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Exploring a rhizobium to fix nitrogen in non-leguminous plants by using a tumor-formation root pathogen

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Abstract

Over 110 million tons of nitrogen fertilizer every year is used for crop production. Scientists have dreamed of enabling rhizobial nitrogen fixation in non-leguminous crops to mitigate the increasing demand for nitrogen fertilizer. However, despite decades of research, rhizobial nitrogen fixation in non-host plants has not been demonstrated. Here, we reported that an N-fixing rhizobium and a clubroot pathogen *Plasmodiophora brassicae* exhibited a synergistic effect on fixing nitrogen in cruciferous plants. Rhizobia were found to invade *P. brassicae*-infected rapeseed (*Brassica napus*) roots in the field. The colonization of rhizobium on rapeseed roots was confirmed by co-inoculating *Mesorhizobium huakuii* with *P. brassicae* under controlled laboratory conditions. *M. huakuii* infection could alleviate clubroot symptoms and promote the growth of diseased rapeseeds. *M. huakuii* could fix nitrogen in *P. brassicae*-infected plants based on the results of ^{15}N isotope dilution tests. The expression of homologs of legume genes required for symbiosis and early-nodulin genes was significantly upregulated in *Arabidopsis* during early infection by *P. brassicae*. More importantly, *M. huakuii* could even fix nitrogen in *P. brassicae*-resistant rapeseed cultivar and promote plant growth when co-inoculated with *P. brassicae*. Our findings provide a new avenue to understand the interaction of rhizobia with non-host plants, stimulate the exploration of fixing nitrogen in non-leguminous plants by nitrogen-fixing rhizobia, and develop a strategy for both disease control and nitrogen fixation on non-host crops.

Keywords: Rhizobia, Nitrogen fixation, Non-leguminous plants, *Brassica napus*, Plant pathogen, *Plasmodiophora brassicae*

Background

N-fixing rhizobia can establish an endosymbiotic interaction with plants. A diverse group of bacteria defined as rhizobia induce the formation of root nodules in leguminous plants. The nodules contain specialized infected cells to host the bacteria. These intracellular rhizobia are surrounded by a host membrane and are named symbiosomes (Jones et al. 2007; Ivanov et al. 2012). In these nodules, rhizobia can fix nitrogen by reducing atmospheric nitrogen to ammonia, which can then be used by the plant (Jones et al. 2007). However, nitrogen (N)-fixing rhizobia nodulate in leguminous plants almost exclusively, and four *Parasponia* species are the only known exceptions of rhizobium-induced nodule symbiosis with a non-leguminous species (Op den Camp et al. 2012). There has been some research that explores whether mutualistic root-nodule symbiosis also occurs naturally or can be engineered in non-leguminous plants (Peng et al. 2008; Oldroyd et al. 2009; Beatty and Good 2011; Kereszt and Kondorosi 2011; Liang et al. 2013; Rosenblueth et al. 2018; Pankievicz et al. 2019). Rhizobia can

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be isolated from some non-leguminous plants (Peng et al. 2008; Garrido-Oter et al. 2018; Kuzmanović et al. 2018; Yoneyama et al. 2019; Rana et al. 2020; Wasai-Hara et al. 2020; Romero-Perdomo et al. 2021). Certain rhizobia were found to live in the intercellular space of rice roots where they could not induce nodules and fix nitrogen (Perrine-Walker et al. 2007; Chen and Zhu 2013). Nitrogen fixation has been supported by mucilage-associated diazotrophic microbiota in maize (Van Deynze et al. 2018). It is not known if rhizobia could invade a prominent non-host group of cruciferous plants, the *Brassicaceae,* and help fix nitrogen (Op den Camp et al. 2012).

The rhizobium nodule symbiosis may have co-opted the signaling as well as the cellular mechanisms for intracellular survival from arbuscular mycorrhizal (AM) symbioses (Ivanov et al. 2012). Several genes encoding components of the common signaling pathway are essential to induce both endosymbiotic interactions, including SYMRK, a receptor-like kinase located at the plasma membrane (Endre et al. 2002; Stracke et al. 2002), CCaMK, a kinase located in the nucleus, and Cyclops, a transcription factor that is activated by CcaMK (Lévy et al. 2004; Mitra et al. 2004; Gleason et al. 2006; Tirichine et al. 2006; Messinese et al. 2007). Rhizobia require a specific exocytosis pathway for the formation of symbiosomes, and this pathway engages the SNARE protein VAMP72e/d in *Medicago* (Ivanov et al. 2012). Genome sequence analyses showed that several genes encoding components of the common symbiotic signaling pathway (SYM pathway), as well as the symbiosis-specific exocytosis, such as *SYMRK, CcaMK, Cyclops*, and *VAMP72d/e*, were absent in the non-host *Brassicaceae* species, such as *Arabidopsis* and *Brassica* spp. (Delaux et al. 2014; Bravo et al. 2016). The loss of these genes, which are required to establish endosymbiosis with rhizobia, might explain why rhizobia cannot colonize crucifer species.

Clubroot, caused by a protist *Plasmodiophora brassicae*, is a common and very important disease on cruciferous crops (Hwang et al. 2012; Dixon 2014; Liu et al. 2020). Previous research showed that *P. brassicae* invades plants via root hairs and lives intracellularly in cortical cells; the protist can also induce de-differentiation of host cortical cells and subsequent cell division by stimulating the synthesis of auxins, cytokinins, brassinosteroids, and flavonoids in the invaded host cells (Devos et al. 2006; Päsold et al. 2010; Jahn et al. 2013; Ludwig-müller, 2014; Malinowski et al. 2016; Zhao et al. 2017a). Consequently, the pathogen induces the formation of tumors (or galls) and results in the distortion and dysfunction of roots. We sampled symptomatic and non-symptomatic roots of *Brasscai napus* from a naturally *P. brassicae*-infested field and carried out microbiome analyses with deep sequencing techniques. Surprisingly, the DNA of rhizobium species belonging to *Rhizobiaceae* and *Bradyrhizobiaceae* was successfully detected, and the relative abundance of rhizobia in non-symptomatic roots was much higher than that in symptomatic roots (Zhao et al. 2017b). The formation of nodule primordial in the root cortex of rhizobial host plants is preceded by changes in several host physiological processes, including an increase in levels of auxin and other phytohormones (Wang et al. 2014). Since *P. brassicae* and rhizobia share similar host physiological processes, we hypothesized that rhizobia might invade rapeseed together with *P. brassicae.*

In this study, we confirmed the colonization of an N-fixing rhizobium in root hairs and cortex cells of rapeseed by co-inoculating *Mesorhizobium huakuii* and *P. brassicae* under controlled lab conditions. *M. huakuii* infection could alleviate clubroot symptoms and promote the growth of diseased rapeseeds. Interestingly, we found that *M. huakuii* could fix nitrogen in *P. brassicae*-infected plants based on the results of ^{15}N isotope dilution tests. We further demonstrated that nitrogen fixation occurred in the roots of a resistant rapeseed cultivar co-inoculated with *M. huakuii* and *P. brassicae*.

Results

Rhizobia are detected in rapeseed roots naturally infected by *P. brassicae*

Bacteria in the family Rhizobiaceae and Bradyrhizobiaceae were detected by 16S rDNA high-throughput sequencing analysis in root cortical tissues of *P. brassicae*-infected rapeseed plants (Zhao et al. 2017b). PCR amplification with the primer pair RAA-F and RAA-R, which was designed based on the variable region of the 16S rRNA gene of common rhizobia, including *M. huakuii*, *Bradyrhizobium japonicum*, *Sinorhizobium meliloti*, and *Mesorhizobium loti,* confirmed the existence of the family Rhizobiaceae and Bradyrhizobiaceae (Additional file 1: Figure S1a, b). Sequencing results also proved the occurrence of rhizobia in root cortical cells of rapeseed. Further PCR amplification assay showed that rhizobia were found in the roots of 74% *P. brassicae*-infected plants (a total of 50 *P. brassicae*-infected plants were tested).

Chinese milk vetch (*Astragalus sinicus* L.), which forms symbioses with *M. huakuii*, is a leguminous cover crop often used for green manure in China and intercropped in autumn after harvesting the rice in the same field. Therefore, we speculated that *M. huakuii* has a great probability to colonize *P. brassicae*-infected rapeseed roots in such an agricultural setting. Fluorescence in situ hybridization (FISH) analysis was performed with a Cy3-labelled *M. huakuii*-specific probe, and the result indicated the presence of *M. huakuii* in *P. brassicae*-infected root cells (Additional file 1: Figure S1c).

