Nodulation Induces Systemic Resistance of *Medicago truncatula* and *Pisum sativum* Against *Erysiphe pisi* and Primes for Powdery Mildew-Triggered Salicylic Acid Accumulation

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Plants encounter beneficial and detrimental microorganisms both above- and belowground and the health status of the plant depends on the composition of this pan-microbiome. Beneficial microorganisms contribute to plant nutrition or systemically or locally protect plants against pathogens, thus facilitating adaptation to a variety of environments. Induced systemic resistance, caused by root-associated microbes, manifests as aboveground resistance against necrotrophic pathogens and is mediated by jasmonic acid/ethylene-dependent signaling. By contrast, systemic acquired resistance relies on salicylic acid (SA) signaling and confers resistance against secondary infection by (hemi)biotrophic pathogens. To investigate whether symbiotic rhizobia that are ubiquitously found in natural ecosystems are able to modulate resistance against biotrophs, we tested the impact of preestablished nodulation of *Medicago truncatula* and pea (*Pisum sativum*) plants against infection by the powdery mildew fungus *Erysiphe pisi*. We found that root symbiosis interfered with fungal penetration of *M. truncatula* and reduced asexual spore formation on pea leaves independently of symbiotic nitrogen fixation. Improved resistance of nodulated plants correlated with elevated levels of free SA and SA-dependent marker gene expression upon powdery mildew infection. Our results suggest

that nodulation primes the plants systemically for *E. pisi*-triggered SA accumulation and defense gene expression, resulting in increased resistance.

Keywords: bacteria-plant symbiosis, ethylene, fungus-plant interactions, hormones, induced systemic resistance, jasmonic acid, nodulation, pathogens, plant antifungal responses, powdery mildew, rhizobium-legume symbiosis, salicylic acid, symbiosis

In their natural environment, plants interact with a plethora of microbes both above- and belowground. The composition of the plant microbiome ultimately shapes the plant's ability to adapt to prevailing conditions (Müller et al. 2016). Beneficial belowground interactions—for example with plant growth-promoting rhizobacteria (PGPR) or plant growth-promoting fungi (PGPF), as well as root endosymbionts such as rhizobia and arbuscular mycorrhizal fungi—help the plant gain access to nutrients and grow on poor or contaminated substrates (e.g., salt or heavy metals) (Miransari 2010). Some beneficial microbes enhance the capability of plants to defend themselves against invaders (Beneduzi et al. 2012; Gray and Smith 2005; Pieterse et al. 2014). Root-interacting microbes can enhance resistance either directly via competition with pathogens or the secretion of antimicrobial substances, or indirectly via induced systemic resistance (ISR) (Müller et al. 2016, Pieterse et al. 2014). In contrast to ISR, which is mediated by beneficial root interactions, a primary local infection by a pathogen can increase resistance to a secondary pathogen attack in distal tissue, a phenomenon named systemic acquired resistance (SAR) (Fu and Dong 2013; Pieterse et al. 2014). Both ISR and SAR rely on phytohormones. However, whereas ISR largely depends on jasmonic acid (JA) and ethylene (ET) signaling (Pieterse et al. 1998), SAR is salicylic acid (SA) dependent (Vernooij et al. 1994). It has been shown in *Arabidopsis thaliana* that SA-dependent SAR mediates defense against biotrophic pathogens, whereas JA- or ET-dependent ISR is effective against necrotrophic pathogens and herbivores (Glazebrook 2005; Loake and Grant 2007; Pieterse et al. 2014; Pozo and Azcón-Aguilar 2007; Thomma et al. 2001; Ton et al. 2002; van Dam and Heil 2011; Van Loon and Bakker 2006). ISR has been reported to be effective against a plethora of root- or shoot-infecting bacteria and filamentous pathogens in monocots and dicots, including important crops such as barley (*Hordeum*

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vulgare), maize (*Zea mays*), tomato (*Solanum lycopersicum*), French bean (*Phaseolus vulgaris*), cucumber (*Cucumis sativus*), tobacco (*Nicotiana tabacum*), and grapevine (*Vitis vinifera*) (Alabouvette et al. 2009; Cameron et al. 2013; Walters et al. 2013). ISR is induced by PGPR such as *Pseudomonas*, *Serratia*, and *Bacillus* strains (De Vleeschauwer and Höfte 2009; Kloepper et al. 2004), as well as nonpathogenic *Fusarium* spp., *Trichoderma* spp., and *Piriformospora indica* (Alabouvette et al. 2009; Franken 2012; Shores et al. 2010) and arbuscular mycorrhizal fungi (Cameron et al. 2013; Jung et al. 2012). These organisms are regarded as significant biocontrol agents (Beneduzi et al. 2012).

Both ISR and SAR are associated with “priming”; that is, the ability of a plant, after a conditioning treatment, to respond more rapidly and strongly in terms of cellular defense reactions upon challenge by a pathogen than nonconditioned plants (Conrath et al. 2006; Mauch-Mani et al. 2017; Ton et al. 2009). Systemic defense priming requires either functional SA or JA/ET pathways depending on whether SAR or ISR, respectively, are conditioned (Conrath et al. 2015; Pieterse et al. 2014). SAR coincides with the accumulation of SA after a primary infection (Gao et al. 2015; Malamy et al. 1990; Návarová et al. 2012) or upon a secondary challenge (Jung et al. 2009), whereas PGPR-induced ISR priming in *Arabidopsis* seems to be based on an enhanced sensitivity to JA and ET rather than on an increase in their abundance per se (Pieterse et al. 2000, 2014; van Wees et al. 1999). In either case, in *Arabidopsis*, defense priming involves the challenge-induced expression of genes that potentially contribute to defense execution. In SAR, these are SA signaling-induced *pathogenesis-related* (PR) genes (Malamy et al. 1990; Ryals et al. 1996; Van Loon and Bakker 2006), while ISR primes for expression of distinct JA- or ET-responsive genes (Hoffland et al. 1995; Pieterse et al. 1998, 2014; Ryals et al. 1996; van Wees et al. 1999; Verhagen et al. 2004).

Root-nodulating rhizobia, which are occasionally referred to as intracellular PGPR (Gray and Smith 2005), are widely present in natural ecosystems and undergo mutualistic interactions with roots of legumes, many of which are important crop plants; for example, pea (*Pisum sativum*), bean (*Phaseolus vulgaris*), lentil (*Lens culinaris*), and soybean (*Glycine max*); or forage plants such as alfalfa (*Medicago sativa*). In the symbiotic relationship with the host, the bacteria are enclosed in root nodules, where they differentiate into intracellular host membrane-enclosed bacteroids and fix atmospheric nitrogen that becomes available to the plant as ammonium (Desbrosses and Stougaard 2011). Thus, they promote legume growth by enabling nitrogen acquisition under limiting conditions, which makes rhizobia valuable agents for sustainable agriculture (Bohlool et al. 1992). Similar to other beneficial or detrimental plant-infecting microbes, rhizobia facilitate initial colonization by suppressing microbe-associated molecular pattern (MAMP)-triggered plant defense (Zamioudis and Pieterse 2012). Furthermore, similar to arbuscular mycorrhizal fungi, rhizobia repress SA-dependent defense in the roots that would otherwise prevent infection (Foo et al. 2014; Gutjahr and Paszkowski 2009). This suppression requires secretion of bacterial lipo-chitooligosaccharides (LCOs) designated as nod factors, which bind to specific plant receptors and initiate the host's symbiotic program (Martínez-Abarca et al. 1998). Although ISR has been studied extensively with classical PGPR and PGPF, little is known about effects of rhizobial symbiosis on systemic plant defense. Most reports on rhizobial control of plant pathogens tested a direct intermicrobial effect on soilborne root-infecting microbes, rather than on induced resistance (Buonassisi et al. 1986; Dakora 2003; Ehteshamul-Haque and Ghaffar 1993; Tu 1978, 1979). However, arbuscular mycorrhiza, which share components of a common plant

