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Putative role of IAA during the early response of *Brassica napus* L. to *Plasmodiophora brassicae*

**Li Xu · Li Ren · Kunrong Chen · Fan Liu ·
Xiaoping Fang**

Abstract This study examined the role of indole-3-acetic acid (IAA) during the early stage of infection of rapeseed (*Brassica napus* L.) by the obligate biotrophic protist *Plasmodiophora brassicae* Woron., which causes clubroot disease. Treatment of infected *B. napus* seedlings with exogenous IAA promoted the development of clubroot disease, including an increase in the number and size of galls, whereas treatment with N-1-naphthylphthalamic acid (NPA) attenuated the disease and altered the location and size of the root nodules, resulting in a reduced disease index. An increased level of free IAA was detected in the roots at 3 and 7 days after inoculation (DAI) compared with the control, suggesting the activation of IAA signaling in the early stage of *P. brassicae* infection. Moreover, IAA treatment caused no significant change in the germination rate of the resting spores but resulted in enhanced root hair infection. RT–PCR and quantitative real-time PCR (qPCR) were used to analyze the expression of IAA homeostasis-related genes (3, 7, and 10 DAI) to identify gene expression patterns in the roots and leaves of *B. napus* after inoculation. Five IAA biosynthesis-related genes examined in this study were induced in the roots after inoculation with *P. brassicae* but not in the leaves. The rapid induction of *BnAAO4* expression at 3 DAI may be responsible for the overproduction of IAA during the early stages of infection. This study suggests that IAA acts as a signaling molecule that putatively stimulates root hair infection in the early response of *B. napus* to *P. brassicae* infection.

Keywords *Brassica napus · Plasmodiophora brassicae* · Clubroot · IAA · Early response · Root hair infection

L. Xu · L. Ren · K. Chen · F. Liu · X. Fang
Key Laboratory of Biology and Genetic Improvement of Oil Crops, Ministry of Agriculture, Wuhan 430062, China

L. Xu · L. Ren · K. Chen · F. Liu · X. Fang (✉)
Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan 430062, China
e-mail: xpfang2008@163.com

Introduction

Clubroot disease of cruciferous crops is caused by the obligate biotrophic protist *Plasmodiophora brassicae* (Dixon 2009). *Brassica napus* L., one of the most important oilseed crops in the world, has suffered from clubroot disease in recent years, posing a serious threat to the production of *B. napus* in China (Ren et al. 2012). The infection of cruciferous hosts by *P. brassicae* leads to cell elongation and cell division in the infected roots and hypocotyls, resulting in gall formation, with galls of different sizes forming in the roots (Hwang et al. 2011). Gall formation alters the water and nutrient supply to the plant because the vasculature is destroyed, greatly inhibiting plant growth (Ludwig-Muller 2009b).

Clubroot is a soil-borne disease, and resting spores of *P. brassicae* can survive in the soil for years, making control of the disease difficult under field conditions once the soil is contaminated (Kageyama and Asano 2009). Crop rotation based on standard agronomic

recommendations may not significantly reduce the level of clubroot inoculum in the soil (Niwa et al. 2008). Chemical control can alleviate the disease but cannot reduce the viable resting spores in the soil or completely eradicate the disease. To ensure the yield and quality of the crop, the development of varieties resistant to clubroot disease is the most effective economic management strategy (Deora et al. 2012). Few clubroot-resistant *B. napus* cultivars are available for commercial production in China, and the pathogen has evolved increasingly virulent pathotypes. Therefore, understanding the molecular mechanisms of clubroot disease development is essential. It may provide a theoretical basis for breeding resistant varieties and aid in the identification of relevant genes for modulation with genetic engineering methods to develop resistant varieties.

The life cycle of the pathogen consists of two distinct phases: the primary phase, when it is restricted to the root hairs of the infected plant, and the secondary phase, which occurs in the cortex and stele of the hypocotyls and roots, leading to abnormal root growth and clubroot formation in susceptible hosts (Feng et al. 2013). The involvement of auxin and cytokinin as pathogenicity factors has been demonstrated by the correlation of clubroot disease symptoms with an increase in the levels of these plant hormones, which cause increased cell division and cell elongation during the late infection stage (Devos et al. 2005; Devos and Prinsen 2006; Jubault et al. 2013; Siemens et al. 2006). It was reported that the free indole-3-acetic acid (IAA) content fluctuated in infected roots at all the time points tested (10–42 days after inoculation [DAI]), although some studies have shown conflicting results. IAA is generally higher in infected roots than in control roots of the same age, which suggests the involvement of IAA accumulation during the late stage of gall formation (Ludwig-Muller 2009a). However, the level of IAA in infected cabbage roots was much lower than uninfected roots when the pathogen produced resting spores at 28 DAI (Kavanagh and Williams 1981). These complicated results may be explained by differences in the interactions between different hosts and the pathogen.