P. brassicae infection facilitates root colonization of rapeseed by *M. huakuii*

Flavonoids produced by legume roots are chemoattractants of rhizobia (Firmin et al. 1986; Spaink et al. 1987; Peck et al. 2006; Cooper, 2007) and have been detected at high levels in *P. brassicae*-infected galls of *Arabidopsis thaliana* (Päsold et al. 2010; Zhao et al. 2017a). Here, we determined the accumulation of flavonoids (quercetin, naringenin, and kaempferol) in root exudates of *P. brassicae*-inoculated rapeseed at 24 h post-inoculation (hpi) via high performance liquid chromatography (HPLC) and found high levels of quercetin, naringenin, and kaempferol with concentrations of 1.83, 0.18, and 1.46 μg/g fr wt, respectively, whereas the levels of naringenin and kaempferol in root exudates of non-inoculated controls were close to the detection limit (< 0.005 μg/g fr wt) (Fig. 1a). The expression of *Nod D*, a gene used to determine the activation of *M. huakuii*, was highly activated in root exudates of *P. brassicae*-infected plants at 6 hpi, even stronger than that in root exudates of Chinese milk vetch (Fig. 1b, c). Thus, root

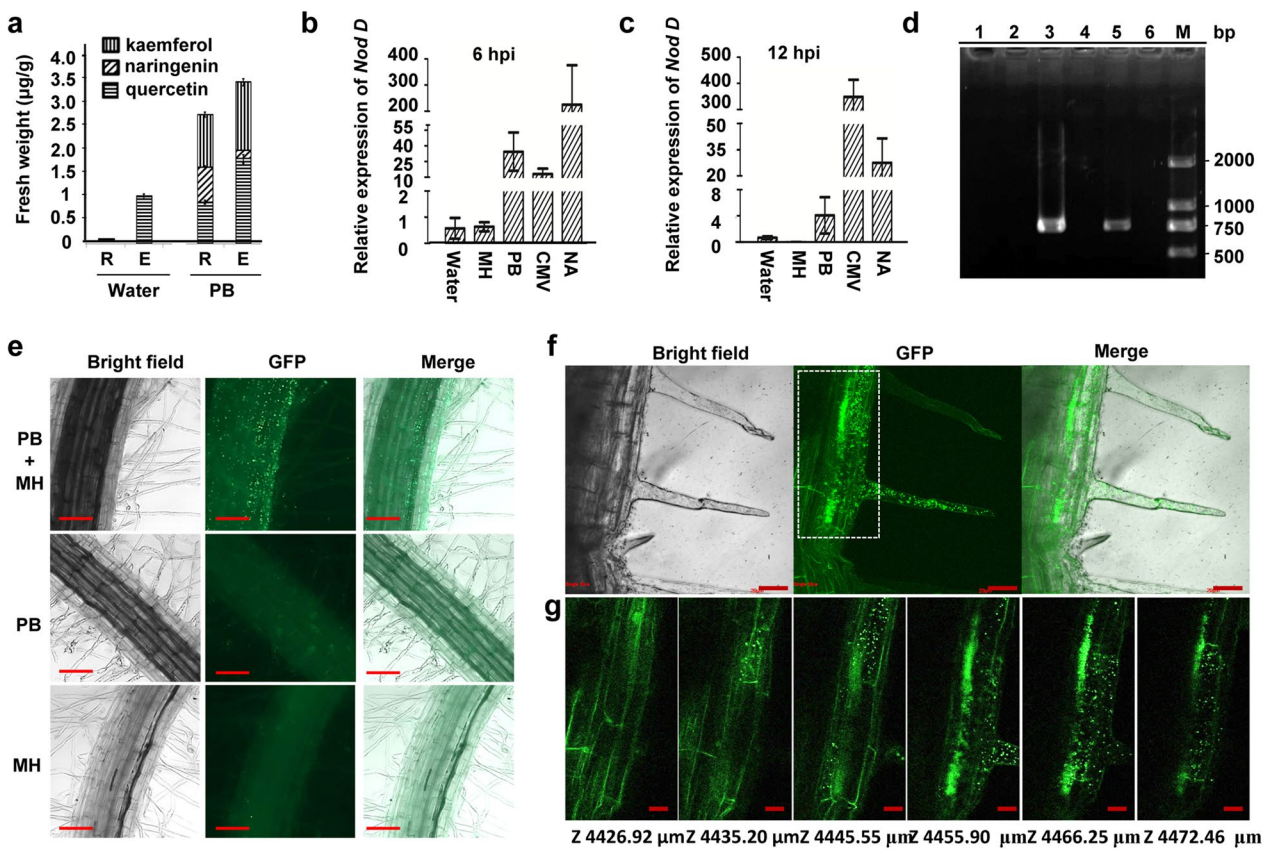

Fig. 1 Attraction of *Mesorhizobium huakuii* to the root cortex of rapeseed by inoculation with *Plasmodiophora brassicae*. **a** Detection of flavonoids in *P. brassicae* (PB)-infected rapeseed roots and root exudates. The rapeseed plants were inoculated with resting spores of *P. brassicae*, and root samples were taken at 24 hpi. R, root; E, root exudate; Water, sterile water-treated rapeseed (control). Fifteen rapeseed plants were used in each treatment and the experiment was repeated three times. **b**, **c** Relative transcript accumulation of *Nod D* of *M. huakuii* in root exudates at 6 and 12 hpi determined by RT-qPCR using the primers NodD-REAL-S and NodD-REAL-A. The transcript level of *Nod D* in the sterile water-treated group was used to normalize the expression levels in different samples. Error bars represent the SE from three replicates. Water, sterile water; MH, *M. huakuii*-inoculated rapeseed; PB, *P. brassicae*-inoculated rapeseed; CMV, Chinese milk vetch; NA, naringenin (0.01 mg/mL)-treated rapeseed. The experiment was repeated three times. **d** Detection of *M. huakuii* in root cortex at 30 dpi by PCR amplification using the primers RAA-F and RAA-R. Lanes 1 and 2, rapeseed seedlings treated with *M. huakuii*-inoculated and *P. brassicae*-inoculated rapeseed root exudates, respectively; Lane 3, Chinese milk vetch seedlings treated with *M. huakui*; Lane 4, rapeseed seedlings treated with naringenin solution (0.01 mg/mL); Lane 5, rapeseed seedlings co-inoculated with *M. huakuii* and *P. brassicae*; Lane 6, rapeseed seedlings treated with sterile water; M, DNA marker. **e** Attachment of *M. huakuii* strain 7653R-GFP on *P. brassicae*-inoculated rapeseed root surface (48 hpi). The GFP signal was observed under a confocal microscope (Olympus, FV1000). MH, *M. huakuii*-inoculated seedlings, PB, *P. brassicae*-inoculated seedlings; PB + MH, *P. brassicae* and *M. huakuii* co-inoculated seedlings, bar = 100 μm. **f**, **g** *M. huakuii* strain 7653R-GFP in root hair and cortical cells of *P. brassicae*-infected root. A set of photographs scanned from the dashed box region in **f** were shown in **g**. The Z stacks are listed at the bottom. Seedlings were sampled at 5–8 dpi. Bar = 20 μm

exudates from *P. brassicae*-infected plants might waken *M. huakuii* and activate the expression of *Nod D*.

Rapeseed seedlings were individually inoculated or co-inoculated with *M. huakuii* strain 7653R-GFP and *P. brassicae* strain ZJ-1, and the roots were examined by confocal microscopy at 48 hpi. Strain 7653R-GFP was found to attach to the surface of co-inoculated roots but not to rapeseed roots without *P. brassicae* (Fig. 1e). These results suggest that rapeseed could only attract the attachment of *M. huakuii* in the presence of *P. brassicae*. Furthermore, *M. huakuii* could not be detected by PCR amplification in the root cortex of rapeseed treated with either *P. brassicae*-inoculated root exudates or flavonoids at 30 days post-inoculation (dpi), while could be detected in the cortex of *P. brassicae* and *M. huakuii* co-inoculated rapeseed roots (Fig. 1d). These results suggested that the colonization of *M. huakuii* in the root cortex of rapeseed is dependent on the existence of *P. brassicae*.

M. huakuii and *P. brassicae* co-inhabit in host cells

To probe if *M. huakuii* could colonize *P. brassicae*-infected rapeseed roots and survive in root cells, a co-inoculation experiment was conducted under controlled laboratory conditions. Rapeseed seedlings were planted in sterilized soil inoculated with *M. huakuii* strain 7653R-GFP (alternatively, 7653R-mCherry) and *P. brassicae* strain ZJ-1 or either, with plants growing in non-inoculated soil as controls. At 5–8 dpi, the rapeseed roots were sampled and analyzed by PCR amplification and fluorescence microscopy. *M. huakuii* was detected in the roots of co-inoculated plants rather than those of the control plants (Additional file 1: Figure S2a, b).