symbiosis pathway with nodulation, trigger systemic priming of defense responses effective against necrotrophic and hemibiotrophic pathogens (Fritz et al. 2006; Lingua et al. 2002; Liu et al. 2007). The plant symbiosis pathway is triggered by perception of bacterial nod factors or fungal (Myc-) LCOs similar to fungal chitooligosaccharides that elicit MAMP-triggered immune signaling (Zipfel and Oldroyd 2017). Interestingly, PGPR-mediated ISR is linked to the perception of MAMPs or avirulence-like protein effectors (Burketova et al. 2015; De Vleeschauwer and Höfte 2009; Shores et al. 2010). Rhizobial nod factors have been reported to induce resistance against pathogens. For example, application of nod factors to soybean roots limited colony size of *Microspora diffusa* powdery mildew on leaves in a dose-dependent manner (Duzan et al. 2005). Notably, powdery mildew fungi are biotrophic pathogens that are strongly susceptible to SA-mediated defense, resulting in reduced colony expansion and asexual reproduction (Chandran et al. 2009; Zimmerli et al. 2004). Accordingly, *Arabidopsis* mutants, or transgenic plants impaired in SA biosynthesis, accumulation, or signaling, display increased susceptibility to adapted powdery mildews (Chandran et al. 2009; Dewdney et al. 2000; Ederli et al. 2015). By contrast, JA-mediated defense is either suppressed or fails to be elicited by adapted powdery mildews (Antico et al. 2012; Kuhn et al. 2016). However, *Arabidopsis* root colonization with the PGPF *Piriformospora indica* reduces powdery mildew asexual spore formation (conidiation) in a JA-dependent manner (Stein et al. 2008), and JA-induced defense responses are effective against adapted powdery mildew fungi when triggered ectopically. For example, by exogenous application of JA, regardless of the biotrophic nature of the subsequent challenge (Ellis et al. 2002a and b; Zimmerli et al. 2004). By contrast, arbuscular mycorrhiza elicited ISR, which is mostly but not exclusively linked to JA/ET signaling (Cameron et al. 2013), was found to be associated with enhanced susceptibility to biotrophic powdery mildews in barley (Gerns et al. 2001; Sonnemann et al. 2005). In conclusion, only a few reports exist on the effectiveness of ISR against biotrophic powdery mildews (Panstruga and Kuhn 2019), and even fewer data are available on nodulation-related ISR, despite the agronomical importance of legumes. In the case of infected pea, powdery mildew can result in yield losses of 25 to 50% (Maharjan et al. 2015). The elicitation of systemic resistance against biotrophic powdery mildews by nod factors applied to roots suggests that ISR might be triggered by rhizobia (Duzan et al. 2005). Whether or not nodulation mediates a systemic effect similar to arbuscular mycorrhiza-induced ISR, and whether SA or JA signaling might be involved in the defense signaling initiated by rhizobia, is not known. The most obvious benefit to the plant of nodulation is the uptake of nitrogen via the nitrogen-fixing microsymbiont (Downie 2014). This improves the nutritional status and fitness of the plant (Kaschuk et al. 2010). Activation of plant defenses by pathogens coincides with high metabolic costs to the plant and limited access to nitrogen can impair defense (Baldwin et al. 1998; van Dam and Baldwin 1998). Accordingly, in addition to systemic resistance effects, nodulated plants might be expected to be able to mount a more robust defense response than plants with inadequate nitrogen due to a growth-defense tradeoff impacting fitness (Huot et al. 2014).

Here, we investigated the impact of an established nodule symbiosis on susceptibility of two distinct legumes, *Medicago truncatula* and pea, against the powdery mildew fungus *Erysiphe pisi*. We show that nodulation quantitatively increases resistance and primes *E. pisi*-triggered accumulation of free SA in leaves. Elevated SA concentrations correlates with enhanced

transcript abundance of SA-responsive genes in leaves, suggesting that SA signaling contributes to powdery mildew resistance and pointing toward an SAR-like mechanism acting from the root to the shoot.

RESULTS

Preestablished nodulation enhances powdery mildew resistance of *M. truncatula* and *Pisum sativum*.

To investigate whether nodulation of *M. truncatula* by *Sinorhizobium meliloti* influences the susceptibility of the host independently of a symbiotic nitrogen nutritional effect, we inoculated 3-week-old nodulated plants and nitrate-fertilized plants with the adapted powdery mildew fungus *E. pisi* (Fig. 1; Supplementary Fig. S1). Because the widely used *M. truncatula* inbred line ‘Jernalong A17’ is resistant to *E. pisi*, we used the highly susceptible line DZA315.16 (Foster-Hartnett et al. 2007). Without nodulation or nitrate supplementation, seedlings made poor growth and yellowed rapidly. Therefore, nonnodulated nonnitrogen-supplemented plants were not used in experiments. By contrast, plants supplied with nitrate did not differ statistically significantly from nodulated plants in their shoot height or dry and wet weight. They further had no statistically significant different nitrogen levels at 1 (3-week-old plants) and 7 (4-week-old plants) days post-fungal inoculation (dpi) (Fig. 1A). Nodule numbers of powdery mildew-inoculated and noninoculated plants were similar, showing that powdery mildew infection did not affect nodule formation (Fig. 1B). Interestingly, despite comparable nitrogen contents, preestablished nodulation of *M. truncatula* resulted in a reduced degree of epidermal

penetration by *E. pisi*. The average penetration success at 1 dpi was reduced statistically significantly ($P < 0.05$) by approximately 10% from 54% on control plants to 43% on nodulated plants (Fig. 1C). By contrast, the degree of sporulation at 7 dpi was the same for both treatments (Fig. 1D). This result indicates that nodulation of *M. truncatula* increases penetration resistance against *E. pisi* but does not influence postpenetration resistance.

To test whether an additional legume would behave correspondingly, *Rhizobium leguminosarum*-mediated nodulation of 2- to 3-week-old pea plants was also tested for possible effects on *E. pisi* infection. Nonnodulated pea plants supplemented with 5 mM nitrate did not differ in their appearance from nodulated plants. Furthermore, the leaf nitrogen content between both groups was not statistically significantly different after 1 or 7 dpi with *E. pisi* (Fig. 2A). Likewise, abundance of nodules was not influenced by inoculation with the fungus (Fig. 2B). However, in contrast to *M. truncatula*, nodulation did not affect fungal penetration at 1 dpi (Fig. 2C) but did result in a statistically significant reduction ($P < 0.05$) in sporulation at 7 dpi (Fig. 2D). Taken together, these data point to an impact of a preestablished nodulation on early prepenetration (*M. truncatula*) or late postpenetration (pea) resistance against *E. pisi*, which is uncoupled from the benefits of symbiotic nitrogen supply.

