PsNinB (PsNinB) were weighed 3 weeks after *Rhizobium* spp. application. Average root weight (white columns), shoot weight (black columns), and root/shoot ratios (hatched columns) of plants from two experiments; bars represent 95% confidence intervals.

inspected to determine whether agroinoculation with pCAPE1/pCAPE2-PsNinA would give rise to a phenotype reminiscent of the *nin* mutant phenotype. Few infection threads were observed in plants agroinoculated with pCAPE1/pCAPE2-PsNinA and many root hairs displayed excessive curling.

Quantification of viral RNA.

As mentioned above, it was unexpected that agroinoculation with pCAPE1/pCAPE2-PsNinA and pCAPE1/pCAPE2-PsNinB induced different nodulation phenotypes. The *PsNin* fragments PsNinA and PsNinB were of equal size and, to our knowledge, there are no previous examples that equal-sized fragments of the same gene causing significantly different phenotypes when inoculated as part of a VIGS vector. Nodulation was reduced to the same extent after agroinoculation with pCAPE1/pCAPE2-PsNinA and pCAPE1/pCAPE2-PsNinC (Fig. 2B). Therefore, targeting of a region encoding a nonconserved region of PsNIN appeared to have the same effect as targeting the PsNinA region encoding the conserved RWP-RK domain, which hypothetically could cause silencing of transcripts from several *Nin* gene family members. Instead, the PSNinB insert may affect virus accumulation and spread, leading to differences in the induction of silencing. In order to compare the amount of RNA2 derived from pCAPE1/pCAPE2-PsNinA and pCAPE1/pCAPE2-PsNinB agroinoculation, RNA2 was quantified by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) on total RNA extracted from whole roots of five plants 5 days after *Rhizobium* spp. application in two experiments (Fig. 4A). Results indicated that RNA2 derived from pCAPE2-PsNinA had accumulated to a significantly higher level than RNA2 derived from pCAPE2-PsNinB ($P < 0.05$). This may explain the difference in nodulation phenotype in plants agroinoculated with pCAPE1/pCAPE2-PsNinA and pCAPE1/pCAPE2-PsNinB, respectively. The secondary structure of the inserts may affect both virus accumulation and induction of silencing; however, analysis of the *PsNin* fragments PsNinA and PsNinB using the RNA secondary structure prediction program at GeneBee Molecular

Biology Server did not produce conclusive results. An siRNA scan (Xu et al. 2006) predicted that fragments PsNinA and PsNinB each would generate 33 efficient siRNAs, whereas PsNinC would generate 30 efficient siRNAs.

Quantification of *PsNin* transcript.

In order to determine whether the effect on nodulation was reflected in alterations of *PsNin* transcript levels, total RNA was extracted from whole roots of 5 to 10 plants agroinoculated with pCAPE1/pCAPE2-PsNinA, pCAPE1/pCAPE2-PsNinB, or pCAPE1/pCAPE2-Con. Roots were harvested either 5 or 21 days after *Rhizobium* spp. application. In control plants, *PsNin* transcription was induced and white nodules were developing 5 days after *Rhizobium* spp. application whereas, 21 days after *Rhizobium* spp. application, *PsNin* transcript levels were high, probably due to expression in mature pink nodules. Therefore, the quantification 5 days after *Rhizobium* spp. application was included to estimate the direct effect of VIGS on *PsNin* transcript levels, whereas the *PsNin* transcript levels 21 days after *Rhizobium* spp. application reflect the lack or reduction of developing and mature nodules. *PsNin* transcripts were quantified by real-time RT-PCR using primers amplifying a region 5' to the *PsNin* fragments PsNinA and PsNinB. For plants agroinoculated with pCAPE1/pCAPE2-PsNinA, *PsNin* transcript levels were significantly lower than in plants agroinoculated with pCAPE1/pCAPE2-Con at both 5 and 21 days after *Rhizobium* spp. application (Fig. 4B). For plants agroinoculated with pCAPE1/pCAPE2-PsNinB, *PsNin* transcripts levels were similar to controls 5 days after *Rhizobium* spp. application but reduced when measured 21 days after *Rhizobium* spp. application. Thus, downregulation of *PsNin* transcript appeared to occur earlier in the pCAPE2-NinA-inoculated plants than in pCAPE2-NinB-inoculated plants, correlating with the different stages at which nodule formation was inhibited by the two constructs.

