Sinorhizobium medicae WSM419 genes that improve symbiosis between *Sinorhizobium meliloti* Rm1021 and *Medicago truncatula* Jemalong A17 and in other symbiotic systems

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S. medicae genes improve *S. meliloti* symbiosis

KEY WORDS

symbiosis, nitrogen fixation, root nodules, model legume, *Medicago truncatula*, *Sinorhizobium meliloti* Rm1021, *Sinorhizobium medicae* WSM419, alfalfa, autoregulation of nodulation (AON)

ABSTRACT

Some soil bacteria called rhizobia can interact symbiotically with legumes in which they form nodules on the plant roots where they can reduce atmospheric dinitrogen to ammonia, a form of nitrogen that can be used by growing plants. Rhizobia/plant combinations can differ in how successful this symbiosis is—*Sinorhizobium meliloti* Rm1021 forms a relatively ineffective symbiosis with *Medicago truncatula* Jemalong A17 but *Sinorhizobium medicae* WSM419 is able to support more vigorous plant growth. Using proteomic data from free-living and symbiotic *S. medicae* WSM419, we previously identified a subset of proteins that were not closely related to any *S. meliloti* Rm1021 proteins and speculated that adding one or more of these proteins to *S. meliloti* Rm1021 would increase its effectiveness on *M. truncatula* A17. Three genes, Smed_3503, Smed_5985, and Smed_6456, were cloned into *S. meliloti* Rm1021 downstream of the *E. coli lacZ* promoter. Strains with these genes increased nodulation and improved plant growth, individually and in combination with one another. Smed_3503, renamed *iseA* (increased symbiotic effectiveness) had the largest impact, increasing *M. truncatula* biomass by 61%. *iseA* homologs were present in all currently sequenced *S. medicae* strains but were infrequent in other *Sinorhizobium* isolates. *Rhizobium leguminosarum* bv. *viciae* 3841 containing *iseA* led to more nodules on pea and lentil. Split root experiments with *M. truncatula* A17 indicated that *S. meliloti* Rm1021 carrying the *S. medicae iseA* is less sensitive to plant induced resistance to rhizobial infection, suggesting an interaction with the plant's regulation of nodule formation.

IMPORTANCE

The legume symbiosis with rhizobia is highly specific. Rhizobia that can nodulate and fix nitrogen on one legume species are often unable to associate with a different species. The interaction can be more subtle—symbiotically enhanced growth of the host plant can differ

47 substantially when nodules are formed by different rhizobial isolates of a species, much like
48 disease severity can differ when conspecific isolates of pathogenic bacteria infect different
49 cultivars. Much is known about bacterial genes essential for a productive symbiosis, but less is
50 understood about genes that marginally improve performance. We used a proteomic strategy to
51 identify *Sinorhizobium* genes that contribute to plant growth differences that are seen when two
52 different strains nodulate *M. truncatula* A17. These genes could also alter the symbiosis between
53 *R. leguminosarum* bv. *viciae* 3841 and pea or lentil, suggesting that this approach may identify
54 new genes that may more generally contribute to symbiotic productivity.
55

56 **INTRODUCTION**

57 Nitrogen is an essential element for plant growth and production. Limited access to bioavailable
58 nitrogen restricts crop productivity and thus food production. However, nitrogen fertilizers are
59 expensive and their use causes additional environmental problems (1, 2). Many legume plants
60 can satisfy their need for nitrogen by establishing a symbiotic association with nitrogen-fixing
61 soil bacteria called rhizobia. In these symbiotic interactions, the rhizobia reduce (fix)
62 atmospheric nitrogen gas to ammonium and release this to the plant. Symbiotic nitrogen fixation
63 (SNF) is a major input of nitrogen into ecosystems. Most current SNF in agricultural ecosystems
64 involves legumes but there is significant interest in the possibility that associative bacterial
65 nitrogen fixation can be established for other crop plants (3).
66 Legume SNF takes place in nodules, novel organs formed on the roots after bacterial infection
67 that provide the specialized environment needed for nitrogen fixation. This environment includes
68 carbon compounds that are used to provide energy and reductant for nitrogen fixation and
69 microaerobic conditions compatible with nitrogenase activity and the active bacterial respiration

needed to generate ATP for the reaction. Forming nodules requires a developmental process based on mutual recognition between the plant and the bacteria that depends on exchanging specific signaling molecules (4–8).

A major goal of nitrogen-fixing symbiosis research is to understand how to increase plant productivity. Increased bacterial ammonium production is clearly a major component of this. However, developing a more productive symbiosis is not simply a matter of choosing a "best" rhizobia and a "best" plant host—matching the bacteria with its host plays an important role in the symbiosis and symbiotic productivity can be limited by sub-optimum interactions in nodule formation, nodule development and the metabolism of nitrogen fixation.

The *Sinorhizobium meliloti* Rm1021 symbiosis with *Medicago truncatula* (barrel medic) Jemalong A17 is one model system for studying symbiosis (6) because of the availability of the genome sequences of the symbionts, transcriptomic and proteomic data of the *Sinorhizobium* symbiont and its host under various conditions, a large-scale plant mutant collection and the ability to manipulate the plant and bacterial genetics (6–8). However, *S. meliloti* Rm1021 was originally studied in the context of its success in interacting with *Medicago sativa* (alfalfa) and it does not form a very effective symbiosis with *M. truncatula* A17 (9–11). In contrast, *Sinorhizobium medicae* WSM419 forms an effective symbiosis with *M. truncatula* A17 but is less effective than *S. meliloti* Rm1021 on *M. sativa*. Thus, these reciprocal differences in performance are not due to a general symbiotic defect in any of the four symbionts but results from problems in a specific paired context. *S. medicae* WSM419 has also been reported to have *M. truncatula* cultivar specific effectiveness—it is more effective with the *M. truncatula* A17 than it is on the *M. truncatula* subsp. *tricycla* cultivar R108 (10). There are physiological differences between *M. truncatula* A17 nodules formed by *S. medicae* WSM419 and *S. meliloti*

Rm1021 (9), but little is known about the genes that make a difference in the higher symbiotic production of *S. medicae* WSM419 on *M. truncatula* A17.

In previous work, a deep proteome of *S. medicae* WSM419 was generated by studying the bacteria in free-living culture and symbiotic association with *M. truncatula* A17 [Yurgel SN, Qu Y, Rice JT, Zink EM, Brown JN, Lipton MS, Kahn ML. Metabolic specialization in a nitrogen-fixing symbiosis: proteome differences between *Sinorhizobium medicae* bacteria and bacteroids. (submitted for publication)]. 1,872 *S. medicae* WSM419 proteins were identified using mass spectrometry. *S. medicae* WSM419 proteins typically have a high level of similarity (>90% identity) to their *S. meliloti* homologs (12) but a subset of 83 unique proteins were identified in this *S. medicae* WSM419 proteome that did not have this high level of similarity with any *S. meliloti* Rm1021 protein. 56 of these were detected only in free-living cells, 9 were nodule specific and 18 were found under both conditions. We speculated that some of these proteins might contribute to the higher symbiotic productivity of *S. medicae* WSM419 on *M. truncatula* A17 and that, by moving the genes coding for these proteins into *S. meliloti* Rm1021, we might improve *S. meliloti* Rm1021 symbiotic production with *M. truncatula* A17.

We selected three genes to test this idea based on their annotated functions potentially relevant to symbiosis and the relative abundance of their proteins in the free-living and symbiotic proteomes. All three of the proteins were found in free-living cells of *S. medicae* WSM419 and, at a lower level, in nodules. These included an annotated ACC deaminase that had the potential to interfere with the production of ethylene, an annotated 1-4-α-glucan glucosidase that might interact with exopolysaccharide biosynthesis and processing, and a gene we call *iseA* that is annotated as encoding a glyoxalase/dioxygenase/bleomycin resistance protein (12). The genes were expressed individually in *S. meliloti* Rm1021 strains and all three strains increased the

116 growth of *M. truncatula* A17 when inoculated plants were compared to plants inoculated with
117 isogenic bacteria containing the empty expression vector. Using compatible plasmids to carry the
118 genes, we co-expressed them in combinations and, in some cases, strains having more than one
119 gene slightly increased dry weight in comparison to strains containing only one. The genes, and
120 especially *iseA*, also stimulated nodulation of *R. leguminosarum* bv. *viciae* 3841 on lentil or pea.

121 Nitrogen fixation is a highly energy demanding process and therefore tightly regulated by the
122 host plant to keep a balance between nitrogen acquisition and energy expenditure. Many legumes
123 control their nodule number after the initial infection by a negative regulatory long-distance
124 (systemic) pathway known as autoregulation of nodulation (AON) pathway (13). Nodulation in
125 split root systems was influenced by the presence of *iseA* in a pattern that suggests that *iseA*
126 might interfere with the AON response (13, 14). These results are consistent with *iseA*
127 overcoming or bypassing the AON response and we suggest that this leads to increased
128 nodulation.
129
130 **RESULTS**
131 **Adding genes from *S. medicae* WSM419 to *S. meliloti* Rm1021 increased symbiotic biomass**
132 **production on *M. truncatula* A17**
133 To analyze the role of *iseA*, Smed_5985, and Smed_6456 in symbiosis with *M. truncatula* A17,
134 each of the genes was cloned downstream of the *lacZ* promoter in two compatible broad host
135 range cloning vehicles, pCPP30 (Tc^R) and pSRKGm (Gm^R), that could be conjugated into *S.*
136 *meliloti* Rm1021 (Table 1). The *lacZ* promoter is considered to be a relatively strong promoter in
137 *Sinorhizobium* (15), although in pSRKGm the LacI repressor protein is also present. We used
138 these plasmids to construct strains carrying each gene and the genes in different pairwise

combinations to test whether there might be additive effects on symbiosis (Table S1). Plant tests also included the strains carrying both of the parent plasmids, pCPP30 and pSRKGm, as vector controls. Shoot dry weight and nodule number are often correlated with the benefit host plants derive from the symbiosis in the field so we limited our analysis to these measures. At 28 days post inoculation (dpi), the average increase in shoot dry weight of plants inoculated with strains containing *iseA*, Smed_5985, and Smed_6456 had higher biomasses (~61%, 24% and ~35%, respectively) when compared with plants inoculated with strains containing the vector lacking an insert (Fig. 1, Fig. S1, Table S2). When both plasmids carried the same gene, growth was slightly higher than when only one of the plasmids contained that gene. When the two plasmids carried different genes, the pairwise combinations of *iseA* and Smed_6456 and of Smed_5985 and Smed_6456 led to slightly higher plant biomass than these genes generated individually (Fig. 1, Table S2). None of the recombinant strains led to growth that was equal to growth when *S. medicae* WSM419 was used as an inoculant.