Nodulation of *M. truncatula* does not alter susceptibility to *Xanthomonas campestris* pv. *alfalfae*.

M. truncatula is a host for the hemibiotrophic bacterial leaf pathogen *Xanthomonas campestris* pv. *alfalfae* (Liu et al. 2007) that causes leaf spot disease of alfalfa (Esnault et al. 1993;

Fig. 1. Nodulation contributes to penetration resistance of *Medicago truncatula* against *Erysiphe pisi*. Three-week-old *M. truncatula* plants that had been nodulated with *Sinorhizobium meliloti* or supplemented with 250 ml of 7.5 mM KNO_3 per nine seedlings as 2-day-old seedlings were either inoculated with *E. pisi* or kept as a control. **A**, Nitrogen content of dry weight (DW) of leaves was determined by gas chromatography. **B**, Abundance of nodules per root weight was evaluated after staining for rhizobial β -glucuronidase activity. **C**, Penetration success of *E. pisi* was scored as percentage of successful penetration events, visible by formation of secondary hyphae. **D**, Fungal spore formation per leaf fresh weight (FW) was assessed by removal of conidia with water and counting in a Neubauer improved chamber. Spore numbers of nitrate-supplemented controls were set to 100%. Data labeled with the same letters are not statistically significantly different ($P \leq 0.05$; tested by one-way analysis of variance and Tukey's honestly significant difference post hoc test (A, B, and C, $n =$ six independent experiments with ≥ 18 plants per time point and treatment) or by Wilcoxon rank sum test (D, $n =$ three independent experiments with ≥ 18 plants per time point and treatment); dpi = days postinoculation with *E. pisi*.

Fig. 2. Nodulation contributes to postpenetration resistance of pea against *Erysiphe pisi*. Two-week-old pea plants that had been nodulated with *Rhizobium leguminosarum* or supplemented with 250 ml of 5 mM KNO_3 per four germinating seeds were either inoculated with *E. pisi* or kept as a control. **A**, Nitrogen content of weight (DW) of leaves was determined by gas chromatography. **B**, Abundance of nodules per root weight was evaluated after staining for rhizobial β -glucuronidase activity. **C**, Penetration success of *E. pisi* was scored as percentage of successful penetration events visible by formation of secondary hyphae. **D**, Fungal spore formation per leaf fresh weight (FW) was assessed by removal of conidia with water and counting in a Neubauer improved chamber. Spore numbers of nitrate-supplemented controls were set to 100%. Data labeled with the same letters are not statistically significantly different ($P \leq 0.05$; tested by one-way analysis of variance and Tukey's honestly significant difference post hoc test (A, B, and C, $n =$ 5 independent experiments with ≥ 9 plants per time point and treatment) or by Wilcoxon rank sum test (D, $n =$ 5 independent experiments with ≥ 9 plants per time point and treatment); dpi = days postinoculation with *E. pisi*.

Riker et al. 1935). To test whether nodulation might also influence this interaction with a hemibiotrophic pathogen, we spray-inoculated nitrate-fertilized or *S. meliloti*-nodulated plants with *X. campestris* pv. *alfalfae* and evaluated bacterial multiplication and nodule abundance at 3 and 7 dpi (Supplementary Fig. S2). We observed the appearance of yellowish (chlorotic) leaf areas and necrotic spots at 7 dpi, indicating successful infection of *M. truncatula*. *X. campestris* pv. *alfalfae* infection did not affect the extent of nodule formation. However, in contrast to powdery mildew infection, nodulation of *M. truncatula* affected neither bacterial infection nor multiplication and had no strong impact on the severity of disease symptoms.

Transcripts of SA-responsive genes accumulate differentially after *E. pisi* inoculation of nodulated *M. truncatula* plants.

Increased resistances of plants against pathogen infection in systemic tissues can be mediated by SAR or ISR. Although SAR mostly depends on SA, ISR often requires the action of JA or ET (Pieterse et al. 1998; Vernooij et al. 1994). To investigate whether SAR or ISR might be involved in the enhanced powdery mildew resistance shown by nodulated plants, we examined steady-state transcript levels of SA- or JA-responsive genes, of which many are well characterized in *Arabidopsis*. To identify SA- and JA-responsive genes in *M. truncatula*, we mined the

genome for candidates with high protein sequence similarity to well-established hormone-responsive genes from *Arabidopsis*. One of the best-characterized SA-responsive genes in *Arabidopsis* is *AtPR-1*, while *AtPR-4* is described as a reporter gene for JA or ET signaling (Lorenzo et al. 2004; Potter et al. 1993; Van Loon and Bakker 2006; Vernooij et al. 1994). *Medtr2g435490* (58% amino acid identity) and *Medtr1g080800* (71% amino acid identity) were identified as potential homologs of *AtPR-1* and *AtPR-4*, respectively, in the Mt3.5v5 genome assembly (Supplementary Fig. S3). Please note that, in the most recent genome version (Mt4.0v2), other *M. truncatula* proteins show higher amino acid identities to *AtPR-1* and *AtPR-4*. To investigate the suitability of the selected candidates as SA or JA reporter genes, we performed hormonal treatments and tested for altered mRNA levels.

Spraying of *M. truncatula* leaves with 10 mM SA statistically significantly increased transcript abundance of the *AtPR-1* homolog *Medtr2g435490* by 13 h after treatment (approximately ninefold, considering the medians) (Fig. 3A). Surprisingly, the mRNA abundance of the *AtPR-4* relative *Medtr1g080800* did not increase in response to treatment with 1 mM methyl jasmonate (MeJA) but accumulated statistically significantly after SA treatment (approximately 33-fold, considering the medians) (Fig. 3B). This finding suggests that the genes are regulated differently in *Arabidopsis* and *M. truncatula* and that, regardless of whether or not the candidates are the functional orthologs of *AtPR-1* and *AtPR-4*, *Medtr1g080800*, in addition to *Medtr2g435490*, can serve as a marker gene for SA signaling. In an alternative approach, the Genevestigator database (Hruz et al. 2008) was mined for genes with JA-responsive expression. *Medtr8g018550* and *Medtr8g018690* showed a strong transcript accumulation in root-derived suspension-cultured cells treated with MeJA (Naoumkina et al. 2007) and were selected as JA-responsive candidate genes. A protein BLAST search revealed their closest homologs in *Arabidopsis* as the lipoxygenase (LOX) *AtLOX1* (*Medtr8g018550*; 56% amino acid identity and *Medtr8g018690*; 55% amino acid identity). Certain LOXs contribute to the biosynthesis of JA and other defense-related compounds (Wasternack and Hause 2013), and many of the respective LOX genes are induced by JA in *Arabidopsis* (Wasternack 2007). However, *AtLOX1* encodes a 9-LOX that is not involved in JA biosynthesis in *Arabidopsis* but is induced after inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 and contributes to local and systemic defense against bacterial pathogens (Vicente et al. 2012) and to JA signaling during *Fusarium graminearum* infection (Nalam et al. 2015). The mRNA of both *M. truncatula* genes accumulated statistically significantly after treatment of leaves with 1 mM MeJA in contrast to mock-treated controls (approximately 27-fold for *Medtr8g018550* and approximately 3,008-fold for *Medtr8g018690*, considering the medians) (Fig. 3C and D).