At 5–8 dpi, GFP or mCherry fluorescence reporting the presence of *M. huakuii* was observed inside root hairs and root cortical cells of co-inoculated plants harboring *P. brassicae* (Figs. 1f, g and 2a). At 30 dpi, resting spores were observed in *P. brassicae*-inoculated roots with clubroot symptom; for co-inoculated roots, mCherry-tagged bacteria (spherical structures) were also observed near the membrane of the resting sporangia of *P. brassicae* (Fig. 2b, c), indicating that *M. huakuii* could live in the same cells colonized by *P. brassicae*. This spherical structure of *M. huakuii* was found to be significantly different from its rod-shaped structure (0.5–3.0 μm) under a free lifestyle (Fig. 2d). At the late stage of clubroot development when resting spores were formed in plant cells, *M. huakuii* was detected between the resting spores inside the resting sporangia of *P. brassicae* by all-electric smart fluorescence microscopy and SEM (Additional file 1: Figure S3).

Expression of *GFP* was detected only from GFP-labeled *M. huakuii* (7653R-GFP) in the co-inoculated root samples (Additional file 1: Figure S2c). This further confirmed that *M. huakuii* could live in co-infected rather than in non-infected rapeseed roots. In addition, *M. huakuii* 7653R-GFP could be recovered from the co-inoculated

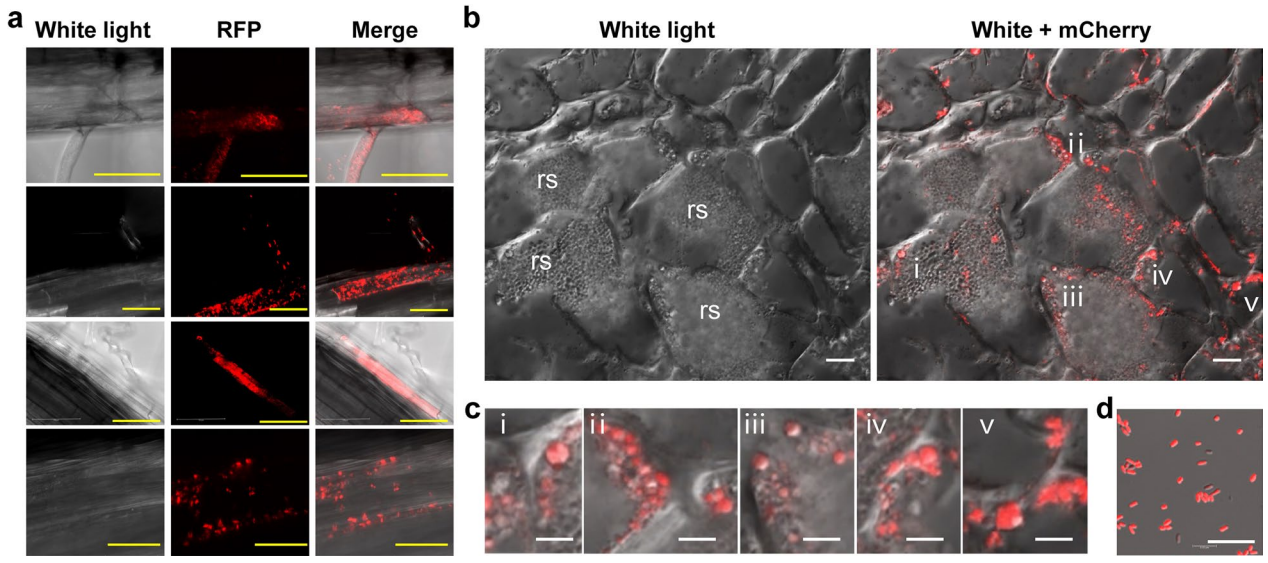

Fig. 2 Co-inhabitation of *Mesorhizobium huakuii* strain 7653R-mCherry and *Plasmodiophora brassica* in cortex cells of rapeseed root. **a** Strain 7653R-mCherry detected in root hair and cortex cells. Co-inoculated seedlings were grown in a growth chamber for 5–8 days. Bar = 50 μm. **b** *M. huakuii* and *P. brassicae* co-inhabit in cortical cells where resting spores (rs) have developed. Co-inoculated seedlings were grown in a growth chamber for 30 days. The 25 μm-thick sections were sliced using a freezing microtome (Lecica CM1950, Germany). Bar = 20 μm. **c** Partically enlarged images of **b**. The corresponding positions are presented as (i) to (V). Bar = 10 μm. **d** The free-lifestyle strain 7653R-mCherry. Images in **b** and **c** were stacked. The red fluorescence was observed under a confocal microscope (Olympus, FV1000). Bar = 10 μm

roots using a medium containing gentamycin sulfate (Additional file 1: Figure S2d). Taken together, the results demonstrated that *M. huakuii* can colonize and persist in rapeseed root cortical cells that are co-infected by *P. brassicae*.

M. huakuii alleviates clubroot symptoms caused by *P. brassicae*

P. brassicae induces club-shaped galls on the infected roots, disrupting root development and function. At late infection stages, diseased plants appear wilted or stunted, show premature senescence, and even die before flowering. To assess whether *M. huakuii* colonization affects the development of clubroot disease, rapeseed seedlings were co-inoculated with *M. huakuii* 7653R-GFP and *P. brassicae* and harvested at 60 dpi. The root system of co-inoculated plants was much more developed than that of the *P. brassicae*-inoculated plants (Fig. 3a, b). The galls on the co-inoculated roots were smaller than those on *P. brassicae*-inoculated roots (Fig. 3a). The colonization of 7653R-GFP could interfere with the gall formation induced by *P. brassicae*, thereby alleviating the disease severity. Inoculation with *M. huakuii* alone had little effect on the development of the rapeseed root system (Fig. 3a, b). Furthermore, both the contents of nitrate nitrogen (19.28 μg/g) and ammonium nitrogen (11.02 μg/g) in the roots of co-inoculated plants were significantly higher than those in *P. brassicae*-inoculated plants (nitrate nitrogen, 12.15 μg/g; ammonium nitrogen, 6.67 μg/g) (Fig. 3c).