DISCUSSION

The main purpose of the present study was to determine whether VIGS could affect expression of a nodulin gene to cause a change in nodulation phenotype in *P. sativum*. To address this, *PsNin* gene transcripts were targeted for degradation through agroinoculation with the PEBV cDNA vector carrying fragments of *PsNin*. When silencing was evident by bleaching of leaves in a set of reference plants agroinoculated with pCAPE1/pCAPE2-PDS, *Rhizobium* culture was applied to the growth medium (Fig. 1A). Three fragments of *PsNin* were tested and, of these, PsNinA and PsNinC (Fig. 1B) almost completely inhibited nodulation while the third fragment, PsNinB, caused a significant reduction in nodulation (Fig. 2). These results indicated that silencing induced by PEBV in roots was more efficient than in green parts, where only a fraction of the flowers were affected upon silencing of *UNIFOLLIATA* and bleaching occurred in patches on leaves upon silencing of *PsPDS* (Constantin et al. 2004). An explanation could be that PEBV is a nematode-transmitted virus (Robinson 2003) and transmission of the virus relies on root infection. However, silencing in roots by TRV, which is also nematode transmitted, was reported to be less efficient in roots compared with leaves (Bhattarai et al. 2007; Valentine et al. 2004).

The fragments PsNinA, PsNinB, and PsNinC were of similar size (430 to 436 bp). To our knowledge, it has not been observed previously that VIGS induced by fragments of similar size from the same gene result in different phenotypes. In the experiments where more than one fragment was tested, no differences were reported in phenotype or transcript reduction (Kim et al. 2005; Kim et al. 2006). Therefore, the difference

Fig. 4. Relative quantification of *Pea early browning virus* (PEBV) RNA2 and *PsNin* mRNA by real-time reverse-transcriptase polymerase chain reaction. Relative levels of PEBV RNA2, *PsNin* mRNA, and *Pisum sativum* 18S RNA were determined by amplification of cDNA derived from total RNA extracted from roots agroinoculated with pCAPE1/pCAPE2-Con (Con), pCAPE1/pCAPE2-PsNinA (PsNinA), or pCAPE1/pCAPE2-PsNinB (PsNinB). Columns display data combined from two experiments. **A**, Relative amounts of PEBV RNA2 5 days after *Rhizobium* spp. application. **B**, Relative amounts of *PsNin* mRNA 5 and 21 days after *Rhizobium* spp. application normalized to relative amounts of 18S RNA. Bars represent the 95% confidence intervals.

between fragments PsNinA and PsNinB were analyzed in more detail. We found that the difference in nodulation phenotype (Fig. 2A) developing after agroinoculation with pCAPE1/pCAPE2-PsNinA and pCAPE1/pCAPE2-PsNinB correlated with the effect on *PsNin* transcript levels (Fig. 4B). The fragment of *PsNin* carried by pCAPE2-PsNinA encodes a region of NIN which is conserved in proteins encoded by the *Nin* gene family. Therefore, it was possible that off-target silencing of genes belonging to this family caused the strong effect on nodulation observed after agroinoculation by pCAPE1/pCAPE2-PsNinA. However, this interpretation was not supported by results from agroinoculation with pCAPE1/pCAPE2-PsNinC. The 433-bp PsNinC insert does not encode a region of PsNIN that is conserved in genes encoded by other members of the *Nin* gene family (Schauser et al. 2005), and agroinoculation with pCAPE1/pCAPE2-PsNinC had an effect on nodulation comparable to agroinoculation with pCAPE1/pCAPE2-PsNinA. An siRNA Scan predicted that fragments PsNinA and PsNinB would both generate 33 efficient siRNAs, but other factors such as target accessibility may influence silencing efficiency (Xu et al. 2006). It was not possible to make conclusions concerning off-targets based on bioinformatic analysis because of the limited sequence information on *P. sativum*.