To investigate whether nodulation might affect transcript levels of SA- and MeJA-responsive genes in leaves of *M. truncatula* plants upon infection, we inoculated 3-week-old nodulated or nitrate-supplemented plants with *E. pisi* as described above and measured transcript accumulation at 1 and 7 dpi (Fig. 4). Although mRNA abundance of the JA-responsive genes *Medtr8g018550* and *Medtr8g018690* did not alter consistently in any of the treatments (Fig. 4C and D), *E. pisi* inoculation induced transcript accumulation of the SA-responsive genes *Medtr2g435490* and *Medtr1g080800* at 7 dpi (Fig. 4A and B). The accumulation of the *Medtr2g435490* and *Medtr1g080800* transcripts in leaves of nodulated plants was slightly, although not statistically significantly, higher than in nonnodulated plants at 7 dpi (Fig. 4A and B). The mRNA of both genes also accumulated marginally at 1 dpi after *E. pisi* inoculation in nodulated compared with nonnodulated plants but

Fig. 3. Effects of salicylic acid (SA) and methyl jasmonate (MeJA) on the transcript abundance of *Medicago truncatula* candidate reporter genes. Three-week-old *M. truncatula* plants were sprayed with either 1 mM MeJA, 10 mM SA, or water as a control and transcript abundance of **A**, *Medtr2g435490*; **B**, *Medtr1g080800*; **C**, *Medtr8g018550*; and **D**, *Medtr8g018690* in leaves was determined at 12 h after the treatment via quantitative reverse-transcription PCR analysis relative to the reference gene *MtTEF1α* (*Medtr6g021800*). Data labeled with the same letters are not statistically significantly different ($P \leq 0.05$; tested by one-way analysis of variance and Tukey's honestly significant difference post hoc test); $n = 3$ independent experiments with ≥ 6 plants per treatment.

this effect was less pronounced as at 7 dpi and not statistically significant (Fig. 4A and B).

Consistent with our findings, Foster-Hartnett et al. (2007) and Samac et al. (2011) observed *E. pisi*-induced transcript accumulation in *M. truncatula* of the PR-4-like *Medtr1g080800* (synonymous with the J. Craig Venter Institute [formerly TIGR] tentative consensus IDs TC125088 and TC101688), together with PR-10-like genes and members of the *chalcone synthase* (*CHS*) gene family. CHSs are involved in phenylpropanoid metabolism contributing to biosynthesis of the isoflavonoid phytoalexin medicarpin in *Medicago* spp. (Dixon et al. 2002; Paiva et al. 1994). Furthermore, *M. truncatula* CHS genes and other phenylpropanoid, flavonoid, and isoflavonoid biosynthesis pathway genes were induced upon infection with the Asian soybean rust *Phakopsora pachyrhizi*; and the isoflavonoid derivative medicarpin, which increases after *P. pachyrhizi* inoculation, contributed to nonhost resistance against this biotrophic fungus (Ishiga et al. 2015). To test whether *E. pisi*-triggered gene expression is enhanced by nodulation, we investigated the transcript accumulation of canonical *MtPR-10* and *MtCHS* genes in our samples. We designed oligonucleotide primer pairs that match multiple isoforms within each gene family (oligonucleotides used for quantitative PCR [qPCR] are listed in Supplementary Table S1). Although the transcript abundance of the respective set of genes increased upon *E. pisi* inoculation (Supplementary Fig. S4), this increase was only statistically significant for the *MtCHS* genes in leaves of

S. meliloti at 7 dpi. Investigation of a possible phytohormone dependency of the transcript elevation, as observed for *Medtr1g080800* and *Medtr2g435490*, suggested an SA-related induction of the *MtPR-10* genes and a JA-triggered repression of the *MtCHS* genes, which was, however, in both cases, not statistically significant.

Enhanced levels of free SA correlate with increased resistance of nodulated plants.

The elevated abundance of SA-responsive transcripts after *E. pisi* inoculation in *M. truncatula* leaves suggested a gain of SA signaling, which was possibly further intensified by nodulation. To test whether the observed increase in transcript abundance of SA marker genes correlates with increased SA content, we measured SA levels in the leaves of nodulated and nonnodulated pea and *M. truncatula*. *E. pisi* inoculation of *M. truncatula* increased the concentration of free SA statistically significantly in leaves of nodulated plants at both 1 and 7 dpi whereas, in nonnodulated plants, an intermediate, not statistically significant increase was only observed at the later time point (Fig. 5A). This result correlates well with the observed greater resistance of nodulated plants at 1 dpi compared with nonnodulated plants whereas, at 7 dpi, the nitrogen-supplemented plants appeared similarly susceptible (Fig. 1C to D). These findings suggest that nodulation allows a faster accumulation of free SA after powdery mildew challenge, which is consistent with the hypothesis that nodulated plants are primed for SA accumulation.

Free SA concentrations were determined in leaves of nodulated and nonnodulated pea plants inoculated with *E. pisi* or not, respectively. Considerably higher amounts of SA were measured in pea (1,200 to 11,900 ng/g fresh weight [FW]) than in *M. truncatula* (20 to 840 ng/g FW) (Fig. 5B). In pea, a statistically significant increase in SA levels was only observed for nodulated plants at 7 dpi. Again, this outcome correlates well with the observation of greater resistance of nodulated compared with nitrogen-supplemented plants only at 7 dpi (Figs. 5B and 2D) but not at 1 dpi, where nodulation did not appear to affect resistance (Fig. 2C). In conclusion, nodulation of pea and *M. truncatula* primed the plants for *E. pisi*-triggered

Fig. 4. Nodulation promotes *Erysiphe pisi*-triggered transcript accumulation of salicylic acid (SA)- but not of jasmonic acid (JA)-responsive *Medicago truncatula* genes. Three-week-old *M. truncatula* plants that had been nodulated with *Sinorhizobium meliloti* or supplemented with 7.5 mM KNO₃ as 2-day-old seedlings were either inoculated with *E. pisi* or kept as a control. Transcript abundance of **A**, *Medtr2g435490*; **B**, *Medtr1g080800*; **C**, *Medtr8g018550*; and **D**, *Medtr8g018690* in leaves was determined at 1 and 7 days postinoculation (dpi) with *E. pisi* via quantitative reverse-transcription PCR analysis relative to the reference gene *MtTEF1α* (*Medtr6g021800*). Data labeled with the same letters are not statistically significantly different ($P \leq 0.05$; tested by one-way analysis of variance and Tukey's honestly significant difference post hoc test); $n = 4$ independent experiments with ≥ 18 plants per time point and treatment and *S. m.* = *Sinorhizobium meliloti*.

Fig. 5. Nodulation primes for *Erysiphe pisi*-triggered salicylic acid (SA) accumulation in *Medicago truncatula* and pea leaves. **A**, The 3-week-old nodulated *M. truncatula* plants; **B**, 2-week-old nodulated pea plants; or the respective KNO₃-supplemented control plants were either inoculated with *E. pisi* or kept as a control. Free SA was extracted from leaves and analyzed by high-performance liquid chromatography at 1 and 7 days postinoculation (dpi). Data labeled with the same letters are not statistically significantly different ($P \leq 0.05$; tested by one-way analysis of variance and Tukey's honestly significant difference post hoc test); $n = 5$ (A) and 3 (B) independent experiments with ≥ 18 (*M. truncatula*) or 9 (pea) plants per time point and treatment. *S. m.* = *Sinorhizobium meliloti* and *R. l.* = *Rhizobium leguminosarum*.

accumulation of free SA that correlated with increased resistance against the powdery mildew fungus.