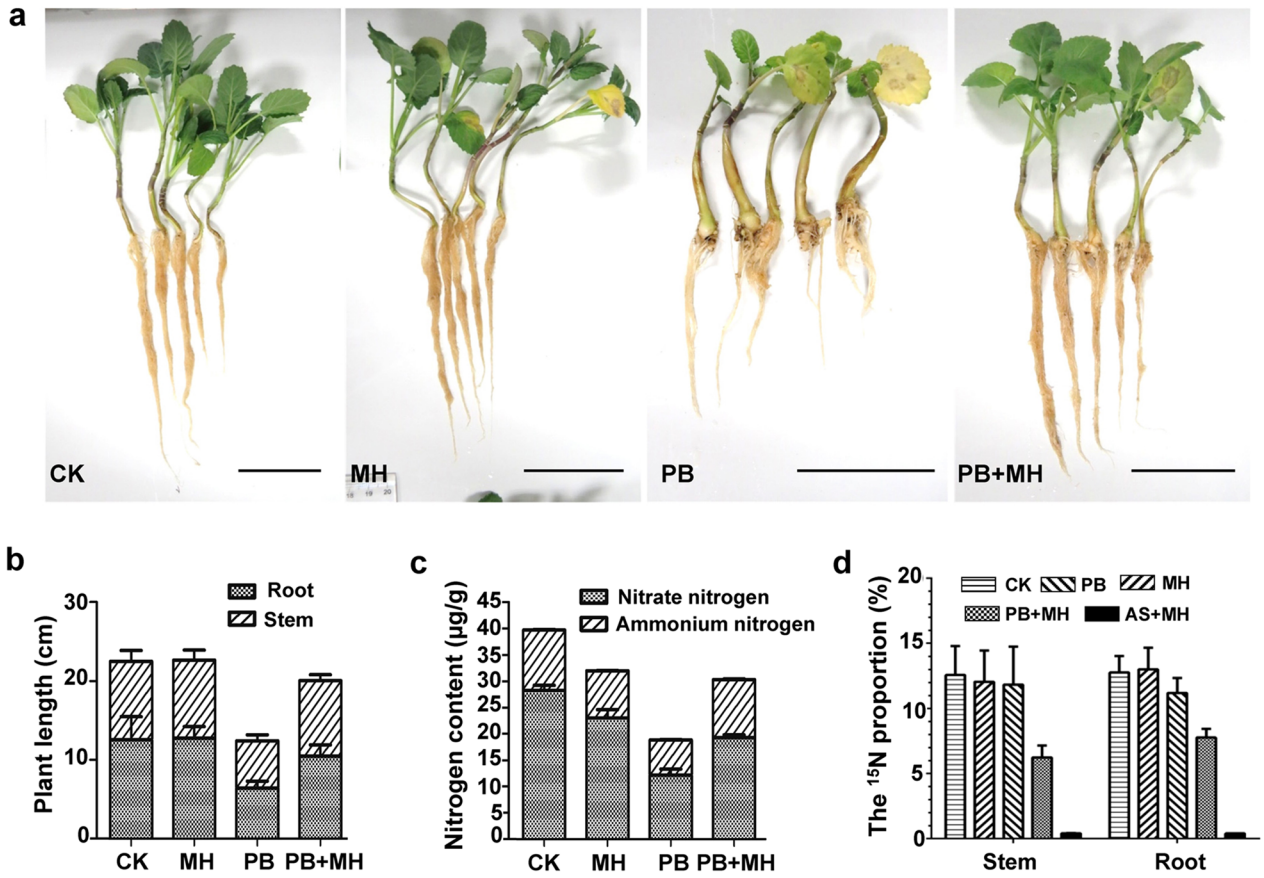

Fig. 3 *Mesorhizobium huakuii* alleviates clubroot symptoms of rapeseed and fixes nitrogen. The seedlings were planted in cups with sterilized garden soil and grown in a growth chamber for up to 60 days without the application of fertilizer during growth. **a** Symptom of rapeseed seedlings. Bar = 5 cm. **b** The average lengths of the root, aerial part, and the whole plant. **c** The nitrogen content in the roots ($P < 0.05$). CK, the non-inoculated seedlings; MH, *M. huakuii*-inoculated seedlings; PB, *Plasmodiophora brassicae*-inoculated seedlings; PB + MH, *P. brassicae* and *M. huakuii* co-inoculated seedlings. **d** The ^{15}N proportion in total nitrogen (%). Rapeseed and *Astragalus sinicus* were planted in pots (4.5 × 4.5 × 5 cm) containing sterilized soil (vermiculite: pearlite = 6:1) and 40 mL of B&D nutrient solution with $K^{15}NO3$ (0.05 g/L) for 45 days. The ^{15}N proportion was determined following a method described by Malik et al. (1987) through rapid combustion using a macro elemental N analyzer (Elementar, vario MAX CN, Elementar Analysensysteme GmbH, Germany). Twenty rapeseed plants were used in each treatment and the experiment was repeated three times

Nitrogen is fixed in co-inoculated roots of *P. brassicae*-susceptible rapeseed

To determine if *M. huakuii* colonization fixes nitrogen in the roots of rapeseed, ^{15}N isotope dilution test was performed according to Malik et al. (1987). Plants either co-inoculated or inoculated alone were grown in $K^{15}NO_3$ (0.05 g/L)-supplemented soil for 45 days, and then the stems and roots were collected for ^{15}N proportion analysis by rapid combustion using a macro elemental N analyzer (Elementar, vario MAX CN, Elementar Analysensysteme GmbH, Germany). The non-inoculated rapeseeds grown under the same condition were used as control. The results showed that the ^{15}N proportion in the co-inoculated rapeseed (6.246 in stem and 7.773 in root) was significantly lower than that in the non-inoculated (12.58 in stem and 12.77 in root), *P. brassicae*-inoculated (11.843 in stem and 11.2 in root), or *M. huakuii*-inoculated (12.053 in stem and 13.023 in root) rapeseeds; in comparison, the lowest ^{15}N proportion (0.416 in stem and 0.406 in root) was examined in *A. sinicus* inoculated with *M. huakuii* (Fig. 3d). Combining the fact that high content of total nitrogen was detected in *P. brassicae* and *M. huakuii* co-inoculated plants (Fig. 3d), it was concluded that *M. huakuii* in co-inoculated plants might fix nitrogen using criteria from Unkovich et al. (2008).

M. huakuii co-colonizes *P. brassicae*-resistant rapeseed and fixes nitrogen

The fact that *M. huakuii* and *P. brassica* could be detected in non-symptomatic roots of rapeseed suggests tumor formation may not be a prerequisite for the colonization of *M. huakuii* in *P. brassicae*-infected roots; thus, we inoculated a resistant rapeseed cultivar Huashuang 5R with both *P. braissicae* and *M. huakuii* to investigate whether *M. huakuii* functions on the resistant cultivar. We found that both the root system and aerial parts of *P. brassicae*-inoculated plants were significantly smaller than those of the non-inoculated plants and *M. huakuii*-inoculated plants despite that *P. brassicae* could not induce tumors in the roots of Huashuang 5R (Fig. 4a–f). PCR analysis results showed that *P. brassicae* could invade the root cortex of the resistant cultivar Huashuang 5R (Fig. 4g), indicating that Huashuang 5R still has a considerable cost to deal with the infection of *P. brassicae*. In comparison, *P. brassicae* and *M. huakuii* co-inoculated plants showed the most vigorous growth of aerial parts and roots, while plants inoculated with *M. huakuii* alone did not show any significant difference from the non-inoculated plants (Fig. 4a–f), demonstrating that *P. brassicae* and *M. huakuii* have synergistic effect on improving the growth of Huashuang 5R. Further PCR analysis and confocal microscope observation suggested that *M. huakuii* inhabited the root cortex of *P. brassicae*-inoculated roots of Huashuang 5R (Fig. 4h–j).

Again, the ^{15}N isotope dilution test was used to determine if *M. huakuii* could fix nitrogen on the roots of Huashuang 5R. All non-inoculated and inoculated Huashuang 5R plants appeared symptoms of nitrogen deficiency, plants inoculated with *P. brassicae* showed the severest symptom, while plants co-inoculated with *P. barassicae* and *M. huakuii* showed mildest symptom (Fig. 5a). The proportion of ^{15}N in stems and roots of *P. brassicae*-inoculated plants was significantly higher than those of the co-inoculated plants (Fig. 5b), suggesting that *M. huakuii* might also fix nitrogen in co-inoculated roots of Huangshuang 5R.

P. brassicae upregulates the expression of legume homologous genes required for symbiosis in *Arabidopsis*

Since *A. thaliana* is also a host of *P. brassicae*, it was used here to probe the possible mechanism underlying the co-colonization of *M. huakuii* with *P. brassicae*. We co-inoculated *M. huakuii* and *P. brassicae* on the roots of *A. thaliana* and found that *M. huakuii* could invade *A. thaliana* roots and grow in the cortex cell (Fig. 6a). In *A. thaliana*, the homologs of legume genes required for nitrogen-fixing symbiosis (for bacteria invasion and nodule formation) (Additional file 2: Table S1) or early-nodulin (Additional file 2: Table S2) were identified, and their expressions were examined in *P. brassicae*-infected *Arabidopsis* roots in comparison with those from control plants using reverse transcription-quantitative PCR (RT-qPCR) analysis. We found that at least four symbiosis-related *Arabidopsis* homologs, namely, *SYMRK*, *NIN*, *NSP1*, and *SYMREM1*, were significantly upregulated during *P. brassicae* infection (Fig. 6b). *Arabidopsis* homologs of legume early nodulin genes encoding EamA-like transporter family protein (such as NMtN21-1, NMtN21-2, NMtN21-3, NMtN21-5, and NMtN21-6) genes and early nodulin-like protein (such as ENODL14, AtENODL8, and AtENODL15) genes were also significantly upregulated (Fig. 6c). Thus, it is likely that a symbiosis-like cellular program is ectopically activated upon infection by the protist pathogen.