The observations that virus vectors carrying small inserts with inverted repeats are more efficient in inducing silencing (Lacomme et al. 2003) and that viral RNA with imperfect duplexes may be the predominant target of DICER (Molnár et al. 2005) suggest that differences in the secondary structure of the *PsNin* fragments could explain the observed difference in nodulation of plants agroinoculated with pCAPE1/pCAPE2-PsNinA and pCAPE1/pCAPE2-PsNinB. However, we could not identify obvious differences in secondary structure of the PsNinA and PsNinB fragments using the RNA secondary structure prediction program at GeneBee Molecular Biology Server. Instead, we found that PEBV RNA2 accumulated to significantly higher levels in plants agroinoculated with pCAPE1/pCAPE2-PsNinA than in plants agroinoculated with pCAPE1/pCAPE2-PsNinB (Fig. 4A), and this was correlated with a larger reduction in *PsNin* transcripts 5 days after *Rhizobium* spp. application (Fig. 4B). A similar correlation was observed in tomato inoculated with a TRV VIGS vector carrying a fragment of *PDS* (Rotenberg et al. 2006). This would suggest that increased virus accumulation improved VIGS and that the virus RNA was better protected from silencing than the target mRNA (Ding and Voinnet 2007). The quantification of PEBV RNA2 showed that, in plants agroinoculated with pCAPE1/pCAPE2-Con, RNA2 accumulated to even higher levels than in plants agroinoculated with pCAPE1/pCAPE2-PsNinA (Fig. 4A). The difference in RNA2 accumulation cannot be explained by differences in the length of the heterologous inserts in pCAPE2-Con, pCAPE2-PsNinA, and pCAPE2-PsNinB and, as in other systems, there does not seem to be a simple correlation between fragment length, virus accumulation, and silencing phenotype (Bruun-Rasmussen et al. 2007; Thomas et al. 2001).

Nin transcripts are highly expressed in developing nodules, suggesting that NIN is required not only at nodule inception but also at later stages of nodule development (Borisov et al. 2003; Schauser et al. 1999). Possibly, the weaker phenotype observed after agroinoculation with pCAPE1/pCAPE2-PsNinB, with a 45 to 70% reduction in visible nodules, can be used for uncovering later functions of NIN. With our present knowledge, we cannot accurately predict the silencing efficiency of a VIGS construct; however, reducing the length of the insert from 430 bp in pCAPE2-PsNinA to 253 bp in pCAPE2-PsNinA253 and 186 bp in pCAPE2-PsNinA186 weakened the

phenotype (Fig. 2B). Still, the total number of nodules was significantly ($P < 0.01$) lower after agroinoculation with pCAPE1/pCAPE2-PsNin186 than after agroinoculation with pCAPE1/pCAPE2-PsNinB, demonstrating that factors other than length were important.

The plants that were agroinoculated with pCAPE1/pCAPE2-PsNin constructs showed clear signs of N starvation, reflected in significantly increased root/shoot ratios compared with plants agroinoculated with pCAPE1/pCAPE2-Con (Fig. 3) (Davidson 1969; Levin et al. 1989). The plants inoculated with pCAPE1/pCAPE2-PsNinB developed nodules and the plants appeared equally N starved as plants inoculated with pCAPE1/pCAPE2-PsNinA, judged from the root/shoot ratio (Fig. 3). However, plants agroinoculated with pCAPE1/pCAPE2-PsNinA maintained only two green leaves compared with three in plants agroinoculated with pCAPE1/pCAPE2-PsNinB, suggesting that the nodules on these plants did provide some N.

Though we cannot explain why agroinoculation with pCAPE1/pCAPE2-PsNinA and pCAPE1/pCAPE2-PsNinC induces a stronger phenotype than agroinoculation with pCAPE1/pCAPE2-PsNinB, our results suggested that it may be possible to modulate silencing strength by choice of fragment as well as by altering the fragment length. Here, agroinoculation with pCAPE1/pCAPE2-PsNinA and pCAPE1/pCAPE2-PsNinC gave silencing phenotypes resembling the mutant *sym35* non-nodulating phenotype (Borisov et al. 2003) whereas agroinoculation with pCAPE1/pCAPE2-PsNinB, pCAPE1/pCAPE2-PsNin186, and pCAPE1/pCAPE2-PsNin253 resulted in a 'leaky' phenotype. An advantage provided by VIGS is the possibility to analyze the effect of targeting a single gene in the same genetic background, in contrast to mutant studies where backcrossing may be needed to eliminate other mutations or T-DNA inserts. In *P. sativum*, application of VIGS can facilitate studies of gene function despite the large genome and recalcitrance to transformation, and makes it possible to take advantage of the knowledge generated previously on nodulation in this species.