DISCUSSION

Systemically induced resistance of *M. truncatula* and pea against the biotrophic powdery mildew fungus *E. pisi* coincides with rhizobia-induced priming of SA accumulation and SA-related gene expression.

Using agronomically important pea and *M. truncatula*, a legume model plant closely related to the widely used forage plant *M. alfalfa*, we found that a preestablished nodulation reduced penetration success of the adapted powdery mildew fungus *E. pisi* in *M. truncatula* and conidiation on pea leaves. (Figs. 1 and 2). Comparable nitrogen contents of symbiotic and control plants (Fig. 1A and 2A) demonstrated that the observed protective systemic effect of the bacteria–root interaction on fungal leaf disease did not primarily depend on symbiotically increased overall nitrogen levels. However, nutritional benefits of the symbiosis that will not manifest in measurements of total plant nitrogen cannot be excluded to contribute to the observed effects.

The phytohormones JA and SA are important mediators of the well-studied systemically triggered resistance mechanisms ISR and SAR, respectively. Using *M. truncatula* reporter genes for both phytohormones (Fig. 3), we noted a trend for enhanced accumulation of SA-related rather than JA-dependent transcripts in leaves of nodulated *E. pisi*-inoculated plants (Fig. 4). The fact that the measured tendency for elevated mRNAs of SA-responsive genes was not robust enough to reflect statistical significance among the independent biological replicates on the one hand might rely on the variability of the two applied biological treatments (nodulation and powdery mildew inoculation). On the other hand, a transcriptome analysis after SA or JA treatment of *M. truncatula* would potentially reveal more strongly regulated reporter genes that might facilitate similar analyses in future studies.

The importance of SA-mediated defense in resistance of legumes against the biotrophic powdery mildews is congruent to findings in *Arabidopsis*, where SA signaling has been shown to play a pivotal role in defense against these fungi (Chandran et al. 2009, 2014; Dewdney et al. 2000; Ederli et al. 2015; Kuhn et al. 2016; Zhang et al. 2015). The involvement of SA-related gene expression in defense of *M. truncatula* against *E. pisi* is further substantiated by upregulation of *M. truncatula* homologs of *Arabidopsis* SA-responsive marker genes upon *E. pisi* inoculation in different *M. truncatula* cultivars (Foster-Hartnett et al. 2007). Microarray experiments of penetration resistant (Jemalong A17), postpenetration resistant (A20) and highly susceptible (DZA315.16; used in this study) *M. truncatula* cultivars revealed equally increased transcript abundance of a common set of genes at 12 h post-fungal inoculation in response to *E. pisi* infection in resistant and susceptible cultivars, probably reflecting onset of basal resistance. These genes comprised *CHS* and PR protein-encoding genes, including a *PR-10* gene, a *PR-1*-like gene, *PR-5*-like thaumatin genes, and the hevein-like *PR-4 Medtr1g080800* (TC101688) that we found to be induced by SA, upregulated upon *E. pisi* infection, and primed by nodulation (Figs. 3 and 4) (Foster-Hartnett et al. 2007). Samac et al. (2011) identified *Medtr1g080800* (TC125088) among the most strongly upregulated genes in *M. truncatula* interacting with the leaf-infecting fungi *E. pisi* and *Colletotrichum trifolii* and the root-infecting oomycete *Phytophthora medicaginis*. The accumulation of the *Medtr1g080800* mRNA in different cultivars and during infection with distinct pathogens, together with our expression data, suggests that, in *M. truncatula*, elicitation of SA-induced basal resistance-related gene expression is triggered by *E. pisi* inoculation and potentially elevated by nodulation.

Consistent with a tendency for induction of SA-related gene expression, SA accumulated in a statistically significant manner to higher quantities in leaves of nodulated plants at 1 dpi with *E. pisi* in *M. truncatula* and at 7 dpi in pea compared with nonnodulated plants that had been infected or not with powdery mildew (Fig. 5). Strikingly, this coincided with the respective time points of enhanced penetration resistance (1 dpi; *M. truncatula*) and reduced spore formation (7 dpi; pea) observed for both nodulated plant species (Figs. 1 and 2). This suggests that nodulation triggers priming for SA accumulation and SA-mediated defense, which is active against powdery mildew fungi. The measured basal levels of free SA are comparable with published data from *M. truncatula* and pea leaves (Jaulneau et al. 2010; Liu et al. 2006), respectively. Interestingly, although we could not find published data on pathogen-induced SA values for pea, the *E. pisi*-triggered SA accumulation of *M. truncatula* leaves is comparable with published data of plants (*M. truncatula* line 210F) inoculated with the hemibiotrophic fungus *C. trifolii* at 3 dpi, before the appearance of necrotic lesions (Jaulneau et al. 2010; Torregrosa et al. 2004). This finding suggests that SA-mediated signaling is involved in defense of *M. truncatula* against fungi during a biotrophic infection phase. Further research on the role of SA biosynthesis and signaling in nodulation-mediated powdery mildew resistance is required to confirm this hypothesis. A potential ortholog of *ISOCHORISMATE SYNTHASE 1 (ICS1)*, the major player of SA biosynthesis in *Arabidopsis*, has been identified in *M. truncatula* (Zhang and Franken 2014). However, its functional characterization is missing, and it is not known whether, in *M. truncatula*, SA is primarily synthesized via the ICS pathway, as in *Arabidopsis*, or via the alternative phenylalanine ammonia lyase pathway, which strongly contributes to SA biosynthesis in soybean (Chen et al. 2009; Shine et al. 2016; Wildermuth et al. 2001).

SA as a mediator of ISR.

The observation that SA concentrations and SA-related gene expression are elevated during a root symbiont-triggered ISR-like effect against a shoot pathogen contrasts with the fact that JA is regarded as the major phytohormone mediating induced resistance during ISR (Pieterse et al. 1998, 2009, 2014; Van Wees et al. 2008). Conversely, priming of free SA levels or SA-mediated gene expression occurs during SAR and is known to contribute to resistance of *Arabidopsis* against secondary infection (Jung et al. 2009). Still, JA is not the only critical defense hormone during ISR. Indeed, several reports confirm a contribution or requirement of SA-dependent signaling for ISR-like effects that partly also involves priming of SA-mediated gene expression (Cameron et al. 2013; Pieterse et al. 2014; Van Wees et al. 2008). Priming of SA-inducible PR genes was reported during arbuscular mycorrhiza-triggered ISR of potato (*Solanum tuberosum*) against the hemibiotrophic oomycete *P. infestans* (Gallou et al. 2011) and during systemic resistance of *Arabidopsis* against *Verticillium dahliae* mediated by *Pae-nibacillus alvei* K165 (Tjamos et al. 2005). In the latter interaction, ISR further required intact SA biosynthesis and signaling (Tjamos et al. 2005). Likewise, *Streptomyces* sp. strain EN27-induced systemic resistance of *Arabidopsis* against *F. oxysporum* required SA (Conn et al. 2008). In addition, resistance against the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* and the insect pest *Spodoptera exigua* triggered by *P. fluorescens* SS101 also depends on SA but not on JA (van de Mortel et al. 2012). Furthermore, a role for SA in the induction of ISR of *Arabidopsis* and tomato against *Botrytis cinerea* has been described for several *Trichoderma* strains (Contreras-Cornejo et al. 2011; Martínez-Medina et al. 2013; Mathys et al. 2012). In conclusion, SA-dependent ISR and

priming for SA-related gene expression has been reported for PGPF and PGPR and can mediate immunity against pathogens of different lifestyles and infection strategies.