Discussion

Human beings increasingly rely on nitrogen fertilizer for crop production to obtain a high yield. The consumption of nitrogen fertilizer was 104 million tons in 2018 and increased to 113.7 million metric tons in 2021 (https://www.statista.com). The growing demand for nitrogen fertilizer has worsened the global energy crisis. In this study, we detected rhizobia in rapeseed roots that had been naturally infected with *P. brassicae* and observed

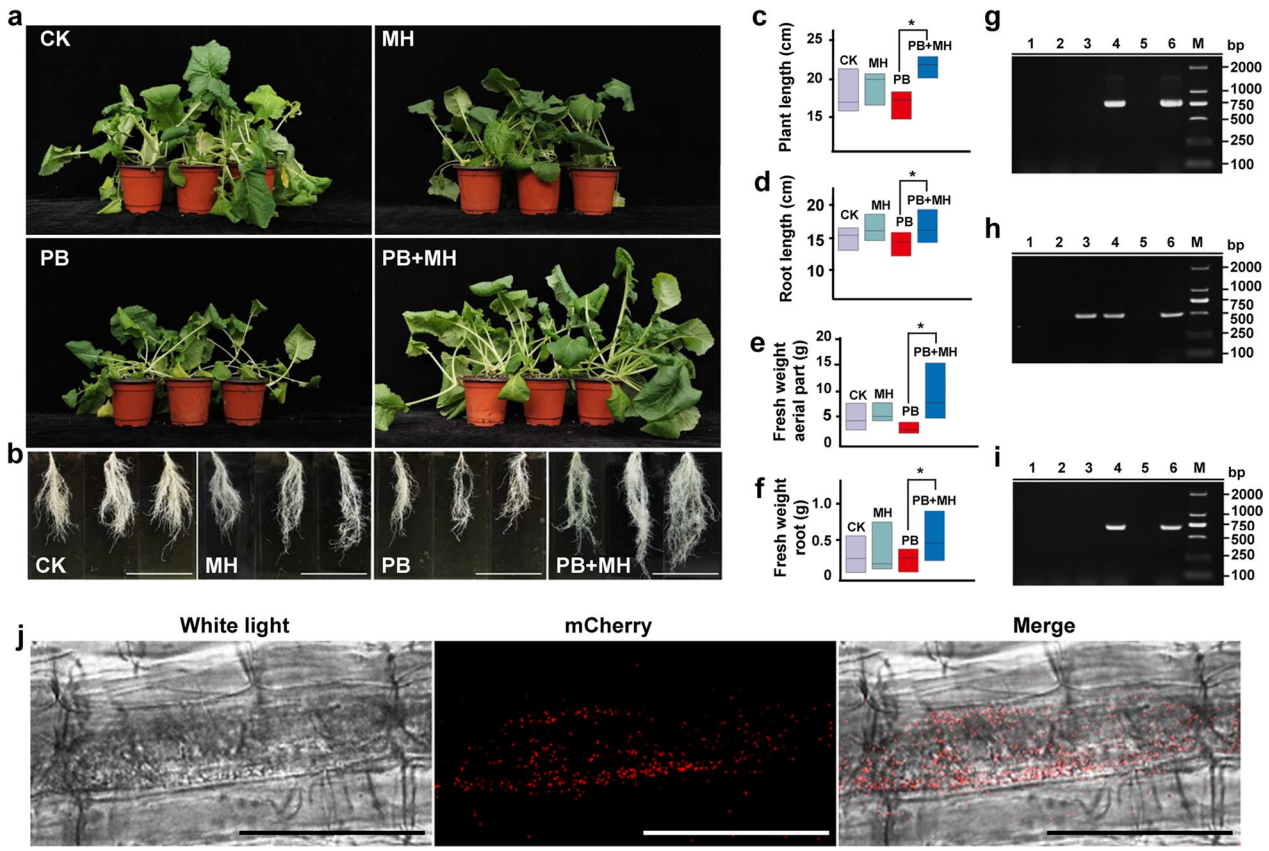

Fig. 4 Growth promotion of resistant rapeseed cultivar Huashuang 5R by *Mesorhizobium huakuii* under *Plasmodiophora brassicae* infection. **a**, **b** Morphologies of aerial parts and roots of treated plants at 30 dpi. No fertilizer was applied during plant growth. **c**–**f** The length and weight of aerial parts and roots; MH, *M. huakuii*-inoculated seedlings; PB, *P. brassicae*-inoculated seedlings; PB + MH, *P. brassicae,* and *M. huakuii* co-inoculated seedlings. **g**, **h** PCR detection of *P. brassicae* and *M. huakuii*. **i** Detection of *GFP* expression by RT-PCR amplification. **g**, **h** and **i** share the same root samples. Lane 1, non-inoculated roots; Lane 2, *M. huakuii*-inoculated roots; Lane 3, *P. brassicae*-inoculated roots; Lane 4, co-inoculated roots; Lane 5, sterile water-treated control; Lane 6, a mixed DNA sample of *M. huakuii* and *P. brassicae*. **j** *M. huakuii* strain 7653R-mCherry lives in *P. brassicae*-inoculated roots of resistant rapeseed cultivar. Seedlings were sampled at 30 dpi. The fluorescence was observed under a confocal microscope (Olympus, FV1000). Bar = 50 μm. Fifteen rapeseed plants were used in each treatment and the experiment was repeated three times

the symbiosis infection of a rhizobium, *M. huakuii,* in *P. brassicae*-infected roots of rapeseed. We further found that *M. huakuii* could also successfully invade rapeseed root cortex and fix nitrogen there when co-inoculated with *P. brassicae*. Thus, we provide a potential way to fix nitrogen with rhizobia on non-leguminous crops; namely, co-inoculation of rhizobia with other microbes that can help rhizobia invade non-leguminous and build a proper environment for living and fixing nitrogen.

The co-infection mechanism of *M. huakuii* and *P. brassicae* is not known, but it may not be tumor-dependent because *M. huakuii* could colonize *P. brassicae*-resistant rapeseed cultivar infected by *P. brassicae* and could live in non-symptomatic roots of susceptible rapeseed that had been infected by *P. brassicae*. How *M. huakuii* invades and fixes nitrogen under such a cellular environment requires further investigation in the future. Furthermore, besides *M. huakuii,* other nitrogen-fixing rhizobia including *B. japonicum*, *S. meliloti*, and *M. loti* could also co-exist with *P. brassicae* on rapeseed roots (Additional file 1: Figure S4), suggesting that the co-invasion of rhizobia and *P. brassicae* may occur widely on cruciferous plants in nature.

P. brassicae initially infects root hairs of cruciferous plants. At this stage, swollen tips of root hair and deformed root hairs are frequently observed (Luo et al. 2014). *P. brassicae* might decrease cell wall integrity of root hairs to allow the entry of zoospores. Auxin homeostasis plays an essential role during *P. brassicae* infection and gall formation, and plant hormones such as cytokinins, auxin, and brassinosteroids are involved in symptom development (Devos et al. 2006; Jahn et al. 2013; Jahn et al. 2014). Flavonoids are highly produced in root galls formed on *Arabidopsis* (Päsold et al. 2010), and the induction of these secondary metabolites was also detected in *P. brassicae*-infected roots in the present

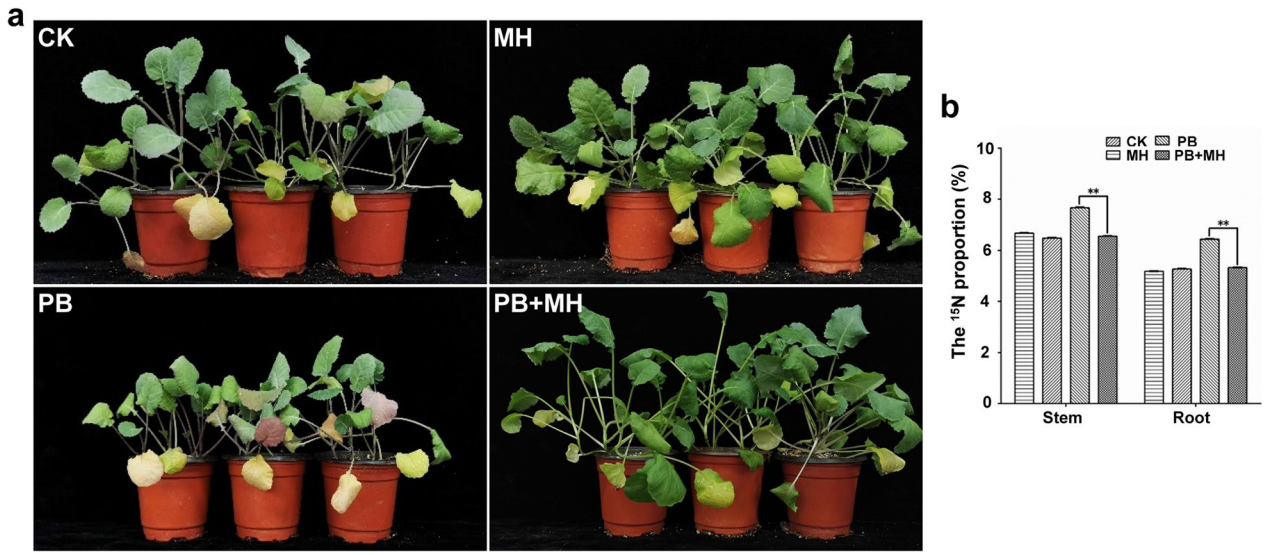

Fig. 5 *Mesorhizobium huaiikui* fixes nitrogen in *Plasmodiophora brassicae*-inoculated resistant rapeseed cultivar Huashuang 4. **a** Morphologies of treated plants. **b** The ^{15}N proportion in total nitrogen (%) of treated rapeseed. Huashaung 5R seedlings were planted in pots containing sterilized soil (vermiculite: pearlite = 6:1) and 40 mL of B&D nutrient solution with $K^{15}NO3$ (0.05 g/L) for 30 days. The ^{15}N proportion was determined following a method described by Malik et al. (1987) through rapid combustion using a macro elemental N analyzer (Elementar, vario MAX CN, Elementar Analysensysteme GmbH, Germany). CK, non-inoculated seedlings; MH, *M. huakuii*-inoculated seedlings; PB, *P. brassicae*-inoculated seedlings; PB + MH, *P. brassicae* and *M. huakuii* co-inoculated seedlings. Fifteen rapeseed plants were used in each treatment and the experiment was repeated three times

study, which were exuded into the environment as early as 24 hpi (Fig. 1a). Flavonoids are also required for the biosynthesis of Nod factors (Long 1996), a key early step of symbiotic interaction with legume hosts, and flavonoid-rich root exudates of *P. brassicae*-inoculated rapeseed were found to activate free-living *M. huakuii* present in rapeseed rhizosphere. Flavonoids have been proven to affect polar auxin transport in plants (Brown et al. 2001; Wasson et al. 2006; Santelia et al. 2008). Therefore, we hypothesized that flavonoids and auxin homeostasis induced by *P. brassicae* might contribute to the invasion of *M. huakuii*.