MATERIALS AND METHODS

Plants growth and bacterial strains.

P. sativum cv. Dark Skinned Perfection (Dæhnfeldt, Odense, Denmark) plants were used in all experiments. To grow plants under sterile conditions, seed were surface sterilized in 1.5% hypochlorite for 20 min, rinsed in sterile water, and placed for germination for 2 days in darkness at room temperature. Germinated seed were transferred to clay granules (Fiboklinker from Maxit, Risskov, Denmark) that had been autoclaved in a semiclosed growth system composed of two Magenta boxes (Sigma, Brøndby, Denmark), with the lower box containing N-free Jensen medium (van Brussel et al. 1982) and a cotton wick from the top box to the bottom box. Plants were grown in climate chambers with 16- and 8-h photoperiod at 200 μ E, 20°C, and 75% humidity.

Plants were agroinoculated 2 weeks after germination as described by Constantin and associates (2004), except that gentamicin (Sigma) at 25 μ g ml⁻¹ replaced rifampicin in the selection media for the bacteria. *R. leguminosarum* bv. *viceae* strain 248 (Josey et al. 1979) was grown to an optical density at 660 of 0.3 to 0.5 in YMB medium (Handberg et al. 1994). For *Rhizobium* spp. inoculation, the culture was diluted 1:19 in Jensen medium and 150 ml was applied to the upper magenta box. Medium containing N consisted of Jensen medium with 1 mM KNO₃.

Secondary lateral roots were inspected in the root hair growth zone using a Leitz Laborlux S Microscope (Leica, Herlev, Denmark) and photographs were taken using Nikon Coolpix 4500 (Nikon, Copenhagen, Denmark). Root fragments were col-

lected from three plants agroinoculated with pCAPE1/pCAPE2-PsNinA or pCAPE1/pCAPE2-Con 3 weeks after *Rhizobium* spp. application. Root sections were stained in 0.05% toluidine blue (Merck, Glostrup, Denmark).

Cloning of silencing constructs.

The binary vectors pCAPE1 and pCAPE2 containing cDNA of PEBV RNA1 and RNA2, respectively, were manipulated by standard cloning methods. Restriction and modifying enzymes (Roche Diagnostics, Mannheim, Germany and New England Biolabs, Ipswich, MA, U.S.A) and *Taq* DNA polymerase (GibcoBRL, Roskilde, Denmark) were used according to the manufacturers' instructions. PCR products were cloned using TOPO-technology (Invitrogen, Taastrup, Denmark). Oligonucleotide primers were provided by MWG (Ebersberg, Germany). To target *PsNin* for VIGS, fragments of *PsNin* cDNA from *P. sativum* cv. Finale (AJ493066) (Borisov et al. 2003) were inserted into pCAPE2-PDS (Constantin et al. 2004), replacing the *PDS* gene fragment. The PsNinA fragment of 430 bp (nucleotides 1,720 to 2,149) was amplified by PCR using forward primer 5'-cactagTTCAGTCTTTTCAGA and reverse primer 5'-GCTGCAGTGCTCAACTCTGGA and inserted as a *SpeI/PstI* fragment generating pCAPE2-PsNinA. The PsNinA253 fragment of 253 bp (nucleotides 1,897 to 2,149) was amplified by PCR using forward primer 5'-ccatggCATAA GCTTGCTGTCTAA and reverse primer 5'-GCTGCAGTGCTCAACTCTGGA and inserted as an *NcoI/PstI* fragment generating pCAPE2-PsNinA253. The PsNinA186 fragment of 186 bp (nucleotides 1,964 to 2,149) was amplified by PCR using forward primer 5'-ccatggGTATGTCCCTACAACTCTGA and reverse primer 5'-GCTGCAGTGCTCAACTCTGGA and inserted as an *NcoI/PstI* fragment generating pCAPE2-PsNinA186. The PsNinB fragment of 436 bp (nucleotides 2,212 to 2,647) was excised as an *NcoI/PstI* fragment and inserted into pCAPE2-PDS generating pCAPE2-PsNinB. The PsNinC fragment of 433 bp (nucleotides 106 to 538) was amplified by PCR using forward primer 5'-gactagtctGGAATATGGTGGT GGGATAGTGGA and reverse primer 5'-gaagatcttCTGCTGA AGATAATGAATTGCCGA and inserted as an *SpeI/BglII* fragment generating pCAPE2-PsNinC. Two control constructs were used in the experiments: pCAPE2-Con (Constantin et al. 2004) and pCAPE2-Con* containing a 400-bp fragment of the *GUS* gene amplified with forward primer 5'-ccatggTTTAACGA TCAGTTCGCCGATGCAGAT and reverse primer 5'-ctgcagCA CCCAGGTGTTTCGGCGTGGTGA and inserted as an *NcoI/PstI* fragment into pCAPE2-PDS replacing the *PDS* gene.