Nitrogen-fixing root nodules (Fix^+) are pink because they contain leghemoglobin, a red protein that becomes visible as the nodule matures and indicates that the microaerobic environment necessary for nitrogen fixation is being established (16). White nodules are immature or aborted (Fix^-). White and pink nodules were counted at 28 dpi on the roots of plants. Plants inoculated with *S. meliloti* Rm1021 expressing the candidate genes individually or co-expressed with each other had significantly more pink nodules than those inoculated with the empty vector control (Table 2). The proportion of white nodules on plants inoculated with *S. meliloti* Rm1021 (pCPP30-*iseA*) was lower than on plants inoculated with the vector control. The three candidate genes (*iseA*, Smed_5985, and Smed_6456) caused an average increase in the number of pink nodules on *M. truncatula* A17 by ~57%, ~36% and ~50%, respectively, when compared with

plants inoculated with the vector controls. We also observed an increase in the number of pink nodules as a result of stacking the target genes in different combinations (Table 2).

Expression pattern of *S. medicae* WSM419 genes

To study expression of the three candidate genes, their potential promoter regions were cloned upstream of the *uidA* gene, which codes for a β-glucuronidase (GUS). Similar fusions were also constructed with the promoter from the *hrrP* gene (P*hrrP*), which has nodule-specific expression (17) and with the promoter (P*fla*) from the *S. medicae* WSM419 Smed_0266 flagellin gene, which was expected to be downregulated within nodules (17). The promoter fusion clones were transformed into *S. meliloti* Rm1021 and tested for *in vitro* and *in vivo* promoter activity. Visible GUS activity was observed in 10 dpi *M. truncatula* A17 root nodules for each of the promoters (*iseA*, Smed_5985 and Smed_6456) (Fig. 2A). In controls, P*hrrP* had prominent GUS expression whereas P*fla* had a very low level of GUS expression that was localized in a small part of the root nodules. In free-living cells, P*fla* was strongly expressed as was the *Salmonella trp* (P*trp*) promoter, which is constitutive in *S. meliloti* Rm1021 (18). The *iseA* promoter (P*iseA*) was expressed at low levels in free-living cells but its expression was higher in minimal media than in complex (LB) media. Expression from the Smed_5985 and Smed_6456 promoters was almost undetectable in the free-living cells (Fig. 2B), a somewhat surprising result because the corresponding proteins were present at a higher level than IseA in the proteomic experiments with free-living *S. medicae* cells.

None of the *S. medicae* WSM419 genes made a major difference in the *S. meliloti* Rm1021 symbiosis with *M. sativa*

M. sativa (alfalfa) has an effective relationship with *S. meliloti* Rm1021 but is only partially effective with *S. medicae* WSM419 (11). We tested whether adding the *S. medicae* WSM419

genes to *S. meliloti* Rm1021, expressed in either pCPP30 or pSRKGm, would increase the growth of alfalfa. Compared to the vector control, plant dry weight and the number of nodules per plant did not change significantly when *S. meliloti* Rm1021 carried any of the three candidate genes (Fig. 3A, B). This may mean that these genes do not contribute to the symbiotic interaction of *S. meliloti* Rm1021 with alfalfa, but it may also be that the symbiosis was already very effective and a marginal contribution to growth was not observable. We note that Ma et al. (18) had previously reported that adding an ACC deaminase to Sm1021 improved the nodulation and biomass in the alfalfa symbiosis.

iseA from _S. medicae_ WSM419 is important for nodulation in _M. truncatula_ A17

The previous experiments show that *iseA* improved plant biomass production in the *S. meliloti* Rm1021 interaction with *M. truncatula* A17 but had little effect on the interaction of *S. meliloti* Rm1021 with *M. sativa.* To examine whether *iseA* is important in the interaction of *S. medicae* WSM419 with *M. truncatula* A17, a *S. medicae* WSM419 *iseA* deletion mutation was constructed using a double recombination marker exchange protocol. *S. medicae* WSM419Δ*iseA* formed a significantly lower number of nodules than *S. medicae* WSM419 wild type on *M. truncatula* A17 and the nodulated plants did not grow as well (Fig. 4). WSM419Δ*iseA* (pCPP30-*iseA*), a strain in which *S. medicae* WSM419Δ*iseA* was complemented with a constitutively expressed version of *iseA* carried on pCPP30, generated a normal number of nodules and restored the plant dry weight to the wildtype level (Fig. 4). These results show that *iseA* contributes to an effective symbiosis between *S. medicae* WSM419 and *M. truncatula* A17**.**

Homologs of the three genes are not distributed evenly in _Sinorhizobium_ strains.

Deploying ACC decarboxylase to increase nodulation is a fairly widespread strategy in rhizobia. We investigated whether homologs to the three genes described here are present in species of

Sinorhizobium using DNA sequence information in the Joint Genome Initiative database (19). The results are shown in Table 3. All 31 isolates of *S. medicae* had genes with very high similarity to the *S. medicae* WSM419 *iseA* gene but only 11 of the other 112 *Sinorhizobium* isolates in the database had *iseA* homologs with sequence identity over 80%. However, 95 of these had some gene with sequence similarity that generated an E value lower than -5, the threshold for our search. Interestingly, seven of the unclassified species isolates had two genes with this lower level of homology, a pattern that was also seen in one of the *S. fredii* isolates. Thus, *iseA* is characteristic of *S. medicae* and found much less frequently elsewhere in *Sinorhizobium*.

Genes with a high degree of similarity to the Smed_5985 glycanase are common in *S. medicae*, occurring in 27 of the 31 strains, and less common in *S. meliloti*, occurring in 12 of the 73 strains. The search for genes similar to the Smed_5985 glycanase also identified several genes with E-values between -07 and -20 that were annotated as thiamine pyrophosphate-dependent enzymes or indolepyruvate decarboxylases in 14 and 7 of the *S. medicae* strains, respectively. In several cases, two adjacent annotated genes had significant similarity to adjacent domains of Smed_5985, suggesting either frameshift mutations or sequencing errors in the original determination. The “break points” in the predicted frameshift sequences were similarly placed in the Smed_5985 alignments, suggesting that these two differences are due to frameshift mutations and were not random errors. Only one of the *S. meliloti* sequences had a similar arrangement and only one strain had an annotated gene with the lower E-value. We again conclude that Smed_5985 is very common in *S. medicae* and less common in *S. meliloti.* One match (E~-47) was found in the single *S. saheli* isolate in the database, but no similar genes were found in the other *Sinorhizobium* strains.

The distribution of genes with similarity to the Smed_6456 ACC deaminase (ACCdA) was interesting. In some species, nearly all of the identified genes had very high similarity to Smed_6456, while in other species genes with significant similarity were found that appear to belong to variant groups and were annotated as D-cysteine desulfhydratases (CysD) or tryptophan synthases (beta chain) (TrpSb). These two groups were distinct in their degree of similarity. As shown in Table 3, the proportion of these annotated groups was distinct by species, even though the same BLAST operation was carried out on each isolate. So, for example, of the 31 *S. medicae* isolates, 10 have genes annotated as ACCdA and 15 have genes annotated as CysD. Some strains, like *S. medicae* WSM419, have both of these genes and some have one or the other. In contrast, 47 of the 71 *S. meliloti* isolates have ACCdA and the 2 that have CysD do not have ACCdA. We have no explanation for these patterns, but the distribution was clearly not random with respect to species.

iseA and Smed_6456 alter the _Rhizobium leguminosarum_ bv. _viciae_ 3841 symbiosis with _Pisum sativa_ (pea) and _Lens culinaris_ (lentil)

To investigate whether the *S. medicae* WSM419 genes could affect efficiency of another symbiotic system, *iseA*, Smed_5985, and Smed_6456 were expressed in *R. leguminosarum* bv. *viciae* 3841 (20) and used to inoculate pea and lentil under greenhouse conditions. There are no close matches in *R. leguminosarum* 3841 to *iseA* or Smed_5985, although we found genes in other *R. leguminosarum* species that have up to 86% identity using search parameters like those above. Smed_6456 has a very close match (98% identity) in *R. leguminosarum* 3841. Growth and nodulation of pea plants were evaluated at 5 weeks post inoculation. *iseA* and Smed_6456 significantly increased the number of nodules on the pea plants, by 45% and ~33% when

253 compared with vector control (Fig. 5A). However, only *iseA* increased pea plant biomass in
254 comparison to the vector control (Fig. 5B).
255 The WSM419 genes were also tested on two lentil cultivars, Pardina and Avondale, by
256 inoculating them with *R. leguminosarum* bv. *viciae* 3841 carrying *iseA*, Smed_5985, or
257 Smed_6456 expressed in pCPP30. Plant dry weight and number of nodules were determined at 5
258 weeks post inoculation. *iseA* increased both the nodule and dry weight of Avondale (Fig. 5C, D)
259 but, while there was some stimulation, the other two genes did not significantly ($p \leq 0.05$)
260 increase these parameters under the statistical tests used. None of the three candidate genes
261 increased yield or nodulation on Pardina by a statistically significant amount ($p \leq 0.05$), although
262 the number of nodules formed when the genes were present was somewhat higher (Fig. S2). The
263 interaction of *iseA* with both pea and lentil nodulation shows *iseA* can have an effect that is not
264 specific to *Medicago* or to *Sinorhizobium*. This implies that their effect is likely to be on some
265 underlying process common to nodulation of these plants, in contrast to highly host specific
266 determinants like lipochitooligosaccharides.
267 **_S. meliloti_ Rm1021 containing _iseA_ was less sensitive to autoregulation of nodulation (AON)**
268 After the initial infection, many legumes limit the number of nodules that are formed on their
269 roots by inducing a systemic AON response (13). To explore the idea of whether *iseA* might
270 increase the nodule number by decreasing the impact of AON, we tested whether *iseA* affected
271 the response of *S. meliloti* Rm1021 to AON by setting up a split root experiment with *M.*
272 *truncatula* A17. In this type of assay, infecting one side of a divided root system decreases the
273 number of nodules formed on the other side when the second side is infected after a delay long
274 enough for signals from the first side to reach it (21). The data (Fig. 6) showed that when the first
275 roots were inoculated with *S. meliloti* Rm1021 (pCPP30), fewer nodules were formed on the

roots inoculated with Rm1021 (pCPP30) after a delay of 3 days. However, when the second root was inoculated with *S. meliloti* Rm1021 (pCPP30-*iseA*) at the delayed time point, the number of nodules formed was still almost as high as a normal infection by a strain that contained *iseA*. These results suggest strains containing *iseA* may partially overcome AON or be less sensitive to it. We also performed both the early and delayed inoculation with *S. meliloti* Rm1021 (pCPP30-*iseA*), where we observed a reduced number of nodules formed after the delayed infection. Suppression of nodulation on the second root inoculated with *S. meliloti* Rm1021 (pCPP30) during the delayed infection after the first set was infected with *S. meliloti* Rm1021 (pCPP30-*iseA*) suggests that *iseA* can also induce AON. When both the early and delayed inoculation used *S. meliloti* Rm1021 (pCPP30-*iseA*), there was a reduced number of nodules formed after the delayed inoculation, but the number of nodules was significantly higher than the nodules formed after delayed infection with *S. meliloti* Rm1021 (pCPP30).