Our finding that *P. brassicae* infection could help the entry of rhizobia into the non-leguminous rapeseed suggests that *P. brassica* may exploit a plant cellular signaling mechanism similar to that of rhizobia for entry and colonization. This finding also suggests that the taxonomic constraints for root colonization by rhizobia might not be insurmountable. Recent research revealed that plant immunity plays a crucial role in rhizobium–legume symbioses and rhizobia need to suppress legume defenses before establishing symbiosis (Gourion et al. 2015). Likely, the successful suppression of rapeseed defenses by *P. brassicae* may also contribute to the colonization of *M. huakuii* in rapeseed root; furthermore, we found that the expressions of *Arabidopsis* homologs of legume genes required for rhizobia symbiosis, early-nodulin genes encoding EamA-like transporter family proteins, and early nodulin-like protein genes were significantly up-regulated after inoculation with *P. brassicae* (Fig. 6b, c), suggesting that *P. brassicae* shares some signal pathways with rhizobia. Further research on the interaction between *P. brassicae* and cruciferous plants, especially those concerning the pathogenic mechanisms of *P. brassicae* and the role of cruciferous homologs of legume genes in *P. brassicae* infection and colonization with rhizobia, may lead to further expansion of the host range of symbiotic rhizobia and high efficiency of nitrogen fixation on non-host plants.

Conclusions

In this study, we detected rhizobia in rapeseed roots naturally infected by *P. brassicae*, observed the symbiosis infection of a rhizobium, *M. huakuii,* in *P. brassicae*-infected rapeseed roots, and proved that rhizobia could also successfully invade the root cortex of rapeseed co-inoculated with *P. brassicae* and fix nitrogen in roots. *M. huakuii* infection alleviated clubroot symptoms and promoted the growth of diseased rapeseeds. Taken together, we provide a potential way to fix nitrogen with rhizobia on non-leguminous crops.

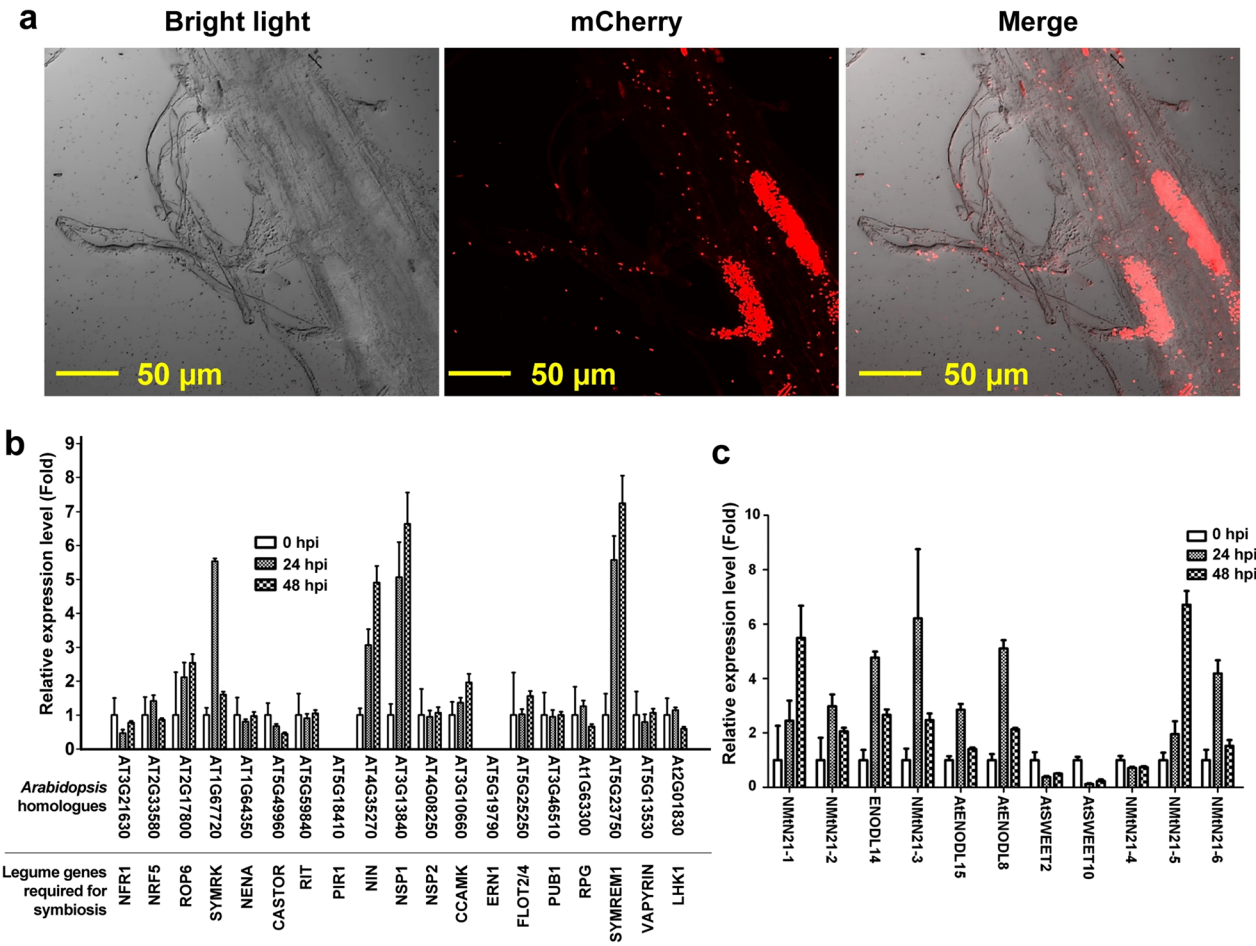

Fig. 6 A symbiosis-like cellular program occurs in rapeseed under *Plasmodiophora brassicae* challenge. **a** *Mesorhizobium huakuii* colonization in root hairs and cortical cells of *P. brassicae*-infected roots of *Arabidopsis thaliana*. *P. brassicae* and *M. huakuii* strain 7653R-mCherry co-inoculated seedlings were incubated in a growth chamber for 5–8 days. The red fluorescence was observed under a confocal microscope (Olympus, FV1000). **b** Relative expression levels of legume homologous genes required for symbiosis in *Arabidopsis* inoculated with *P. brassicae* resting spores. The *Arabidopsis* homologs along with their corresponding legume genes (abbreviated names) were listed on the x-axis, and the full names of legume genes were shown in Additional file 2: Table S1. **c** Relative expression levels of legume homologous genes for early formation of the nodule in *Arabidopsis* inoculated with resting spores of *P. brassicae*. The abbreviations on the x-axis represent *Arabidopsis* homologs of the corresponding legume early-nodulin genes, which are listed in Additional file 2: Table S2. The gene expression was detected with RT-qPCR. The expression level of each gene at 0 hpi was set as 1.0. The expression level of *actin* was used to normalize each gene in different samples. The primer pairs are listed in Additional file 2: Table S4

Methods

P. brassicae, rhizobia, plants and growth conditions

P. brassicae strain ZJ-1 was originally isolated from a naturally diseased rapeseed plant in Zhijiang County, Hubei Province, China. The resting spores were extracted and purified following the method described by Castlebury et al. (1994). *M. huakuii* is a nitrogen-fixing bacterium that forms symbioses with a leguminous plant, Chinese milk vetch (*A. sinicus*). 7653R, a streptomycin-resistant mutant of strain 7653 and a well-studied *M. huakuii* strain (Wang et al. 2014), was originally isolated from Chinese milk vetch in Yangzhou, Jiangsu Province in the early 1970s. Strain 7653R labeled with a green fluorescent protein (GFP) and a red fluorescent protein (mCherry) was named 7635R-GFP and 7635R-mCherry, respectively. Other rhizobial strains, including *B. japonicum* USDA110, *S. meliloti* Sm2011, and *M. loti* MAFF303099, were kindly gifted by Prof. Zhongming Zhang (Huazhong Agricultural University).