Nodulation modulates plant SA levels.

When invading the plant, microbes must overcome the first layer of plant defense triggered by the recognition of MAMPs and referred to as MAMP-triggered immunity, which can render the plant resistant against invasion if not impeded by microbial effectors (Dangl and Jones 2001; Jones and Dangl 2006). MAMP-triggered responses include the accumulation of SA, which elicits SA-dependent defense upon microbial recognition (Tsuda et al. 2008). Legumes respond to the recognition of rhizobial MAMPs, similarly as to the recognition of pathogenic bacteria, by initiating an immune response (El Yahyaoui et al. 2004; Kouchi et al. 2004). However, this is quickly suppressed by nod factors that trigger the symbiotic program, eventually leading to nodule formation (Geurts and Bisseling 2002; Liang et al. 2013). As with biotrophic pathogens, high SA concentrations inhibit rhizobial infection (Ferguson and Mathesius 2014). Consequently, together with other MAMP responses, rhizobial nod factors mediate repression of transient SA accumulation that would otherwise inhibit root colonization (Blilou et al. 1999; Foo et al. 2014; Gutjahr and Paszkowski 2009; Martínez-Abarca et al. 1998; Stacey et al. 2006; Subramanian 2013; van Spronsen et al. 2003). Generally, successful establishment and maintenance of nodules is orchestrated by a fine-tuned interplay of distinct phytohormones (Ferguson and Mathesius 2014; Puppo et al. 2005). Therefore, it is not surprising that phytohormone levels are impacted by nodulation. However, whereas alterations in phytohormone homeostasis are well documented for root systems, only a few studies investigated phytohormone concentrations in shoots of nodulated plants and no reports on shoot levels of SA exist to our knowledge (Ferguson and Mathesius 2014; Puppo et al. 2005). Our results demonstrate that, while nodulation alone does not or only slightly increases SA values in the leaves of *M. truncatula* and pea plants, it primes the plants for accumulation of SA after powdery mildew challenge (Fig. 5).

Nodulation differentially affects defense of *M. truncatula* and pea against *E. pisi*.

As mentioned above, the protective effect of rhizobial symbiosis differed depending on the host plant: whereas, in *M. truncatula*, the fungal penetration success but not sporulation was impaired, *E. pisi* formed fewer conidia on pea leaves but showed unaltered penetration (Figs. 1 and 2). A discrepancy between impaired penetration and successful further fungal development, as observed for *M. truncatula*, has been reported also for distinct powdery mildew pathosystems. For example, oat powdery mildew (*Blumeria graminis* f. sp. *avenae*) penetration success on different oat (*Avena sativa*) genotypes does not necessarily correlate with development of secondary hyphae (Sánchez-Martín et al. 2011) and, in wild grapevine (*Vitis rupestris*), highly different penetration rates by *E. necator* can lead to very similar rates of sporulation (Barba et al. 2015).

This heterogeneous impact on penetration (*M. truncatula*) and sporulation (pea) that we observed (i) might reflect distinct degrees of adaptation of the fungus to the two different host species or (ii) may depend on the microsymbiont–host combinations used in our experiments.

(i) Different cultivars or accessions of both investigated hosts, *M. truncatula* and pea, showed different degrees of susceptibility against *E. pisi*, ranging from complete susceptibility through intermediate infection phenotypes to complete resistance. However, most pea and *M. truncatula* cultivars are susceptible to most *E. pisi* isolates (Fondevilla and Rubiales

2012; Prats et al. 2007; Sun et al. 2016). Moreover, the accessions we used (*Pisum sativum* Frisson and *M. truncatula* DZA315.16) are highly susceptible against *E. pisi*, indicating that the fungus is well adapted to both lines.

(ii) ISR-eliciting factors of PGPR comprise surface lipopolysaccharides, exopolysaccharides, flagella, biosurfactants, N-acyl-homoserine lactones, N-alkylated benzylamines, antibiotics, and siderophores, including bacterial SA (Beneduzi et al. 2012). Although rhizobia have not been reported to produce SA, rhizobial strains differ in their capacity to synthesize other siderophores, phytohormones such as gibberellic acid, distinct nod factors, lipopolysaccharides, exopolysaccharides, and effector proteins (Ghosh and Maiti 2016; Tatsukami and Ueda 2016; Wang et al. 2012). They also vary in their ability to fix nitrogen in different plant–rhizobial combinations (Schumpp and Deakin 2010). However, these characteristics are known to mainly affect host specificity, because rhizobial strains specifically colonize certain host plant groups (Laguerre et al. 2012; Wang et al. 2012). Due to their chitooligosaccharide backbone, nod factors closely resemble fungal chitooligomers (Dénarié and Cullimore 1993; Lerouge et al. 1990). MAMPs such as chitooligomers or bacterial flagellin are well-described elicitors of induced resistance (Zipfel et al. 2004) and can also act as a trigger for systemic resistance (Burketova et al. 2015). Therefore, it is conceivable that nod factors and further rhizobial molecules, similarly to the aforementioned PGPR factors (De Vleeschauwer and Höfte 2009; Shores et al. 2010), might act as inducers of ISR. This notion is supported by the study of Duzan et al. (2005), who found that purified nod factors of *Bradyrhizobium japonicum* 532C applied to soybean roots limited colony size of the powdery mildew fungus *Microspora diffusa* on leaves in a dose-dependent manner. Because nod factors and additional potential ISR elicitors such as lipopolysaccharides are also detectable in differentiating or differentiated bacteroids and root cells of nodules (Kannenberg et al. 1994; Skorupska et al. 2006; Timmers et al. 1998), they might contribute to the protective effect of established nodulation that we observed for powdery mildew resistance of *Medicago truncatula* and pea. Because these molecules differ depending on the rhizobial strain (Ghosh and Maiti 2016; Skorupska et al. 2006; Tatsukami and Ueda 2016; Wang et al. 2012), and also the plant receptors or targets of these factors differ in distinct hosts, it is possible that specific rhizobia–host combinations elicit differentially induced defense responses during penetration and postpenetration resistance. An example for this is the effect of PGPR lipopolysaccharides and flagellin on *F. oxysporum*. The respective purified molecules of *Pseudomonas* PGPR strains induced resistance in plants; however, the effect varied depending on the plant species to which these molecules were applied (Alabouvette et al. 2009; Bakker et al. 2007).

Nodulation does not affect proliferation of the hemibiotrophic bacteria *X. campestris* pv. *alfalfae*.