DISCUSSION

Forming a successful nitrogen-fixing symbiosis between rhizobia and a legume requires the two symbiotic partners to be compatible with each other throughout the process of infection and nodule development. This is particularly obvious for interactions where both the plant and bacteria are capable, in the sense that each can form a productive symbiosis with some partner, but have difficulty forming a productive symbiosis with other, closely related partners. We took advantage of the known interactions between two *Sinorhizobium* species and two *Medicago* species in which one set of interactions [{*S. medicae/ M. truncatula*} and {*S. meliloti/ M. sativa*}] is very effective but the reciprocal set [{*S. meliloti/ M. truncatula*} and {*S. medicae/ M. sativa*}] is only partially effective (10, 11). Nitrogen fixation still occurs in the partially effective pairings but the nodulated plants show clear signs of nitrogen stress. Because each symbiont can

do well in one relationship, the lower effectiveness with the other partner is clearly due to some mismatch in the host-symbiont interaction. The availability of genetic resources for all the four organisms makes this an appropriate model system to study host-symbiont interactions and to investigate the implications of effectiveness on the evolutionary and ecological development of this type of symbiosis.

We had previously shown [Yurgel SN, Qu Y, Rice JT, Zink EM, Brown JN, Lipton MS, Kahn ML. Metabolic specialization in a nitrogen-fixing symbiosis: proteome differences between *Sinorhizobium medicae* bacteria and bacteroids. (submitted)] that 83 *S. medicae* WSM419 proteins expressed in *S. medicae* WSM419 did not have close homologs in *S. meliloti* Rm1021. We tested three candidate *S. medicae* WSM419 genes—*iseA*, Smed_5985, and Smed_6456—for their effect on the less productive *S. meliloti* Rm1021/*M. truncatula* A17 interaction by expressing them in *S. meliloti* Rm1021 individually and in combination and found that all three stimulated nodulation and growth. The genes significantly increased the dry weight and the number of pink (Fix^+) nodules *S. meliloti* Rm1021 formed on *M. truncatula* A17. Learning the exact mechanism by which these genes function when expressed in *S. meliloti* Rm1021 is beyond the scope of this study. However, hypothetical connections can be made between two of these three genes, annotated as coding for a 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Smed_6456) and coding for a glucan-1-4-α-glucosidase (Smed_5985), since ethylene suppression and polysaccharide production and modification are two known elements in nodule formation. There does not appear to be a precedent in the literature for an *iseA*-like gene, which is annotated in *S. medicae* WSM419 as coding for glyoxalase/bleomycin resistance protein/dioxygenase protein, influencing the symbiosis. *iseA* is especially interesting since it is

present in all 31 of the *S. medicae* sequences in the Joint Genome Initiative (JGI) database but in few other sinorhizobia.

ACC deaminases can degrade ACC, the immediate precursor of ethylene. In legumes, the phytohormone ethylene mediates an induced defense response and induction of ethylene inhibits nodule formation (22–24). Many rhizobia have developed ways of interfering with ethylene signaling as a way of increasing their nodulation ability, such as making ACC deaminase, which cleaves ACC (25). *S. meliloti* Rm1021 does not make an ACC deaminase but adding the *R. leguminosarum* bv. *viciae* 3841 ACC deaminase increases *S. meliloti* Rm1021 nodulation in alfalfa (25). Thus, it was not surprising that expressing Smed_6456 in *S. meliloti* Rm1021 increased its symbiotic productivity with *M. truncatula* A17.

Smed_5985 was a candidate for improving *S. meliloti* Rm1021 productivity on *M. truncatula* A17 because of its predicted involvement in breaking down polysaccharides. Glucosidases play a role in making sugars available to rhizobia (26). Processing succinoglycan is important for symbiotic function of this polysaccharide during nodulation of alfalfa (27, 28).

iseA, which is a member of a class of genes not previously described as being involved in nodulation, gave the strongest response in increasing the nodule number and plant biomass of *M. truncatula* A17 (Fig. 1, Table 2). IseA is one of 27 predicted proteins in *S. medicae* WSM419 that is classified at EMBL-BL (https://www.ebi.ac.uk) as belonging to the vicinal oxygen chelate (VOC) protein superfamily (29). *S. meliloti* Rm1021 has 33 VOC proteins, according to EMBL-BL (https://www.ebi.ac.uk). The VOC family is characterized by a motif in which several sites that can bind a metal are brought together in the folded protein structure and then interact with O_2 or with molecules that contain oxygen atoms, often on adjacent carbons. The level of overall homology between these proteins in *S. meliloti* Rm1021 is low, but the binding sites are fairly

well conserved. None of these are closely related to IseA (Fig. S3 A). Homology searches using NCBI BLASTP program and other tools indicate that IseA is most closely related to the extradiol dioxygenase group within the VOC family (30). Extradiol dioxygenases cleave aromatic rings containing adjacent hydroxyl groups at a position external to the OH groups. When the 132 amino acids of *iseA* were used to do a BLASTP search with *S. meliloti* Rm1021, the only significant homology was ~30% amino acid identity with a region near the carboxyl terminus of SMa0723, a hypothetical 262 amino acid VOC protein on the pSymA megaplasmid (Fig. S3 B). The classification of *iseA* suggests it is involved in the metabolism of small molecules, either in the rearrangement of structures that contain oxygen or in oxidizing these compounds using molecular oxygen but the range of activities of these proteins precludes a more specific assignment.

Although these VOC proteins in *S. medicae* WSM419 have conserved substructures, they presumably differ in their functions. Only four of these belong to the same diol dioxygenase subgroup as *iseA*. To test whether *iseA* has a unique role in nodulation in comparison with the other genes sharing the same annotation, the *iseA* gene was disrupted in *S. medicae* WSM419 genome and the resulting mutant formed fewer nodules on *M. truncatula* A17 and the nodulated plants had a lower dry weight. Complementation of the *S. medicae* WSM419Δ*iseA* strain with pCPP30 *iseA* restored the nodule number and dry weight (Fig. 4). These results indicate that *iseA* participates in normal nodule formation and nitrogen fixation and, significantly, that other *S. medicae* VOC proteins are not able to substitute for it in order to complement its activity. *iseA* might plausibly have been identified in a screen for WSM419 mutants with impaired symbiosis. The versatility of VOC proteins and the lack of a close and functionally characterized homolog make it difficult to speculate about *iseA* function. It might degrade diol-containing compounds of

interest in symbiosis like caffeic acid, quercetin, or luteolin. Caffeic acid is a precursor of G-and S-lignin subunits. Luteolin is the prototypical flavonoid inducer of Nod factor synthesis in *S. meliloti* (31). Another diol-containing flavonoid, quercetin, inhibits nodulation in pea by interfering with Nod factor synthesis (32). IseA might also be involved in catabolism of diol-containing molecules like catechol, an intermediate in breakdown of many aromatic compounds.

To gain some insight into whether the three *S. medicae* WSM419 genes might be significant for nodulation in other systems, we constructed derivatives of *R. leguminosarum bv. viciae* 3841 expressing the three candidate genes separately and tested the effect in pea and lentil. *iseA* increased both nodule number and dry weight in the lentil cultivar Avondale under greenhouse conditions but had no significant effect on Pardina. Both *iseA* and Smed_6456 increased nodulation in pea plants but only *iseA* led to increased pea plant biomass. These results suggest that if *iseA* and Smed_6456 interact with signaling during nodule formation, they do not have the high level of specificity that are characteristic of flavonoid or EPS signals and they may be acting more like ethylene in interacting with more general aspects of nodulation control. For the lentil cultivar Avondale, the biomass accumulated under greenhouse conditions also increased. We interpret the symbiotic growth stimulation conferred by adding the three *S. medicae* WSM419 genes to *S. meliloti* Rm1021 as at least partially due to the relatively ineffective nature of the interaction between *S. meliloti* Rm1021 and *M. truncatula* A17. However, if the results with Avondale can be translated to the field, it may be that *iseA,* or *iseA* homologs in other *R. leguminosarum* strains (Table 3), could be useful in increasing productivity in less compromised situations and that *iseA* might be useful in rhizobial inoculants.

Interestingly, none of the three genes had a substantial symbiotic phenotype when the *S. meliloti* Rm1021 strains containing them were tested on *M. sativa*. Possible explanations are that

alfalfa does not respond to the activities they generate or that *S. meliloti* Rm1021 is able to trigger a comparable response in alfalfa through some other mechanism. It is also possible that any potential growth response is masked by the already productive interaction in this symbiosis.

We also analyzed the expression level and spatial expression pattern of the *iseA*, Smed_5985, and Smed_6456 promoters by creating promoter-GUS fusion constructs. All three genes were expressed well in the nodules and *iseA* was also expressed in free-living bacteria. These results are consistent with the original *S. medicae* WSM419 proteome data that prompted us to examine these proteins (Yurgel et al. (submitted)]. In this study, *iseA* was also found in both free-living and symbiotic conditions with higher abundance in the nodule and ACC deaminase produced from Smed_6456 was found in higher amounts in symbiotic than in free-living bacteria.

Nodule formation for nitrogen fixation is a highly energy-demanding process and therefore tightly regulated by the host plant to keep a balance between nitrogen acquisition and energy expenditure. Many legumes control their nodule number after the initial infection by AON (13), a negatively acting regulatory systemic response that represses nodule formation by producing mobile peptides in the root that are mobilized to the shoot and then generate an unknown inhibitory signal that is perceived by the root. While there may be other explanations, our current thinking is that *iseA* can partially overcome or bypass the AON response and that this leads to increased nodulation. This idea is consistent with the results of split root experiments that show *S. meliloti* 1021 with *iseA* is relatively unaffected by AON (Fig. 6). If *iseA* interferes with the SDI, this could explain both the increase in nodulation and partial resistance to AON. Whether this is accomplished by affecting local interactions at a single root hair or by modulating the receptiveness of the entire root system is unknown. The AON response has some mechanistic similarity to environmental interactions that modify nodulation, such as sensitivity to nitrate and

acidity (33). It would be interesting to determine if *iseA* affects these other plant-mediated pathways for controlling rhizobial nodulation since its presence might allow nodulation to occur under otherwise unfavorable conditions.