The three fragments PsNinA, PsNinB, and PsNinC all were analyzed by the siRNA Scan program (Xu et al. 2007) to determine the number of efficient siRNAs predicted from each fragment.

Real-time RT-PCR.

Samples were collected from five randomly selected plants per treatment per experiment. For each sample, total RNA was extracted from 100 mg of root tissue using RNeasy plant mini kit (Qiagen, Hilden, Germany) with on-column DNase treatment following the manufacturer's recommendations. An additional DNase treatment was performed for 15 min at 37°C with 0.8 U of RQ1 RNase-free DNase (Promega Corp., Madison, WI, U.S.A.) per 40 µl of RNA eluate. The DNase reaction was terminated by addition of RQ1 DNase Stop Solution (Promega Corp.) and subsequent incubation for 10 min at 65°C. RNA concentration was measured on a Nanodrop ND-1000 Spectrophotometer (Saveen Werner, Malmö, Sweden) and 0.5 µg was used for first-strand cDNA synthesis with random hexamer primers for the RT reaction, as described previously (Constantin et al. 2004).

For relative quantification of RNA by real-time RT-PCR, 18S (U43011) primers 18SF 5'-GACTACGTCCCTGCCCT TTG (nucleotides 1,571 to 1,590) and 18SRV 5'-AACACTT CACCGGACCATTCA (nucleotides 1,618 to 1,638), PEBV RNA2 (X78455) primers PE2FW 5'-TCGGTTTGCTGACCT ACTGG (nucleotides 461 to 480) and PE2RV 5'-CCACTTC GACCCAAGCACTA (nucleotides 566 to 585), and PsNin (AJ493066) primers NinFW 5'-GGATTCCAAGAAGCTTGC AG (nucleotides 1,094 to 1,113) and NinRV 5'-CTACACTGG CTCTGCAGCTG (nucleotides 1,282 to 1,301) were designed using Primer3 (Rozen and Skaletsky 2000). Real-time PCR was performed using the 7500 Real Time PCR System (Applied Biosystems, Naerum, Denmark). Each 20 µl of PCR reaction contained 4 µl of RT reaction, 12 µl of SYBR Green Master Mix (Applied Biosystems), and 500 nM each primer. Samples were heated to 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each RT reaction was analyzed in duplicate. The 7500 System Software program calculated relative amounts of RNA based on PCR cycle threshold values obtained from a fourfold dilution series of a standard RT sample from a plant inoculated with pCAPE1/pCAPE2-Con. Calculated relative amounts of target gene RNA were normalized by calculated relative amounts of 18S RNA.

Statistical analysis.

Data were analyzed by InStat 3.0 software (GraphPad Software, San Diego, CA, U.S.A.). The nonparametric Mann-Whitney test was applied to analysis of nodule numbers presented in Figure 2. Other statistical analyses were performed using an unpaired *t* test. Data without a normal distribution were transformed by converting to a natural logarithm.

ACKNOWLEDGMENTS

We thank B. Olsen and M. Læssøe Steffensen for expert technical assistance with laboratory work and maintenance of plants in the greenhouse. This work was supported by the Danish Agricultural and Veterinary Research Council (grant no. 53-00-0330 to O. S. Lund and grant no. 23-03-0118 to M. Grønlund) and by The Danish National Research Foundation (Ph.D. stipend to G. Constantin).

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AUTHOR RECOMMENDED INTERNET RESOURCES

GeneBee Molecular Biology server: www.genebee.msu.su/genebee.html
 Samuel Roberts Noble Foundation's SiRNA Scan:
bioinfo2.noble.org/rnaiscan.htm
 Association of Applied Biologists, Descriptions of Plant Viruses website:
www.dpvweb.net/dpv/index.php