In contrast to the protective effect of nodulation against powdery mildew infection, nodulated *M. truncatula* plants remained fully susceptible against the hemibiotrophic bacterial pathogen *X. campestris* pv. *alfalfae*. Notably, the well-documented protection of arbuscular mycorrhizal plants, which is mainly effective against necrotrophs, also systemically impairs infection by *X. campestris* pv. *alfalfae* (Liu et al. 2007). This seeming discrepancy between our data and published findings might either be related to the different inoculation methods used (syringe infiltration versus spray inoculation) or be attributed to distinct effects of rhizobia and arbuscular mycorrhiza-triggered ISR. Because syringe infiltration bypasses natural ingress into the apoplast, possibly enabling an

earlier switch of the bacteria to a necrotrophic lifestyle, we chose spray inoculation to imitate a naturally occurring infection. The differential effectivity of nodulation-induced ISR against *E. pisi* and *X. campestris* pv. *alfalfae* might be due to the different modes of pathogenic infection and accommodation—penetration of epidermal cells (*E. pisi*) versus colonization of the intercellular spaces via stomata (*X. campestris* pv. *alfalfae*)—or depend on distinct effects on pathogens with different lifestyles. Remarkably, bacterial disease progression quickly led to occurrence of chlorotic or necrotic leaves at 7 dpi, indicating that *X. campestris* pv. *alfalfae* rapidly changes lifestyle from biotrophy to necrotrophy and, therefore, JA- or ET-related ISR mechanisms of arbuscular mycorrhizal plants might be more effective against this pathogen.

ISR: An additional advantage of nodulated legumes?

In conclusion, we found that nodulation mediates increased resistance of two legumes against an adapted biotrophic powdery mildew parasite. Based on our experiments, we hypothesize that this observation potentially relies on priming of the shoot for SA-mediated defense. Interestingly, although the efficiency of nitrogen fixation in the symbiotic association between *M. truncatula* (Jemalong A17) and *S. meliloti* Sm2011 (or the closely related strain Sm1021) is suboptimal, the symbiosis is still persistent (Meade et al. 1982; Schumpp and Deakin 2010; Sulieman and Schulze 2010; Terpolilli et al. 2008). Remarkably, this phenomenon is common, because many legumes and rhizobia form inefficient nodules unable to provide the plant with sufficient nitrogen (Schumpp and Deakin 2010). In addition to regulatory difficulties to suppress inefficient nodulation (Schumpp and Deakin 2010), the beneficial effect on plant protection, despite a lacking nutritional advantage, might be one reason for the evolutionary advantage of the persistence of suboptimal nitrogen-fixing bacteria within nodules. Future investigations of the ability of rhizobial mutants defective in the installation of symbiosis and nitrogen fixation to facilitate ISR will answer the question of whether a functional symbiosis is required for the initiation of nodulation-mediated systemic resistance.

MATERIALS AND METHODS

Plant preparation and growth.

Seeds of the *E. pisi*-susceptible (Foster-Hartnett et al. 2007) *M. truncatula* line DZA315.16 (HM005/L000734; kindly provided by Jean-Marie Prosperi at INRA-Montpellier) were scarified and surface sterilized with sulfuric acid and germinated as described by J. Garcia, D. G. Barker, and E.-P. Journet in the *M. truncatula* handbook. After 2 days of germination on inverted water agar (9%) plates, nine seedlings were transferred to one Sterivent container (107 by 94 by 96 mm; Duchefa, Haarlem, The Netherlands) containing sterile vermiculite that had been washed with water, supplemented with 250 ml of nutritional solution per container (1 mM CaCl₂, 100 μM KCl, 800 μM MgSO₄, 10 μM Fe-EDTA, 35 μM H₃BO₃, 0.3 μM CuSO₄, 8 μM ZnSO₄, 9 μM MnCl₂, 0.5 μM Na₂MoO₄, 20 μM KH₂PO₄, and 20 μM Na₂HPO₄) and autoclaved. Each seedling was inoculated with 500 μl of a suspension of β-glucuronidase (GUS) expressing *S. meliloti* Sm2011 (provided by J. Prell; RWTH Aachen University, Aachen, Germany) at an optical density at 600 nm (OD₆₀₀) = 0.1 in sterile water that had been grown overnight in morpholineethanesulfonic acid-buffered (10 μM; pH 5.7) tryptone-yeast medium (per liter: 5 g of tryptone, 3 g of yeast extract, and 0.9 g of CaCl₂ dihydrate) complemented with streptomycin at 50 mg per liter. Seedlings that were not inoculated with rhizobia were supplemented with nutrient solution that additionally contained 7.5 mM KNO₃.

Height, wet weight, and dry weight of *M. truncatula* plants were determined after 3 weeks.

Seeds of *P. sativum* 'Frisson' (JL2491/id#25879; provided by M. Ambrose, Germplasm Resources Unit, John Innes Centre, Norwich, U.K.) were surface sterilized for 5 min with a 2% sodium hypochlorite solution, washed thoroughly with sterile water, and kept at 4°C in sterile water overnight. Four seeds were transferred into one container and inoculated with GUS expressing *R. leguminosarum* bv. *viciae* 3841 (kindly provided by Jürgen Prell; RWTH Aachen University) at 1 ml/seed, as described for *M. truncatula* and *S. meliloti*. Seeds that were not inoculated with rhizobia were supplemented with 250 ml of nutrient solution that additionally contained 5 mM KNO₃. Pea and *M. truncatula* plants were grown in a daily cycle of 16 h of light (23°C; approximately 125 μmol m⁻² s⁻¹) and 8 h of darkness (20°C).

E. pisi infection assays.

For each independent experiment, 24 containers with nine *M. truncatula* or four pea plants each were prepared. In all, 12 of these containers were supplemented with KNO₃ and 12 were treated with rhizobia. After 2 (pea) or 3 (*M. truncatula*) weeks, half of the plants of each treatment (six containers) were inoculated in an inoculation tower with *E. pisi* conidia that had been generated on susceptible pea plants. Leaves and roots were sampled at 1 (three containers) and 7 dpi (three containers) with *E. pisi* or after mock treatment. Roots were weighed and stained for GUS activity of rhizobia to evaluate nodule numbers per fresh weight. The sampled leaves were pooled and one fraction was frozen in liquid nitrogen and stored at -80°C until further analysis of nitrogen content, transcript accumulation, or free SA content (depending on the experiment). Another fraction was used to analyze the percentage of successful penetration events (1 dpi) and the sporulation (7 dpi). Penetration success was determined as described previously for *Arabidopsis* (Consonni et al. 2006) and asexual spore formation per gram FW was analyzed after collecting the conidia by thoroughly vortexing the leaves with water and counting in a Neubauer improved chamber (Weßling and Panstruga 2012).