In this paper, we showed that transferring into *S. meliloti* Rm1021three *S. medicae* WSM419 genes that do not have close relatives in *S. meliloti* Rm1021 improved the strain's symbiotic productivity with *M. truncatula* A17. One of these genes, *iseA*, was present in all *S. medicae* genomes that are available, and it may be especially useful for symbionts of *M. truncatula*. *S. meliloti* WSM1022, which does well on *M. truncatula* (11), is one of a few *S. meliloti* strains that contains an *iseA*. *iseA* promoted nodulation in two different grain legumes, pea and lentil. Moreover, *iseA* allows the bacteria to promote nodule formation even when the AON regulatory circuit is operating, suggesting that it can bypass or overcome this regulation in some way. In legume species, nodulation is hampered by several external factors such as low pH, the presence of nitrate, and salt stress (13, 33, 34). There are general similarities between the circuitry that controls systemic plant responses to these challenges, and it might be that *iseA* can influence nodulation under stress conditions. Understanding how field conditions limit nodulation is becoming more important, both because of changing conditions in cultivated fields and because agriculture is moving into more marginal environments. The identification and expression of genes that confer improvements to nodule formation or symbiotic productivity may be able to allow legumes to take more advantage of symbiotic nitrogen fixation in the field.

MATERIALS AND METHODS

Strains, media, and culture conditions

The bacterial strains and plasmids used are listed in Table 1. Rhizobia were grown at 30ºC in liquid YMB or minimal medium supplemented with mannitol (1% wt/vol) ($MMNH_4$) with

antibiotics when appropriate (35). *Escherichia coli* strains were grown at 37 ºC in LB or LB agar with the indicated antibiotics (36). Antibiotics used as needed were: 10 µg/mL tetracycline, 12.5 µg/mL gentamicin for *E. coli*, 100 µg/mL gentamicin for *S. meliloti*, 75 µg/mL gentamicin for *R. leguminosarum*, 40 µg/mL kanamycin for *E. coli*, and 40 µg/mL neomycin for *S. meliloti.*

Plasmid and strain construction

Standard techniques were used for PCR amplification of DNA, DNA isolation, restriction enzyme digestion, ligations, and transformations (36). All enzymes used for cloning were obtained from New England BioLabs (Ipswich, MA, USA). Custom oligonucleotides used in this paper are listed in Table 4. Plasmid DNA was conjugated into rhizobia by biparental mating using *E. coli* host S17-1 (35, 37) as a donor strain. All the plasmid integrations and genomic deletions were screened and confirmed by PCR and DNA sequencing. Briefly, the ORF of the three candidate genes were amplified from the *S. medicae* WSM419 (12) genome using gene specific primers. The PCR products were digested, purified, and cloned downstream of the *lacZ* promoter in the pSRKGm or pCPP30 plasmids, which are Gm^R and Tc^R, respectively (38, 39). *S. meliloti* Rm1021 strains co-expressing two genes in two different plasmids were constructed by biparental mating, selecting for dual antibiotic resistance. Individual genes cloned in both pSRKGm and pCPP30 were also transformed into *R. leguminosarum* bv. *viciae* 3841 (20) to test the effect of these genes in pea and lentil. The *iseA* deletion mutants were constructed using an insertion/excision strategy (35). Briefly, 0.5-kb and 0.6-kb chromosomal regions flanking the target gene (*iseA*) were amplified, digested, and ligated together. The resulting composite flanking regions were then amplified and ligated into pK19 *mob sacB* (40). The plasmid was named pK19 *mob sacB*-Δ*iseA* and introduced to *E. coli* S17-1, then conjugated into *S. medicae* WSM419. Since pK19 *mob sacB*-Δ*iseA* cannot replicate in *S. medicae* WSM419, it must

integrate by recombination into one of the regions flanking *iseA* to yield neomycin resistant colonies. Single recombinants were subcultured on $MMNH_4$ agar supplemented with 5% sucrose to select for a second recombination event that eliminated the *sacB* gene. When this second recombination occurs in the other flanking region, an allele with Δ*iseA* remains. Neomycin sensitive colonies were identified and the Δ*iseA* mutation was confirmed by sequencing.

To construct promoter-GUS fusions, ~150 bp from the regions upstream of the start codon of each of the three candidate genes was amplified from the *S. medicae* WSM419 genome (Table 1) and fused with the *E. coli* β-glucuronidase gene (GUS) in the replicative plasmid pPG012, Km^R/Nm^R. The plasmid pPG012 was built by joining different fragments, pVS1 *repA*, *staA*, and *oriV*, followed by km^R and p15A *oriV*, and RK2 *oriT*. Finally, a multiple cloning site was. added, and the plasmid was sequence verified [Benedict A, Ghosh P, Scott S, Griffitts JS. A conserved rhizobial peptidase that interacts with host-derived symbiotic peptides. (under review)].

Similar fusions also were made with the *hrrP* promoter (P*hrrP*), which is normally up regulated in the nodule (17); the flagellin promoter from *S. medicae* WSM419 Smed_0266, which was expected to be down-regulated in the nodule; and the tryptophan promoter (P*trp*) from *Salmonella*, which is a constitutive promoter in *S. meliloti* (17). These reporter plasmids were transformed into *S. meliloti* Rm1021 and GUS expression was monitored in liquid media ($MMNH_4$, LB) and *in planta*.

Plant growth, inoculation and nodulation

Plants and their sources are listed in Table 5. *M. truncatula* A17 seeds were surface sterilized by using concentrated sulfuric acid, rinsed several times with sterile water and soaked briefly in bleach (5% sodium hypochlorite). The seeds were again rinsed thoroughly with sterile water and kept in water at 4 °C for 2 days then allowed to germinate in Petri plates at room temperature.

The germinated 2-day old seedlings were planted in Magenta boxes (Sigma GA-7 vessel) filled with a 4:1 mixture of Turface and Vermiculite (Turface Athletics; Thermo-O-Rock West Inc.). The plants were grown for 4 days before inoculation and maintained in a growth chamber with a 16h/8h light/dark cycle at 20°C. Alfalfa *(M. sativa* cv Ladak) seeds were sterilized, germinated and planted in sand filled Magenta boxes (Sigma GA-7 vessel) as described previously (35). Plants were grown in a growth chamber with 24 h of light at 20°C. At 28 dpi, nodule number and plant shoot dry weight were determined for both *M. truncatula* A17 and *M. sativa*. Sterilization of pea (*P. sativum* cv. Green Arrow) seeds, plant inoculation, and growth conditions were done as described previously (41). Plant shoot dry weight and nodule numbers were quantified at 5 weeks post inoculation.

Seeds of two lentil (*Lens culinaris*) cultivars, Pardina and Avondale, were sterilized by washing with 100% ethanol for 1 min followed by washing with sterile water. The seeds were then soaked in bleach (5% sodium hypochlorite) for 5 min, washed with sterile water, dried in a laminar flow hood, and scarified mechanically before germination. For germination, seeds were soaked in sterile water for 5 h and incubated in moist filter paper in the dark at room temperature. After 3 days, the germinated seedlings with radicle length greater than 2 cm were selected and transplanted into Turface. The plants were maintained in a greenhouse with 16 h light/8 h dark cycle at 20°C. Data were collected after 5 weeks post inoculation.

Rhizobia strains used for inoculation were grown on solid LB agar with appropriate antibiotics for 48 h then resuspended in sterile water to a final OD_{600} of 0.5. Each plant in the growth box was inoculated with the tested strains by applying 0.5 ml of the cell suspension. All plant boxes were supplemented with standard nitrogen-free nutrient solution (35)(42). To determine shoot dry weight of plants, plant materials were dried at 60°C for 48 h in an oven and

505 weighed. Shoot dry weight and nodule number are often correlated with the benefit host plants
506 derive from the symbiosis in the field so we have limited our analysis to these measures. All the
507 experiments were replicated at least three times with similar conditions and data point from one
508 representative experiment was presented.

509 **Histochemical GUS staining and quantification**

510 For colorimetric quantification of GUS reporter activity, *S. meliloti* Rm1021 cultures were grown
511 to log phase in $MMNH_4$ media or LB (OD_{600} 0.6 – 1.1). β-glucuronidase reactions were carried
512 out in a GUS master mix buffer (15 mM Na_2HPO_4, 10 mM NaH_2PO_4, 5 mM KCl, 1 mg/mL p-
513 nitrophenyl-β-D-glucuronide (PNPG), 0.01% Triton X-100, and 0.5% β-mercaptoethanol, pH
514 7.0) at 37 °C (17). The reactions were stopped using an equal volume of 1M Na_2CO_3 stop buffer.
515 Miller units were calculated [(1000*OD_{410})/ (OD_{600}*volume in milliliters*time in minutes)].
516 Bacterial culture used to measure GUS activity were performed in three biological replicates. To
517 stain for β-glucuronidase in nodules, 10 dpi whole nodules of *M. truncatula* A17 were incubated
518 in GUS reaction buffer [100 mM sodium phosphate (pH 7.0), 2 mM potassium ferricyanide, 2
519 mM potassium ferrocyanide, 10 mM EDTA, 0.1% Triton X-100 and 1 mM X-Gluc (5-bromo-4-
520 chloro-3-indolyl β-D-glucuronide) (Gold Biotechnology Inc., St. Louis, MO)] for 8 h at 37°C in
521 the dark (17). After staining, the nodules were washed with 75% ethanol and photographed.
522 Images were captured under a Leica EZ4D dissecting microscope (Leica Microsystems, Inc.).

523 **Split root development and inoculation**

524 *M. truncatula* A17 split root experiments were performed as described previously (21). *M.*
525 *truncatula* A17 seeds were prepared as described above. Five one-day old seedlings were placed
526 on 9 cm Petri dishes containing Harrison Modified Farhaeus (HMF) media inserted between two
527 Whatman filter papers (Whatman, catalog # 1001090) and stored vertically for 4 days in a

growth chamber (25°C and a 16h/8h light/dark cycle). On the 6th day, the tip of the main root was cut to induce lateral root development. The plants were again placed in the HMF media between two sheets of Whatman filter paper. After 3-5 days, those plants with split roots of approximately equal length were selected. Lateral roots from the same plantlet were directed to grow into two independent compartments made by adjacent Perlite-filled Magenta boxes. When inoculation was staged, the delayed inoculation of the second root was performed 3 days after the first inoculation. After 4 weeks from the delayed inoculation total number of nodules per root was counted. The plants were maintained in a growth chamber with 16h/8h light/dark cycle at 20°C.