Transcriptomic and proteomic experiments have provided evidence for the involvement of IAA pathway-related genes during gall formation in clubroot disease (Cao et al. 2008; Siemens et al. 2006, 2009; Sundelin et al. 2011). The expression levels of nitrilase (Ando et al. 2008; Grsic-Rausch et al. 2000; Siemens et al. 2006), myrosinase (Searle et al. 1982; Sundelin et al. 2011), aldehyde oxidase (Ando et al. 2006), auxin-conjugate hydrolase (LudwigMuller et al. 1996; Schuller and Ludwig-Muller 2006), and other enzymes involved in auxin homeostasis are induced during the late stage of the infection process. The importance of nitrilase in gall development was shown with a *nit1* mutant of the model plant *Arabidopsis*, and antisense plants have shown reduced gall size that correlated with reduced gall IAA content (Grsic-Rausch et al. 2000; Neuhaus et al. 2000). Thus, the involvement of IAA in gall formation during the secondary phase of the pathogen's life cycle was demonstrated.

There has been little research on IAA in the primary stage of infection, including the germination of the resting spores, zoospore release and penetration, the formation of the primary plasmodium, and the release of secondary zoospores, which do not produce visible symptoms in the root system. In this study, we focused on the primary stage of the infection process and the initiation of gall formation (3–10 DAI). By treating *B. napus* with exogenous IAA or N-1-naphthylphthalamic acid (NPA), which inhibits the polar transport of IAA in the plant, we clarified the effect of IAA on clubroot disease. The level of free IAA during the early stage of infection was also investigated. RT-PCR and qPCR were used to examine the expression of IAA-biosynthesis-related genes to determine the gene expression patterns in the roots and leaves of *B. napus* soon after *P. brassicae* inoculation (3, 7, and 10 DAI), defined as the 'root hair infection stage'. The general role of IAA in *B. napus* clubroot disease was thus clarified and may provide the basis for identifying the molecular mechanism underlying clubroot disease.

Materials and methods

Plant material and pathogen isolate

The clubroot-susceptible *B. napus* cultivar 'zhongshuang 11' was used as the plant material. The root galls used in this study were collected from clubroot-infested field plots in Zhi'jiang, Hubei, China. The strain of the resting spores isolated from the galls was identified as pathotype 4 (Ji et al. 2013), according to the differential classification of Williams (1966).

The resting spores of *P. brassicae* were isolated as the procedure described by Castlebury et al. (1994).

Approximately 5 g of fresh galled roots was homogenized in a blender in 50 ml of sterile distilled water (sdH$_2$O) for 2 min at high speed, and the resultant homogenate was filtered through eight layers of cheesecloth. The filtrate was centrifuged at 4 °C in a 50-ml centrifuge tube at $4000 \times g$ for 10 min in a swinging-bucket rotor. The supernatant was discarded, and the pellet was resuspended in 5 ml of 50 % sucrose and centrifuged again at $2000 \times g$ for 10 min. The resulting supernatant was transferred to a new 50-ml tube and diluted with 30 ml of sdH$_2$O before centrifugation at $4000 \times g$ for 10 min. The pellet was resuspended in 5 ml of sdH$_2$O and centrifuged as above to remove any remaining sucrose. The supernatant was removed, and the resulting spore pellet was resuspended in 5 ml of sdH$_2$O. The spore suspension was adjusted to a final concentration of 5×10^6 spores/ml with sdH$_2$O.

Inoculation of resting spores and disease investigation

Seeds of "zhongshuang 11" were sown in commercial sterilized soil (a complex of soil, peat, and composted pine bark) at 22 °C/18 °C day/night temperatures with a photoperiod of 14 h light and 10 h dark. The soil was inoculated with resting spores of *P. brassicae* 1 week after sowing by the addition of 1 ml of 5×10^6 spores/ml in sdH$_2$O to the soil at the base of each plant. The control plants were not inoculated but were treated with distilled water in the same way.