X. campestris pv. *alfalfae* infection assays.

Eight containers with nine *M. truncatula* plants each were prepared for each independent experiment for inoculation with *X. campestris* pv. *alfalfae*. Of the eight containers, four were supplemented with KNO₃ and four were inoculated with *S. meliloti*. *X. campestris* pv. *alfalfae* XA-1 (kindly provided by Steven Beer, Cornell University, Ithaca, NY, U.S.A.) was grown in Luria-Bertani (LB) medium and a suspension at OD₆₀₀ = 0.2 was prepared in 10 mM MgCl₂ and 0.04% Silwet. Three-week-old plants were spray inoculated with *X. campestris* pv. *alfalfae* 1 day after incubation of the containers in a closed tray to obtain high humidity and induce opening of stomata. Leaves of *X. campestris* pv. *alfalfae*-inoculated plants were sampled at three different time points: 0 (3 to 6 h after spraying), 3, or 7 dpi. Sampling of the roots was performed as described for *E. pisi*-inoculated plants to determine the number of nodules per gram FW. Sampled leaves were combined into four to six technical replicates per time point, each of which consisted of two distinct leaves of two individual plants (four leaves in total). The leaf material was weighed, surface sterilized in 70% EtOH, and washed twice with water. Afterward, the leaves were ground with a micropestle in a 1.5-ml reaction tube in 500 μl of 10 mM MgCl₂. For each replicate, a dilution series (10⁻¹ to 10⁻⁵) was prepared. Each dilution (10 μl) was plated on an LB plate as technical triplicates. After 2.5 days at 28°C, the colonies were counted and the CFU per gram of FW were calculated.

Phytohormone treatment of *M. truncatula* plants.

Three-week-old *M. truncatula* plants (one container with six or more plants per treatment for each independent experiment, prepared as described above) were sprayed with 10 mM SA, 1 mM MeJA (both in aqueous solution), or water as a control treatment. In all cases, the pH was adjusted to 5.7 and 0.01% Tween-20 was added. The leaves were sampled at 12 h after the treatment, frozen in liquid nitrogen, and stored at -80°C .

Evaluation of nitrogen content by gas chromatography.

The nitrogen content of at least 18 (*M. truncatula*) or nine (pea) plants per time point and treatment as a percentage of dry weight was measured in 1- to 2-mg duplicates/sample of freeze-dried, ground leaf material encapsulated in tin boats, with a Purge and Trap gas chromatography unit equipped with a Vario EL III Element Analyzer (Elementar Analysensysteme, Hanau, Germany) (Dhaliwal et al. 2014). The instrument was calibrated against three samples of acetanilide, which was used as a standard (10.36% nitrogen content).

RNA extraction and quantitative reverse-transcription PCR.

Total RNA was isolated from frozen *M. truncatula* leaves of 18 or more plants per time point and was treated with the innuPREP Plant RNA Kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol. Quality of the RNA was checked spectrophotometrically and via agarose gel electrophoresis. RNA was digested with DNase I and tested via PCR for absence of genomic DNA. cDNA was synthesized with the High-Capacity RNA-to-cDNA Kit and oligodT primers according to the manufacturer's protocol (Thermo Fisher Scientific, Darmstadt, Germany). qPCR was performed with the Takyon No ROX SYBR Mastermix blue dTTP (Eurogentec, Köln, Germany) in 10- μl reactions in a 384-well-plate according to the manufacturer's two-step protocol, with melting curve analysis in a LightCycler 480 (Roche Diagnostics, Mannheim, Germany). Amplicons were sequenced to confirm the product identity. Transcript abundance was calculated with the $\Delta\Delta\text{Ct}$ method (Livak and Schmittgen 2001) relative to the reference gene *ELONGATION FACTOR 1 α* (*MTTF1 α* ; *Medtr8g021800*), which is widely used as a *M. truncatula* reference gene during symbiosis and disease and showed great transcript stability upon JA treatment (Genevestigator database) (Hruz et al. 2008; Nyamsuren et al. 2003; Wulf et al. 2003).

SA extraction and analysis by high-performance liquid chromatography.

Free SA was extracted from approximately 200 mg of frozen leaf material (of at least 18 (*M. truncatula*) or (nine) pea pooled plants per time point and treatment) and quantified after a modified method of Meuwly and Métraux (1993) described by Kiefer and Slusarenko (2003). *o*-Anisic acid was used as an internal standard. In short, after extraction of ground leaves with methanol and partitioning against 50:50:0.5 ethyl acetate-cyclohexane-isopropanol, the organic phases were separated and the solvent removed in a vacuum centrifuge. The residue was dissolved in 100 μl of methanol per 100 mg fresh weight of the primary plant material for further high-performance liquid chromatography (HPLC) analysis. The HPLC was conducted using water-acetic acid (950:50) as eluent A and methanol-acetic acid (950:50) as eluent B to perform the following gradient: 60% A (prerun), 53.5% A (5.5 min), 40% A (7 min), 35% A (17 min), 10% A (20 min), 10% A (25 min), 60% A (26 min), and 60% A (30 min). Under these conditions, *o*-anisic acid and SA were detected after 4.3 and 9.1 min, respectively, using a fluorescence detector set at excitation wavelength 300 nm and emission wavelength 416 nm. The HPLC-system (Jasco, Groß-Umstadt, Germany) consisted of a DG-2080-53

3-Line-Degasser, a LG-980-02 ternary gradient unit, a PU-980 intelligent HPLC pump, a CO-2060Plus Intelligent column thermostat, a AS-1555 intelligent sampler, a FP920 fluorescence detector, and an LC-Net II/ADC. The ChromPass Chromatography Data System, version 1.8.6.1, was used for control and analysis.

Molecular phylogenetic analysis of SA- and JA-responsive marker genes.

BLASTP analysis against *M. truncatula* and *Arabidopsis* was performed with amino acid sequences encoded by *Medtr8g018550*, *Medtr8g018690* (*LOX* genes), *AtPR-1*, and *AtPR-4*. BLAST hits with an E value $< 10^{-4}$ were used for alignment by multiple sequence comparison by log-expectation (MUSCLE) (Edgar 2004). Molecular phylogenetic analysis was conducted in MEGA7 (Kumar et al. 2016). The evolutionary history was inferred by using the maximum-likelihood method based on the most suitable substitution model to correct for multiple changes at the same site during the evolutionary history of the sequences. The models used were the Whelan and Goldman model for AtPR-1 BLAST hits (Whelan and Goldman 2001), the JTT matrix-based model for AtPR-4 BLAST hits (Jones et al. 1992), and the Le and Gascuel model for LOXs (Le and Gascuel 2008). Alignment gaps were partially deleted. The bootstrap consensus tree inferred from 1,000 replicates was calculated (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log-likelihood value. A discrete γ distribution was used to model evolutionary rate differences among sites. All positions with less than 95% site coverage were eliminated.

Statistical analysis and evaluation of priming.

Statistical analysis of quantitative normally distributed experimental data was tested by Levene's test to confirm homogeneity of variance followed by one-way analysis of variance and Tukey's honestly significant difference post hoc test using R/Bioconductor (Gentleman et al. 2004). A Wilcoxon rank sum test was performed for nonnormally distributed data (spores per gram FW) that had been normalized to the respective control sample. Plants were defined as primed for a particular response if the response value of the rhizobium-*E. pisi* treatment was statistically significantly larger ($P \leq 0.05$) than the response values of nitrate-*E. pisi*, rhizobium-mock, and nitrate-mock treatments. Box plots were generated with R/Bioconductor (Gentleman et al. 2004): center lines show the medians, box limits indicate the 25th and 75th percentiles as determined by R software, whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, and outliers are represented by dots.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

M. truncatula genome resource: <http://www.medicagogenome.org>
M. truncatula handbook: <https://www.noble.org/medicago-handbook/>

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