Statistical analyses

Statistical analysis was performed using Student's t-test. P values < 0.05 was considered to be statistically significant. All experiments were done at least three times and data points in the figures are from one representative experiment, with the number of plants used in each treatment represented there.

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552 **Table 1** Bacterial strains and plasmids used in this study

553

Bacterial strains or plasmids	Relevant features [a]	Reference
Plasmids		
pCPP30	incP1 replicative plasmid derived from pRK2/pRP4, Tc^R	(39)
pCPP30-*iseA*	pCPP30 P*lacZ-iseA*, Tc^R	This work
pCPP30-Smed_5985	pCPP30 P*lacZ*-Smed_5985, Tc^R	This work
pCPP30-Smed_6456	pCPP30 P*lacZ*-Smed_6456, Tc^R	This work
pSRKGm	pSRKGm, derived from pBBR1 Gm^R	(38)
pSRKGm-*iseA*	pSRKGm-P*lacZ-iseA*, Gm^R	This work
pSRKGm-5985	pSRKGm-P*lacZ*-Smed_5985, Gm^R	This work
pSRKGm-6456	pSRKGm-P*lacZ*-Smed_6456, Gm^R	This work
pPG012	A broad host range plasmid, p15A and pVS1 origins, Km^R/Nm^R	Benedict A, Ghosh P, Scott S, Griffitts JS.
pK19 *mob sacB*	pUC19 derivative, *lacZ_ mob sacB*, Km^R	(37)
Strains		
S. meliloti Rm1021	Wild type rhizobial strain	(43)4/11/22 1:13 PM
S. medicae WSM419	Wild type	(12)
R. leguminosarum bv. *viciae* 3841	Wild type	(20)
E. coli (S17-1)	pro hsdR recA [RP4-2(Tc::Mu) (Km::Tn7)]	(40)
Rm1021 (pPG012::GUS)	pPG012 carrying *gus* gene, Km^R/Nm^R	Benedict A, Ghosh P, Scott S, Griffitts JS.
Rm1021 (P*iseA*::GUS)	pPG012 carrying *iseA* promoter (158bp)-*gus*, Km^R/Nm^R	This work
Rm1021 (PSmed_5985::GUS)	pPG012 carrying Smed_5985	This work

	promoter (360 bp)-*gus*, Km^R/Nm^R	
Rm1021 (PSmed_6456::GUS)	pPG012 carrying Smed_6456 promoter (360 bp)-*gus*, Km^R/Nm^R	This work
Rm1021 (PSmed_0266::GUS)	pPG012 carrying Smed_0266 (flagellin) promoter (337 bp)-*gus*, Km^R/Nm^R	This work
Rm1021 (P*hrrp*::GUS)	pPG012 carrying hrrP promoter (474 bp)-*gus*, Km^R/Nm^R	Benedict A, Ghosh P, Scott S, Griffitts JS.
Rm1021 (P*trp*:: GUS)	pPG012 carrying *trp* promoter (474 bp)-*gus*, Km^R/Nm^R	Benedict A, Ghosh P, Scott S, Griffitts JS.
Rm1021 (pCPP30) (pSRKGm)	Tc^R, Gm^R	This work
Rm1021 (pCPP30-*iseA*) (pSRKGm)	Tc^R, Gm^R	This work
Rm1021 (pCPP30) (pSRKGm-*iseA*)	Tc^R, Gm^R	This work
Rm1021 (pCPP30- Smed_5985) (pSRKGm)	Tc^R, Gm^R	This work
Rm1021 (pCPP30) (pSRKGm- Smed_5985)	Tc^R, Gm^R	This work
Rm1021 (pCPP30- Smed_6456) (pSRKGm)	Tc^R, Gm^R	This work
Rm1021 (pCPP30) (pSRKGm- Smed_6456)	Tc^R; Gm^R	This work
Rm1021 (pCPP30-*iseA*) (pSRKGm-*iseA*)	Tc^R, Gm^R	This work
Rm1021 (pCPP30- Smed_5985) (pSRKGm- Smed_5985)	Tc^R, Gm^R	This work
Rm1021 (pCPP30- Smed_6456) (pSRKGm- Smed_6456)	Tc^R, Gm^R	This work
Rm1021 (pCPP30-*iseA*) (pSRKGm- Smed_5985)	Tc^R, Gm^R	This work
Rm1021 (pCPP30-*iseA*) (pSRKGm- Smed_6456)	Tc^R, Gm^R	This work
Rm1021 (pCPP30- Smed_5985) (pSRKGm- Smed_*iseA*)	Tc^R, Gm^R	This work
Rm1021 (pCPP30- Smed_6456) (pSRKGm-*iseA*)	Tc^R, Gm^R	This work
Rm1021 (pCPP30- Smed_6456) (pSRKGm- Smed_5985)	Tc^R, Gm^R	This work
Rm1021 (pCPP30- Smed_5985) (pSRKGm- Smed_6456)	Tc^R, Gm^R	This work
R. leguminosarum bv. *viciae* 3841 (pCPP30-*iseA*)	Tc^R	This work
R. leguminosarum bv. *viciae* 3841 (pCPP30- Smed_5985)	Tc^R	This work
R. leguminosarum bv. *Viciae* 3841 (pCPP30- Smed_6456)	Tc^R	This work
R. leguminosarum bv. *viciae* 3841 (pSRKGm-*iseA*)	Gm^R	This work
R. leguminosarum bv. *viciae* 3841 (pSRKGm- Smed_5985)	Gm^R	This work

R. leguminosarum bv. *viciae* 3841 (pSRKGm-Smed_6456)	Gm^R	This work
S. medicae WSM419Δ*iseA*		This work
S. medicae WSM419Δ*iseA (*pCPP30)	Tc^R	This work
S. medicae WSM419Δ*iseA (*pCPP30-*iseA*)	Tc^R	This work

554

555 [a] Tc^R, tetracycline resistance; Gm^R, gentamycin resistance; Km^R, kanamycin resistance; Nm^R, neomycin
556 resistance

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562 **Table 2** Numerical values for *M. truncatula* A17 nodule numbers in symbiosis infected with *S.*

563 *meliloti* Rm1021 strains containing the indicated plasmids

564

Combination of genes [a]	**Pink nodules (avg)**	**White nodules (avg)**	**Pink nodules (SEM)**	**White nodules (SEM)**
S. meliloti Rm1021				
Vector control [(pCPP30, Tc^R) (pSRKGm, Gm^R)]	17.9	8.4	1.6	1.0
(pCPP30-*iseA,* Tc^R) (pSRKGm, Gm^R)	28.7^{***}	5.3	1.8	0.5
(pCPP30, Tc^R) (pSRKGm-*iseA,* Gm^R)	27.5^{***}	5.7	1.9	0.8
(pCPP30-*iseA,* Tc^R) (pSRKGm-*iseA,* Gm^R)	30.1^{***}	6.3	1.6	0.7
(pCPP30-Smed_5985, Tc^R) (pSRKGm, Gm^R)	25.4^{**}	5.9	1.7	1.1
(pCPP30, Tc^R) (pSRKGm-Smed_5985, Gm^R)	23.2^{**}	6.4	0.9	1.0
(pCPP30-Smed_5985, Tc^R) (pSRKGm-Smed_5985, Gm^R)	22.6^{**}	4.7	1.4	0.7
(pCPP30-Smed_6456, Tc^R) (pSRKGm, Gm^R)	26.7^{***}	6.7	1.6	0.8
(pCPP30, Tc^R) (pSRKGm-Smed_6456, Gm^R)	27.2^{***}	6.4	2.8	1.0
(pCPP30-Smed_6456, Tc^R) (pSRKGm-Smed_6456, Gm^R)	23.9^{**}	2.9	1.2	0.4
(pCPP30-*iseA,* Tc^R) (pSRKGm-Smed_5985, Gm^R)	30.5^{***}	5.8	1.6	0.6
(pCPP30-Smed_5985, Tc^R) (pSRKGm-*iseA,* Gm^R)	31.8^{***}	6.3	2.0	0.8
(pCPP30-*iseA,* Tc^R) (pSRKGm-Smed_6456, Gm^R)	29.6^{***}	5.7	2.3	0.5
(pCPP30-Smed_6456, Tc^R) (pSRKGm-*iseA,* Gm^R)	31.4^{***}	4.6	2.2	0.7
(pCPP30-Smed_6456, Tc^R) (pSRKGm-Smed_5985, Gm^R)	28.2^{***}	4.9	2.3	0.5
(pCPP30-Smed_5985, Tc^R) (pSRKGm-Smed_6456, Gm^R)	27.7^{***}	5.7	2.4	0.9
S. medicae WSM419	32.4^{***}	5.4	2.3	0.8

565

566 [a] Each of the genes was cloned in two different plasmids, pCPP30; Tc^R and pSRKGm; Gm^R

567 were expressed in *S. meliloti* Rm1021 in various combinations. The *S. medicae* WSM419 control

568 shows that more productive associations are possible. The experiments were performed in

569 triplicate and the values present here are from one experiment. All data were collected at 28 dpi

570 (n = 18). Significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) for Student's t test vs.

571 vector control. The vector control contains comprises of pCPP30, Tc^R and pSRKGm, Gm^R.