All rhizobial strains were cultured in TY medium (yeast powder 3 g, tryptone 5 g, $CaCl_2$ 0.6589 g in 1000 mL ddH_2O with a pH value of 7.0) with gentamycin sulfate (10 μg/mL) at 28 °C. For inoculation, the rhizobial cultures ($OD_{600}=0.5$) were precipitated at 4000 g for 10 min

and re-suspended with sterilized water. Clubroot-susceptible rapeseed cultivar Zhongyou 821, clubroot-resistant cultivar Huashaung 5R, Chinese milk vetch cultivar Heshengzi 1, and *A. thaliana* Ecotype Columbia (Col-0) used in this study were cultured and propagated in a plant-growth chamber (20 ± 1 °C, 14 h/10 h light/dark, and 100 mmol photons/m^2.s (Wuhan Ruihua Instrument & Equipment Co. Ltd, Wuhan, China).

PCR detection

The diseased rapeseed plants were randomly sampled from a *P. brassicae*-contaminated field where Chinese milk vetch was inter-planted. Root samples were carefully washed with tap water, and their surface layers were peeled off with a sterilized razor. Then, approximately 500 mg of root cortical tissues were sampled for DNA extraction with CTAB. *P. brassicae* detection was performed following the protocol described by Wallenhammar and Arwidsson (2001). The expected size of PCR products using the primer pair PbITS6/PbITS7 is 512 bp. To detect rhizobia, the primer pair RAA-F/RAA-R was designed based on the 16S ribosomal RNA sequences of *M. huakuii*, *B. japonicum*, *S. meliloti*, and *M. loti*, with an expected PCR product of 761 bp. The conditions for PCR amplification included denaturation at 95 °C for 5 min and 32 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min. The PCR products were sequenced by Beijing Genomics Institute (BGI). Rapeseed plants individually inoculated or co-inoculated with *P. brassicae* and *M. huakuii* were used as controls. The primers were listed in Additional file 2: Table S3.

Co-inoculation of rapeseed with *P. brassicae* and *M. huakuii*

Seeds of susceptible rapeseed cultivar Zhongyou 821 were surface-sterilized with sodium hypochlorite (5.5%) for 3 min and sown in each pot (4.5 × 4.5 × 5 cm) with sterilized garden soil (Zhenjiang Beilei Organic Fertilizer Co., Ltd., China). Ten-day-old seedlings were inoculated with 2 mL of *P. brassicae* resting spore suspension (1×10^7 spores/mL) following the instruction of Päsold et al. (2010) and 2 mL of cell suspension ($OD_{600} = 0.6$) of *M. huakuii* strain 7653R-GFP or strain 7653R-mCherry. Rapeseed seedlings inoculated with the same volume of resting spores, bacterial suspension, or ddH_2O were used as controls. Seedlings of Chinese milk vetch inoculated with strain 7635R were also used as a control. Forty plants were used for each treatment. The plants were grown in the chamber for 30 days to allow for gall formation without fertilizer during growth. The co-inoculated rapeseed seedlings were subjected to (RT)-PCR detection and re-isolation of the inoculated bacteria, and green or red fluorescence observation was performed under a confocal microscope, an all-electric smart fluorescence microscope, or scanning electron microscope (SEM).

Co-inoculation of rapeseed with *P. brassicae* and other rhizobia

Seeds of susceptible rapeseed cultivar Zhongyou 821 were surface-sterilized and sown following the method described above. Ten-day-old seedlings were inoculated with 2 mL of resting spores of *P. brassicae* and 2 mL of the cell suspension ($OD_{600} = 0.6$) of strain *B. japonicum* USDA110, *S. meliloti* Sm2011, or *M. loti* MAFF303099. Seedlings inoculated with the same volume of *P. brassicae*, bacterial suspension, or ddH_2O were used as controls. The plants were grown in the chamber for 30 days to allow gall formation without fertilizer during the growth. The co-inoculated rapeseed seedlings were subjected to (RT)-PCR detection of *P. brassicae* and rhizobial strains.

Fluorescence in situ hybridization (FISH)

FISH was used to further confirm the co-infection of rapeseed roots by *P. brassicae* and *M. huakuii*. Root tissues from the *P. brassicae*-contaminated field were carefully washed with tap water and embedded in a frozen embedding medium (Sakura Finetek USA, Inc., Torrance, CA, United States) at -23 °C overnight. The root tissues (25-μm thick) were sliced using a freezing microtome (Lecica CM1950, Germany).

For *M. huakuii*-specific probe, fragments amplified with the primer pair RAA-F/RAA-R using genomic DNA of *M. huakuii* as a template were labeled with Biotin-14-dUTP (containing CY3 Label) via the Random Primer method of BioPrime DNA Labeling System (Invitrogen™ Thermo Fisher Scientific (China) Co., LTD) following the instructions. Hybridization and immunological detection were conducted according to the methods of Speel (1999) with minor modifications using a laser scanning confocal microscope (Olympus FV1000, Japan); the excitation wavelengths were 559 nm for Cy3 and 405 nm for 4',6-diamidino-2-phenylindole (DAPI).

Fluorescence observation

To detect GFP-labelled or mCherry-labelled rhizobia (7653R-GFP or 7653R-mCherry) in co-inoculated roots, a freezing microtome (Leica CM1950, Germany), a laser scanning confocal microscope (Olympus FV1000, Japan) and an all-electric smart fluorescence microscope (Olympus BX63, Japan) were used. Non-inoculated roots, *P. brassicae*-inoculated roots, and rhizobia-inoculated roots were also examined as controls.

To observe GFP-labelled rhizobia (7653R-GFP) attaching to the surface of co-inoculated rapeseed roots, the seedlings were taken out from the soil at 2 dpi. After

washing with sterile water three times, the roots were observed under a laser-scanning confocal microscope.

To observe the colonization of strains 7653R-GFP or 7653R-mCherry (RFP-labelled *M. huakuii*) on rapeseed roots at the early infection stage, co-inoculated rapeseed seedlings were taken out from soil at 5–8 dpi. The root hairs and cortical cells were observed under a laser-scanning confocal microscope.

To observe the colonization of 7653R-GFP or 7653R-mCherry on rapeseed roots at the late infection stage (30–45 dpi), root tissues, embedded in Tissue-Tek O.C.T. Compound medium (Sakura Finetek USA, Inc., Torrance, CA) at -23 °C overnight and sliced using a freezing microtome, were observed under a laser scanning confocal microscope or an all-electric smart fluorescence microscope. The experiment was repeated three times.

Scanning electron microscopy (SEM) observation

To further observe *M. huakuii* growing in rapeseed root galls induced by *P. brassicae*, rapeseed roots co-inoculated with *P. brassicae* and *M. huakuii* were collected, carefully washed and cut into small segments of 1–2 cm in length. The root segments were transversely sectioned with a sharp razor and subjected to SEM (Hitachi SU8010, Japan), and the images were made at an acceleration voltage of 10 kV. *P. brassicae*-inoculated or *M. huakuii*-inoculated roots were also examined as controls.

Re-isolation of *M. huakuii* from rapeseed roots

To confirm that *M. huakuii* indeed colonized rapeseed roots infected by *P. brassicae*, roots of co-inoculated rapeseed seedlings were surface-sterilized with 70% ethanol for 30 s, treated with 0.1% mercuric chloride ($HgCl_2$) for 2 min and rinsed with sterilized water for 3 times. Then, the root surface layer was peeled with a sterilized razor and the root cortex was finely ground with a mortar and pestle. The resulting root juice was diluted with a proper volume of sterilized water, spread on TY medium amended with gentamycin sulfate (10 μg/mL) and incubated at 28 °C for 72 h. The colonies that appeared were assessed under a fluorescence microscope (emission wavelength of 488 nm) (Olympus SZX16, Japan). Rapeseed seedlings inoculated with either *P. brassicae* or *M. huakuii* and Chinese milk vetch seedlings inoculated with strain 7653R-GFP were used as controls. Five plants were examined for each treatment.