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574 **Table 3** Distribution of *iseA*, Smed_5985 and Smed_6456 by species[a]

		***iseA* (Smed_3503)**		**Smed_5985**		**Smed_6456**		
Sinorhizobium	**Number of genomes**	**High**	**Low**	**High**	**Low**	**High ACCdA**	**Med CysdS**	**Low TrypSb**
Sinorhizobium americanum	4	0	4	0	0	4	5	4
Sinorhizobium arboris	1	1	2	0	0	1	0	0
Sinorhizobium fredii	17	0	6	0	0	2	0	14
Sinorhizobium kostiensis	1	0	0	0	0	0	0	0
Sinorhizobium medicae	31	31	0	27	38	10	15	0
Sinorhizobium meliloti	73	6	67	11	1	47	2	0
Sinorhizobium saheli	1	0	0	0	0	1	0	0
Sinorhizobium sp	14	4	17*	0	0	9	0	8
Sinorhizobium terangae	1	0	0	0	0	1	0	0
Rhizobium leguminosarum	69	0	62	38	12	20	19	67

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577 [a]The distribution of DNA sequences with some similarity to *iseA* (Smed_3503), Smed_5985, and
578 Smed_6456 was examined in *Sinorhizobium* species and *Rhizobium leguminosarum* biovars
579 listed in the JGI database by using the *S. medicae* WSM419 protein sequences to search for
580 homologs using BLAST-P, with a maximum cutoff of E>-05 (19). The number of Finished,
581 Permanent Draft, and Draft Genome DNA sequences is shown in the second column. For each of
582 the three genes, the number of matches within the indicated genomes that had a value of E<–80
583 is shown in the high similarity columns. These are speculated to be homologs. The low
584 similarity column contains matches of much poorer quality, typically E>-15. As explained in the
585 text, the sequences found using the Smed_6456 protein sequence were in three groups: proteins

with very high similarity to ACC deaminase (ACCdA), proteins that had been annotated as resembling cysteine desulfhydrase proteins (CysdS), which typically had -55< E<-35, and proteins that had been annotated as tryptophan synthase, beta chain, with -15< E<-05. The 69 *Rhizobium leguminosarum* genomes studied comprised all of the sequences listed on March 8, 2021 and included several species biovars.

602 **Table 4** Primers used in this study

Primer	Sequence [a]	Usage
oMK01	F: GCGTCTAGAGGAGGTACTGAATGCTATCTAAAACA	Clone *iseA* gene into pCPP30
oMK02	R: GCGGGATCCTCAACCCTTGGCTGCCCT	Clone *iseA* gene into pCPP30
oMK03	F: GCGTCTAGAGGAGGTACTGAATGACAGTTGCATTTCGC	Clone Smed_5985 gene into pCPP30
oMK04	R: GCGGAGCTCTCACGAACTTCGGCTGCG	Clone Smed_5985 gene into pCPP30
oMK05	F: GCGGAGCTCGGAGGTACTGAATGTCACTGTTGGAAAAG	Clone Smed_6456 gene into pCPP30
oMK06	R: GCGGAATTCTCAACCGTCCCTGTAGTA	Clone Smed_6456 gene into pCPP30
oMK07	F: GCGGAGCTCGGAGGTACTGAATGCTATCTAAAACA	Clone *iseA* gene into pSRKGm
oMK08	R: GCGGGATCCTCAACCCTTGGCTGCCCT	Clone *iseA* gene into pSRKGm
oMK09	F: GCGGAGCTCGGAGGTACTGAATGACAGTTGCATTTCGC	Clone Smed_5985 gene into pSRKGm
oMK10	R: GCGTCTAGATCACGAACTTCGGCTGCG	Clone Smed_5985 gene into pSRKGm
oMK11	F: GCGGAGCTCGGAGGTACTGAATGTCACTGTTGGAAAAG	Clone Smed_6456 gene into pSRKGm
oMK12	R: GCGGGATCCTCAACCGTCCCTGTAGTA	Clone Smed_6456 gene into pSRKGm
oMK13	F: GCGGGATCCGGAGGTACTGAATGGTCCGTCCTGTAGAA	Clone *gus* gene into pPG012
oMK14	R: GCGTCTAGATTATTGTTTGCCTCCCTG	Clone *gus* gene into pPG012
oMK15	F: GCGGAGCTCCGGATACGGCACCG	Clone *iseA* promoter in pPG012-*gus*
oMK16	R: GCGGAATTCGAAGGGCAGTGCTT	Clone *iseA* promoter in pPG012-*gus*
oMK17	F: GCGGAGCTCTTTGCGAAGCTCTACAAC	Clone Smed_5985 promoter in pPG012-*gus*
oMK18	R: GCGGAATTCTGGCTGACTCCAAAATCG	Clone Smed_5985 promoter in pPG012-*gus*
oPG19	F: GCGGAGCTCCCATCGCGCCGAGGCCTA	Clone Smed_6456 promoter in pPG012-*gus*
oMK20	R: GCGGAATTCCGATCAGGGCCTCCGTGC	Clone Smed_6456 promoter in pPG012-*gus*
oMK21	F: CGCGAGCTCTCCATGAGCCGTCGGCAT	Clone Smed_0266 promoter in pPG012-*gus*
oMK22	R: CGCGAATTCGGTTTAGTGCCCCTTGG	Clone Smed_0266 promoter in pPG012-*gus*
oMK23	F: CGCGAGCTCGGGATGTGCTGCAAGGCG	Clone *trp* promoter in pPG012-*gus*
oMK24	R: CGCGAATTCTATCAGGAAGTGCGCCACC	Clone *trp* promoter in pPG012-*gus*
oMK25	F: CGCGAGCTCTCTCGTCGAGAACGT	Clone *hrrP* promoter in pPG012-*gus*
oMK26	R: CGCGAATTCGACGGAATATCCGCG	Clone *hrrP* promoter in pPG012-*gus*
oMK27	F: GCGAAGCTTCTGGTTTCGCGGCGTC	Clone right border (500bp) of *iseA*
oMK28	R: GCGTCTAGATGATTGCTCCCTTCTG	Clone right border (500bp) of *iseA*
oMK29	F: GCGTCTAGATGCGGCTTGGAGCACT	Clone right border (600bp) of *iseA*
oMK30	R: GCGAAGCTTCCGCACGCGCTGCGCG	Clone right border (600bp) of *iseA*

603 [a] Restriction sites used for cloning are underlined.

Table 5 Legume plants used in this study

Plants	Cultivar	Source
Medicago truncatula (Barrel medic)	Jemalong A17	USDA-ARS Washington State University
Medicago sativa (Alfalfa)	Ladak	Bruce Seed Farm, Inc., Townsend, MT
Pisum sativum (Pea)	Green Arrow	USDA-ARS Washington State University
Lens culinaris (Lentil)	Pardina	Dr. Rebecca McGee, USDA-ARS
Lens culinaris (Lentil)	Avondale	Dr. Rebecca McGee, USDA-ARS

FIGURE LEGENDS

FIG 1. Genes from *S. medicae* WSM419 increase the symbiotic productivity of S. *meliloti* Rm1021 with *M. truncatula* A17. The shoot dry weight of *M. truncatula* A17 inoculated with *S. meliloti* Rm1021 expressing the candidate genes (*iseA*, Smed_5985, and Smed_6456) independently and in different combinations. All data were collected at 28 dpi, the time of harvest ($n = 18$). Error bars indicate SEM. Significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) for Student's t test vs. vector control (pCPP30, Tc^R and pSRK, Gm^R) The plasmids pCPP30 (Tc^R) and pSRKGm (Gm^R) are represented as pC and pS respectively.

FIG 2. Expression pattern of *iseA*, Smed_5985 and Smed_6456 promoters

(A) Histochemical β-glucuronidase (GUS) staining of *M. truncatula* A17 root nodules from 10 dpi. The GUS staining of nodules induced by *S. meliloti* Rm1021 carrying no promoter-GUS, P*fla* (flagellin)-GUS, P*hrrP*-GUS, P*iseA*-GUS, PSmed_5985-GUS, and PSmed_6456-GUS, respectively. Scale bar 100 μm. (B) GUS expression levels in free living *S. meliloti* Rm1021 cells after grown to log phase in $MMNH_4$ and LB. The constitutive *Salmonella*-derived *trp* promoter (P*trp*) was used as a positive control. Error bars represent standard errors of the mean of three biological replicates.

FIG 3. These *S. medicae* WSM419 genes do not significantly improve the *S. meliloti* Rm1021 symbiosis with *M. sativa*. (A) Nodule number (B) and plant shoot dry weight data 28 dpi for *M. sativa* inoculated with *S. meliloti* Rm1021 containing the pCPP30 vector control, pCPP30-*iseA*, pCPP30-Smed_5985, and pCPP30-Smed_6456, respectively ($n = 16$). Error bars indicate SEM. No significant differences were observed between transgenic strains and vector control (pCPP30, Tc^R) using Student's t test. Similar results were obtained with the pSRKGm, Gm^R plasmid carrying the genes (data not shown).

FIG 4. Deleting the *iseA* gene from *S. medicae* WSM419 lowers symbiotic productivity with *M. truncatula* A17 (A) Nodule number (B) and plant shoot dry weight data at 28 dpi for *M. truncatula* A17 plants infected with *S. medicae* WSM419, WS419Δ*iseA*, WS419Δ*iseA* (pCPP30) and WS419Δ*iseA* (pCPP30-*iseA*) (n = 16). Error bars indicate SEM. Significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) for Student's t test.

FIG 5. *S. medicae* WSM419 genes increased pea and lentil performance when expressed in *R. leguminosarum* bv. *viciae* 3841

Number of nodules(A) and plant shoot dry weight (B) at 5 weeks post inoculation for pea (*P. sativum* cv Green Arrow) inoculated with *R. leguminosarum* bv. *viciae* 3841 containing the pCPP30 vector control, pCPP30-*iseA*, pCPP30-Smed_5985 and pCPP30-Smed_6456, respectively (*n* = 16).

Number of nodules (C) and plant shoot dry weight (D) at 5 weeks post inoculation for lentil (*Lens culinaris* cv Avondale) inoculated with *R. leguminosarum* bv. *viciae* 3841 containing the pCPP30 vector control, pCPP30-*iseA*, pCPP30-Smed_5985 and pCPP30-Smed_6456, respectively (*n* = 16). Error bars indicate SEM. Significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) for Student's t test vs. vector control (pCPP30, Tc^R). Similar results were obtained with the pSRKGm, Gm^R plasmid carrying the genes (data not shown).

FIG 6. *iseA* altered nodulation in a *M. truncatula* A17 split root system

M. truncatula A17 roots were manipulated to produce equal lateral roots partitioned in two sections, represented by two bar graphs. The early infected roots (represented by red) were inoculated 3 days earlier than the delayed infected roots (represented by blue). The split roots

were inoculated with *S. meliloti* Rm1021 expressing pCPP30 and pCPP30-*iseA* in different combinations (n = 16). The number of nodules per root from both the early and delayed inoculated roots were counted 4 weeks after the delayed inoculation. Error bars indicate SEM. Significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) for Student's t test.