HPLC flavonoid analysis

To determine if *P. brassicae* could induce rapeseed to produce flavonoids at an early stage of infection, 10-day-old rapeseed seedlings growing were transplanted into 2 mL EP centrifuge tubes, and 1 mL of resting spore suspension (1×10^7 spores/mL) was then added. The inoculated seedlings were placed in a growth chamber for 24 h and the flavonoids in the roots and exudates (spores removed) were detected with HPLC following the method of Päsold et al. (2010). Sterilized water was used instead of the resting spore suspension as a control. Three flavonoids, namely, quercetin, naringenin, and kaempferol (Sigma), were used as the standard samples. The experiment was performed twice.

Effect of *M. huakuii* on *P. brassicae*-inoculated rapeseed

Seeds of susceptible cultivar Zhongyou 821 or resistant cultivar Huashuang 5R were surface-sterilized with sodium hypochlorite and placed on wet filter paper for pre-germination, and then were sown in pots ($4.5 \times 4.5 \times 5$ cm) filled with sterilized garden soil at a density of three seeds per pot. After incubation in a plant growth chamber for 10 days, the rapeseed seedlings were inoculated with 2 mL of resting spore suspension (1×10^7 spores/mL) of *P. brassicae* and 2 mL of cell suspension ($OD_{600} = 0.6$) of strain 7653R-GFP. Seedlings inoculated with the same volume of resting spores, *M. huakuii* cell suspension, or treated with the same volume of ddH_2O were used as controls. After an additional 6–7 weeks, the plants were gently washed and the root morphology was observed. The experiment was performed twice.

Nitrogen detection in roots

A salicylic acid colorimetric analysis was used to detect the nitrate nitrogen and a Nesslerization method to detect the ammoniate nitrogen in rapeseed roots following Cataldo et al. (1975) and Golterman (1991) with minor modifications. Fresh roots were gently washed and weighed after removing the remaining water. The roots were then ground into a powder with liquid nitrogen and suspended in 3 mL of ddH_2O per gram of roots in a sterile centrifuge tube. After a boiling water bath for 30 min, the supernatant was collected by centrifugation at 7500 g for 10 min. For nitrate nitrogen detection, 0.1 mL supernatant was transferred into a new centrifuge tube containing 0.4 mL of 5% salicylic acid-sulphuric acid solution (5 g salicylic acid dissolved in 100 mL of sulphuric acid solution), mixed well, and then placed at room temperature for 20 min. Next, 9.5 mL of 8% sodium hydroxide solution was added slowly and cooled to room temperature, and then the absorbance at a wavelength of 410 nm was measured using an automatic microplate reader (BOX 998, BioTek® Instruments, Inc. USA). To detect the content of ammoniate nitrogen in the roots, 100 μL of supernatant was mixed with 448 μL of solution A (20 g sodium hydroxide, 26.8 g disodium hydrogen phosphate heptahydrate, and 50 g potassium sodium tartrate dissolved in 1000 mL deionized water), 152 μL of solution

B (150 g sodium salicylate dissolved in 500 mL deionized water), 100 µL of solution C (0.2 g sodium dichloroisocyanurate dissolved in 100 mL deionized water) and 50 µL solution D (0.4 g sodium nitroprusside dissolved in 50 mL deionized water). The solution was then left at room temperature for 20 min and the absorption value at a wavelength of 660 nm was measured with an automatic microplate reader. Deionized water instead of the sample was used for the blank control. Potassium nitrate and ammonium chloride were used to make a standard curve for determining the nitrate and ammoniate nitrogen contents, respectively. The experiment was performed 3 times.

Nitrogen fixation activity measurement by ^{15}N isotope dilution

Seeds of Zhongyou 821 and Huashuang 5R were surface-sterilized with sodium hypochlorite and pre-germinated on wet filter paper before being sown into pots ($4.5 \times 4.5 \times 5$ cm) containing sterilized soil (vermiculite: pearlite = 6:1) at a density of three seeds per pot. Forty mL of B&D nutrient solution (Broughton and Dilworth 1971) with $K^{15}NO_3$ (0.05 g/L) was added to each pot. The seedlings were cultivated in a plant growth chamber for 10 days and inoculated with 2 mL of resting spore suspension (1×10^7 spores/mL) of *P. brassicae* and 2 mL of cell suspension ($OD_{600} = 0.6$) of strain 7653R-mCherry. Seedlings inoculated with the same volume of resting spores, bacterial suspension, or ddH_2O, and *A. sinicus* inoculated with *M. huakuii* were used as controls. The inoculated plants were incubated in the chamber for an additional 45 days.

The nitrogen fixation activity was measured by ^{15}N isotope dilution following a method described by Malik et al. (1987). Forty-five days after inoculation, six rapeseed roots were gently washed and mixed as a sample. The samples were dried for one week at 80 °C to constant weight for N determination. The ^{15}N proportion was determined by rapid combustion using a macro elemental N analyzer (Elementar, vario MAX CN, Elementar Analysensysteme GmbH, Germany). The experiments were repeated twice.

RNA extraction and RT-qPCR analysis

Previously, many leguminous plant genes required for mutualistic symbiosis with rhizobia were identified. Homologs of these leguminous plant genes (Additional file 2: Tables S1 and S2) were identified in *Arabidopsis* and their expressions in *P. brassicae*-infected *Arabidopsis* roots were examined using RT-qPCR.

Twenty-day-old plants of *A. thaliana* Clo-0 grown in sterilized soil were inoculated with 2 mL of the resting spore suspension of *P. brassicae* (1×10^7 spores/mL) and incubated in a growth chamber for either 24 or 48 h. Plants treated with 2 mL of sterilized water were used as a negative control. The RNA and cDNA samples were prepared as described above. For quantitative real-time PCR (qPCR), 10-µL reaction systems were analyzed in triplicate using the CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The program was as follows: denaturation at 95 °C for 2 min followed by 49 amplification cycles of 95 °C for 5 s and 60 °C for 30 s. The melt curve was generated to verify the specificity of the amplification (from 65 to 95 °C with an increment of 0.5 °C per cycle, with each cycle held for 5 s). The expression level of each gene at 0 hpi was set as 1.0. The expression level of *actin* was used to normalize each gene in different samples. The primer sequences are provided in Additional file 2: Table S3.

The *Nod D* gene was used to determine if *M. huakuii* could be activated by *P. brassicae*-inoculated root exudates. *M. huakuii* suspension collected as described above was stored at 4 °C for 12 h to reduce bacterial metabolic activity. One milliliter of inactivated suspension was inoculated into 1 mL of root exudate from *P. brassicae*-inoculated rapeseed seedlings and incubated in an incubator at 20 °C for either 6 h or 12 h. The bacterial cells were collected and immediately chilled with liquid nitrogen before RNA extraction and qPCR analysis. *Nod D* expression levels in sterilized water (negative control), root exudates of water-treated rapeseed seedlings, root exudates of Chinese milk vetch seedlings, and 0.01 mg/mL Naringenin solution-treated root exudates were also examined. The experiment was performed 3 times.

Statistical analysis

Data from each experiment were analyzed using ANOVA in SAS 8.0 (SAS Institute, Inc., Cary, NC, USA) to look for significant treatment effects and, when present, to determine the least significant differences ($P \leq 0.05$) among the treatments.

Abbreviations

AM: Arbuscular mycorrhizal; DAPI: 4′,6-Diamidino-2-phenylindole; FISH: Fluorescence in situ hybridization; RT-qPCR: Reverse transcription-quantitative PCR; SEM: Scanning electron microscopy.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-022-00154-w.

Additional file 1. Figure S1. Detection of rhizobia in the clubroot-gall of naturally infected rapeseed. **Figure S2.** Detection of *Plasmodiophora brassicae* and *Mesorhizobium huakuii* in artificially inoculated rapeseed roots. **Figure S3.** *Mesorhizobium huakuii* lives intercellularly between resting spores of *Plasmodiophora brassicae* at the late stage of clubroot development. **Figure S4.** Detection of *Plasmodiophora brassicae* and rhizobia in artificially inoculated rapeseed roots

Additional file 2. Table S1. *Arabidopsis* homologues of legume genes required for symbiosis. **Table S2.** *Arabidopsis* homologues of legume early-nodulin genes. **Table S3.** The primers used for PCR, RT-PCR, and RT-qPCR detection of *Plasmodiophora brassicae* and *Mesorhizobium huakuii*. **Table S4.** The primers used for RT-qPCR detection of the *Arabidopsis* homologs of legume genes involved in symbiosis

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Authors' contributions
YZ and DJ designed the experiments. YZ, LG, ZG, BT, TC, JX, JC, and YL performed the experiments and analyzed the data. YZ, YF, and DJ wrote the manuscript. SX and TB reviewed and edited the manuscript. DJ conceived the project and finalized the manuscript. All authors read and approved the final manuscript.

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Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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