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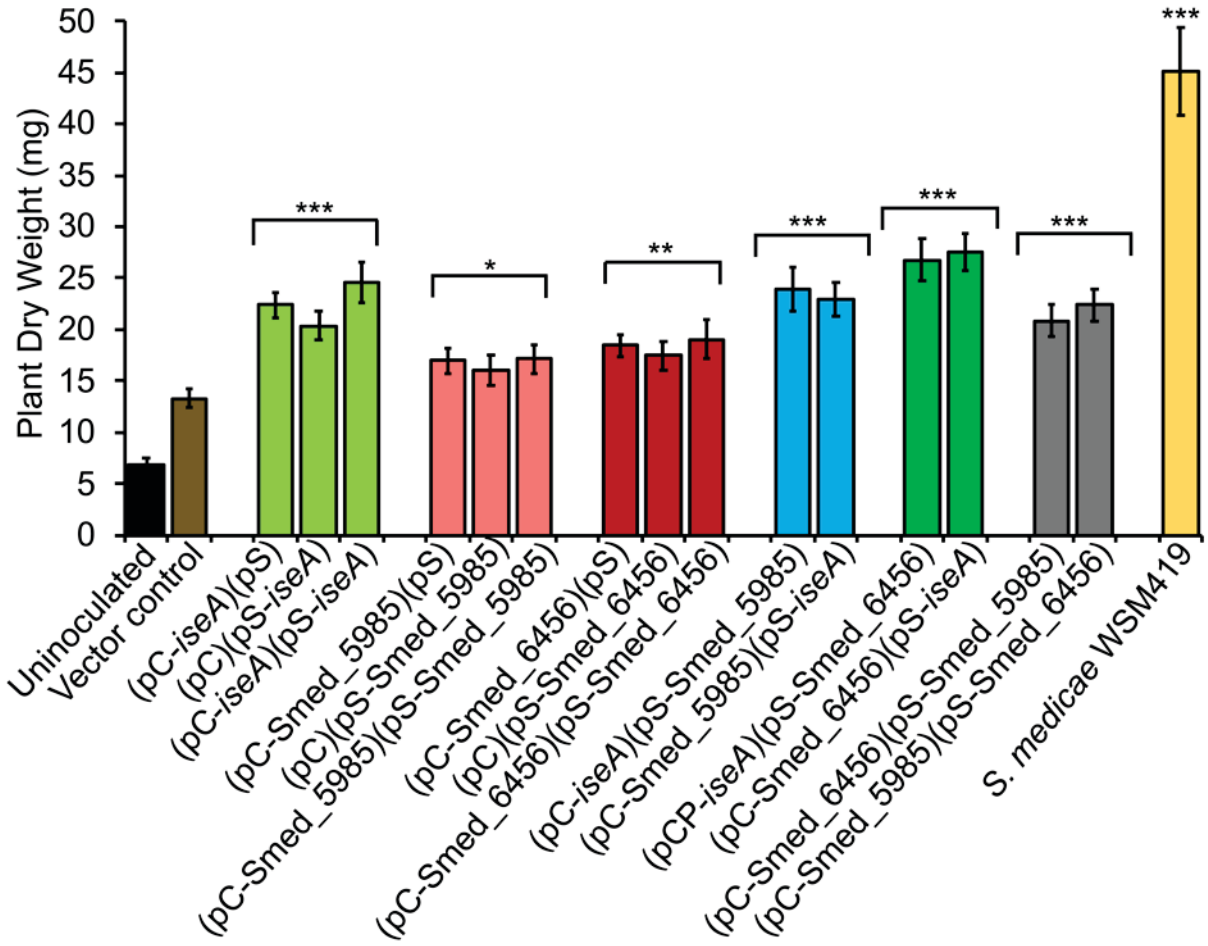
FIGURES.



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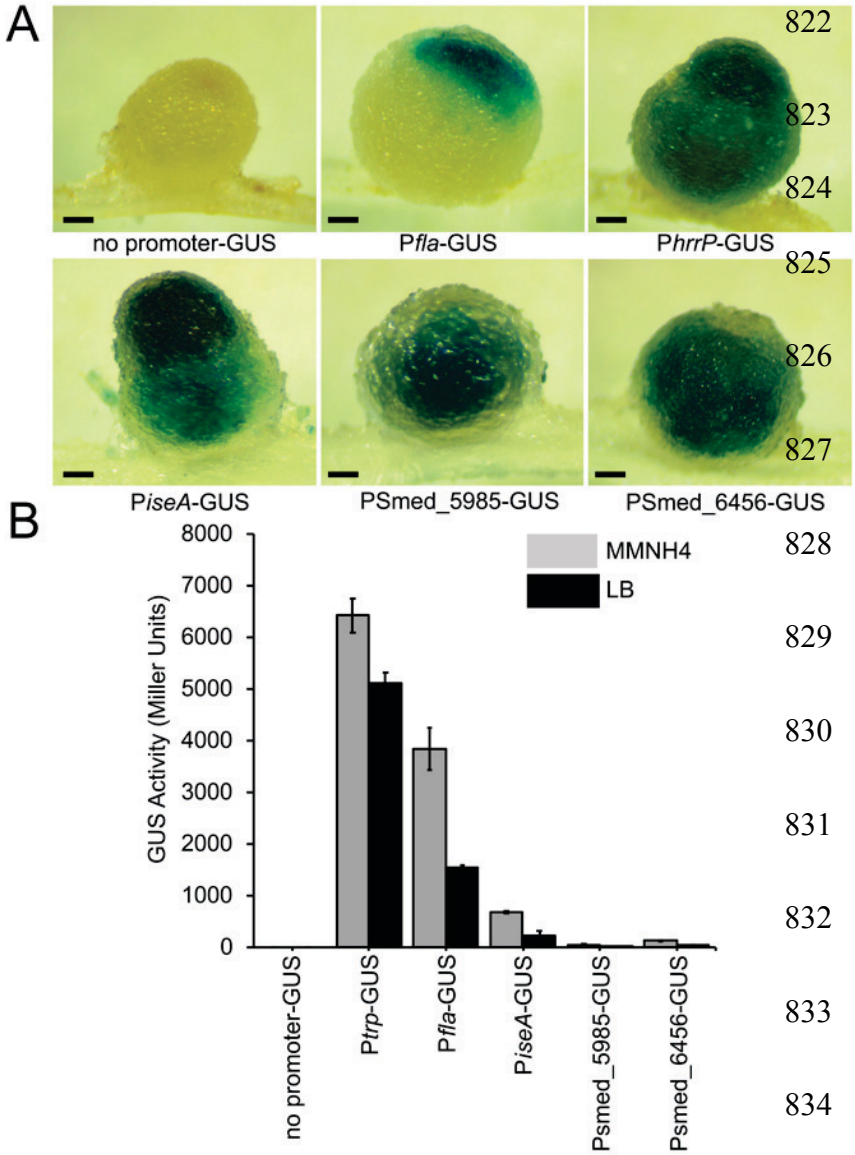

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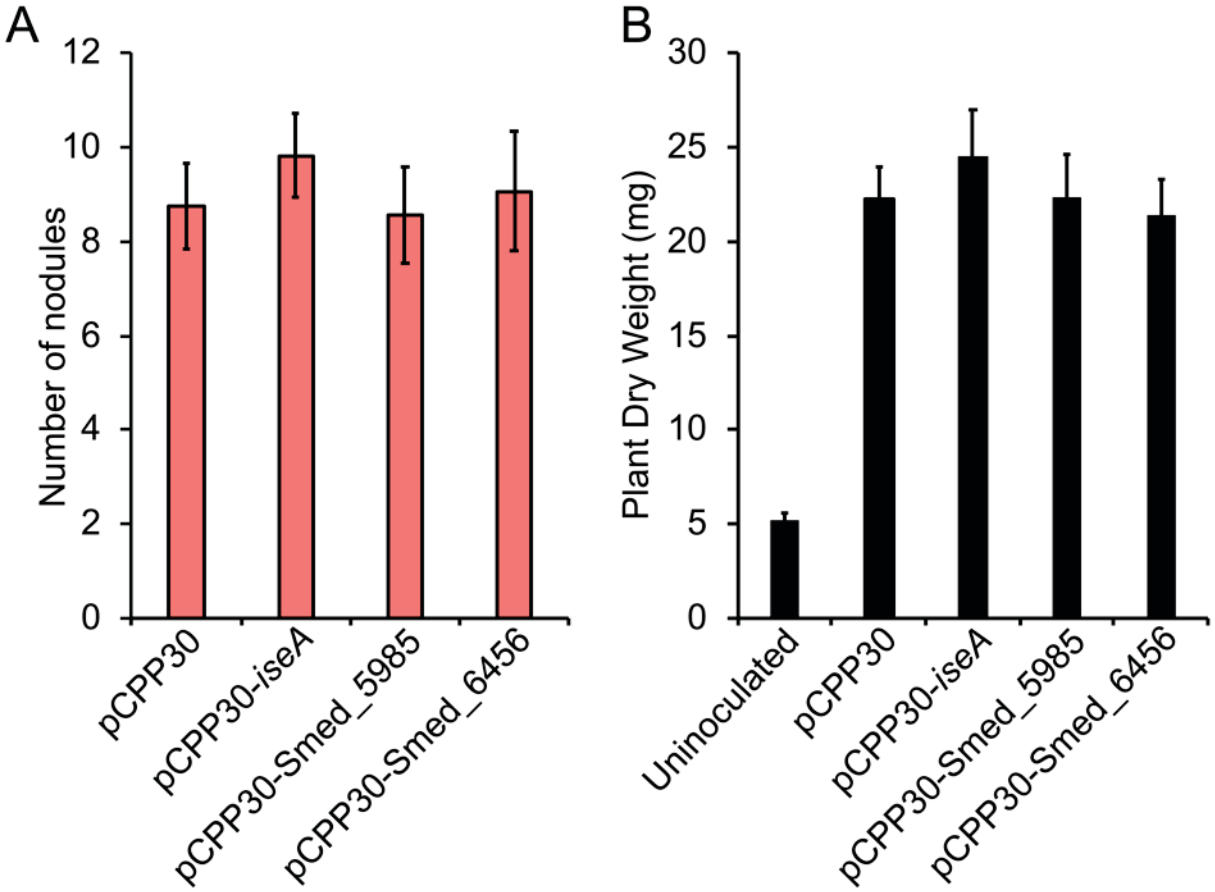

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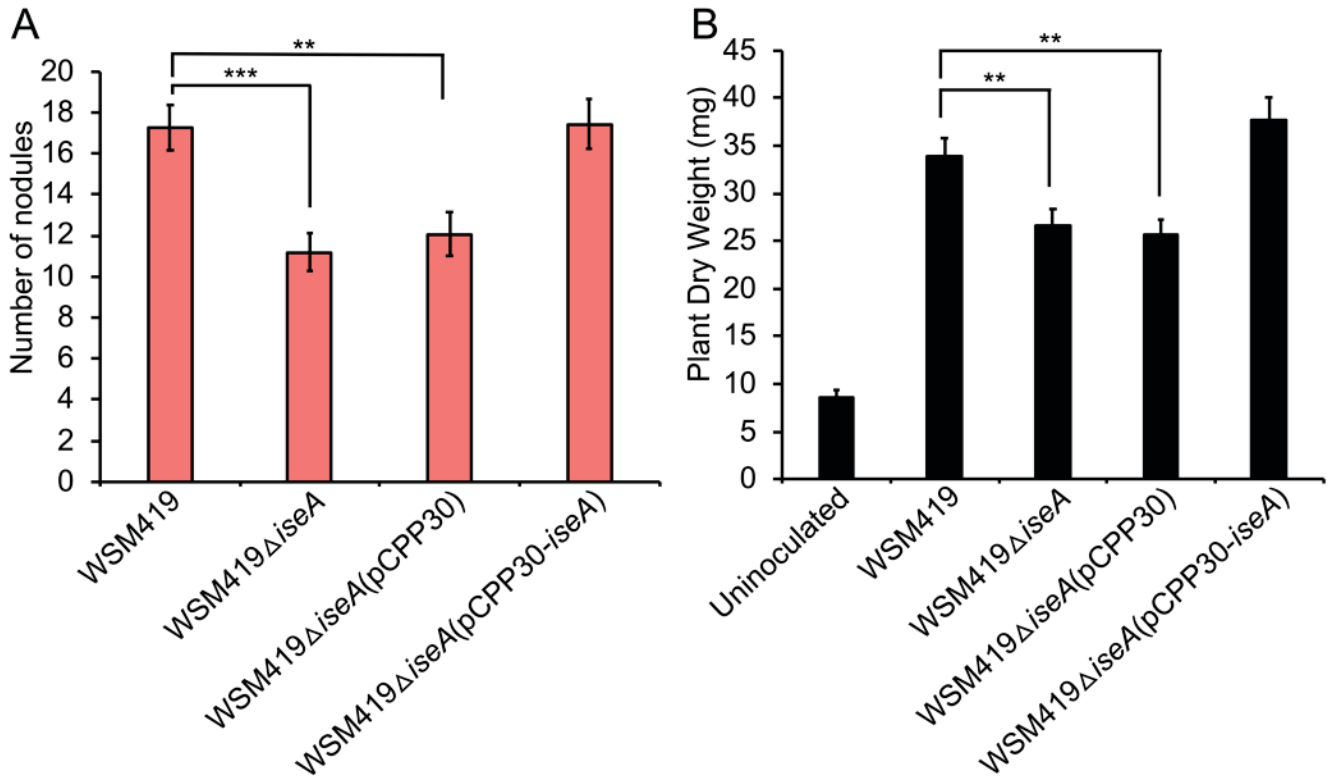

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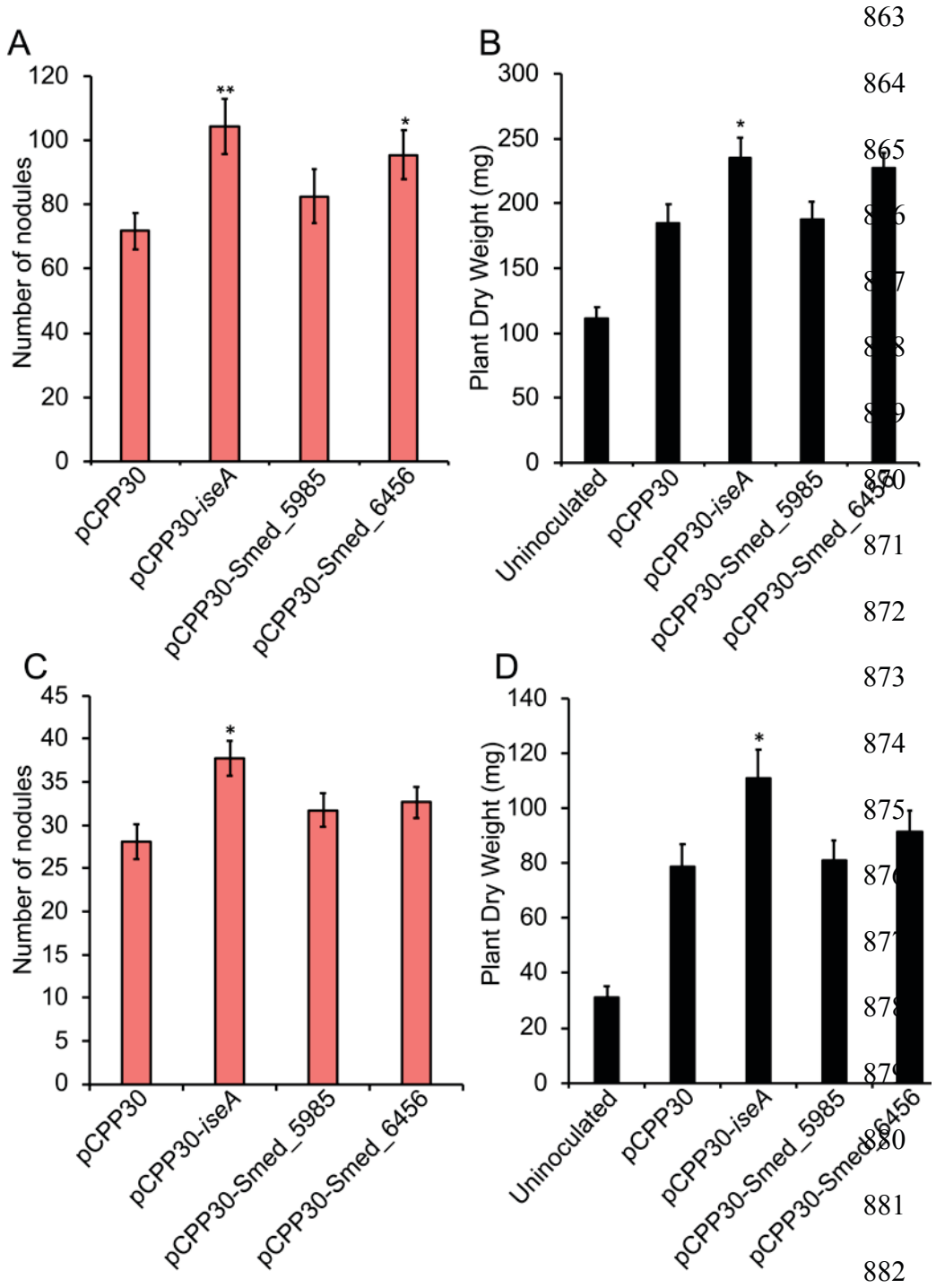
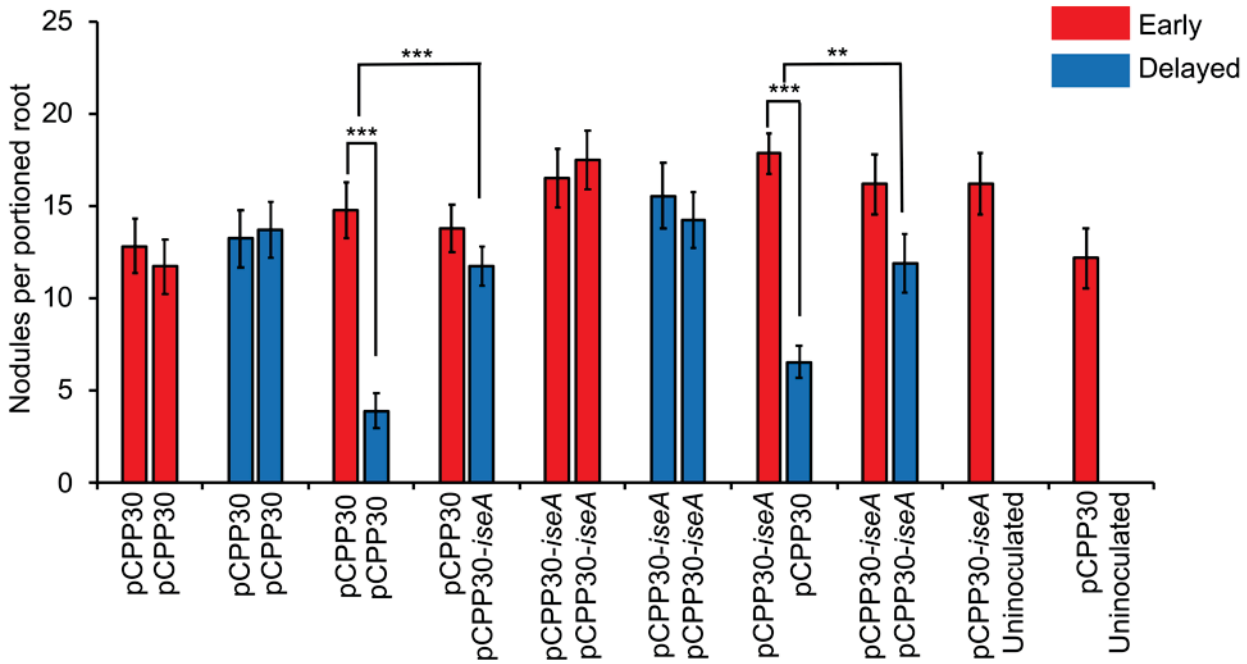


FIG 5. ***S. medicae*** **WSM419 genes increased pea and lentil performance when expressed in** ***R. leguminosarum*** **bv.** ***viciae*** **3841**

(A) Number of nodules (B) and plant shoot dry weight at 5 weeks post inoculation for pea (*P. sativum* cv Green Arrow) inoculated with *R. leguminosarum* bv. *viciae* 3841 containing the pCPP30 vector control, pCPP30-*iseA*, pCPP30-Smed_5985 and pCPP30-Smed_6456, respectively ($n = 16$).

(C) Number of nodules (D) and plant shoot dry weight data for lentil (*Lens culinaris* cv Avondale) at 5 weeks post inoculation inoculated with *R. leguminosarum* bv. *viciae* 3841 containing the pCPP30 vector control, pCPP30-*iseA*, pCPP30-Smed_5985 and pCPP30-Smed_6456, respectively ($n = 16$). Error bars indicate SEM. Significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) for Student's t test vs. vector control (pCPP30, Tc^R). Similar results were obtained with the pSRKGm, Gm^R plasmid carrying the genes (data not shown).



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