The disease was investigated by pulling up the seedlings at 35 DAI. Disease severity was assessed qualitatively based on the disease index (Hwang et al. 2012): 0 = no visible root galls; 1 = very slight swelling of the lateral roots; 2 = moderate swelling of the roots; and 3 = presence of large galls on the primary roots. The disease index was calculated from the four-grade scale as follows: disease index = $(1n1 + 2n2 + 3n3 + 4n4) \times 100/4Nt$, where n1–n4 represents the number of plants in the indicated grade and Nt represents the total number of plants tested. The data presented are the mean ± standard errors (SE) of at least three replicates.

Treatment of inoculated seedlings with exogenous IAA or its inhibitor NPA

Rapeseed seedlings were treated with different concentrations (10^{-7} and 10^{-10} mol/L) of IAA (Sigma-Aldrich, St Louis, MO, USA) by adding IAA to the soil on different days (0, 2, 4, 6, and 8) after inoculation with

P. brassicae. Seedlings were treated with NPA (Sigma-Aldrich) (10^{-7} mol/L) by spraying the leaves after inoculation of the seedlings with *P. brassicae*. The leaves were covered with film to retain moisture. At least 30 plants were analyzed for each line and each treatment. The disease was investigated as described above.

Treatment of germinating resting spores with exogenous IAA

The adjusted resting spore suspension was disinfected with freshly prepared 2 % (w/v) chloramine-T solution plus 0.05 % (v/v) Tween 20 at room temperature for 25 min and then washed twice with sdH$_2$O by centrifugation, as described previously (Asano et al. 1999). The resulting pellet was suspended in an antibiotic solution containing 1 g/L colistin sulfate, 1 g/L vancomycin hypochlorite, and 6 g/L cefotaxime sodium and incubated at 25 °C in the dark for 1 day. The suspension was then washed twice by centrifugation in sdH$_2$O. Finally, the spores were suspended in sdH$_2$O and adjusted to a concentration of 5×10^6 spores/ml. The optimal conditions of pH 5.5 and temperature of 25 °C were provided for spore generation (Luo et al. 2014). Different concentrations of IAA were continuously added to the disinfected resting spore suspension on different days (0, 2, 4, 6, and 8) after inoculation at final concentrations of 10^{-7}, 10^{-8}, and 10^{-9} mol/L.

Spore germination was analyzed every second day from 1 to 9 DAI. A droplet of spore suspension was placed on a microscope slide and stained with orcein. The proportion of germinated spores was determined under a light microscope by counting the empty spores (Friberg et al. 2005). The statuses of 200 spores (germinated or non-germinated) on three different microscope slides were analyzed for each replicate.

Treatment of root hair infection with exogenous IAA

An improved solution culture technique was employed to observe root hair infection (Luo et al. 2014). Seeds of rapeseed were germinated on wet filter paper for 3 days (25 °C, 16 h photoperiod). Culture solution was added to a $10 \times 20 \times 35$ cm plastic tray and consisted of half strength Hoagland nutrient solution. A 0.5 cm thick plank with holes (2 cm diameter) covered the plastic tray. The seedlings were fastened to round pieces of foam (1 cm thick), which were placed in the holes. The spore suspension was added to the solution to give

a final concentration of 10^6 spores/ml. IAA was then added to the solution on different days (0, 2, 4, 6 and 8) after inoculation to give a final concentration of 10^{-7} mol/L.

Roots were rinsed clean with tap water, and a segment (0.5 cm long) was cut from the top 0 to 1 cm of taproot at different days (3, 5, 7 and 9 days) after inoculation. The segment was stained with FAA Phloxine B and was mounted on glass with a coverslip to be observed and imaged with an optical microscope with a×20 objective lens (Donald et al. 2008). The number of total root hairs and root hairs with primary infection were counted in a field of view at 7 DAI. At least 20 segments were analyzed for each treatment.

Extraction and determination of free IAA

Infected and control roots and leaves were harvested at 3, 7, and 10 DAI, washed thoroughly, and dried on filter paper. Approximately 0.1 g of fresh roots and leaves was frozen in liquid nitrogen and ground into powder. Free IAA was extracted and quantified as previously described (Li et al. 2011). A 0.5 ml aliquot of 1-propanol:H_2O:concentrated HCl (2:1:0.002, $v/v/v$) was added, and the solution was shaken for 30 min at 4 °C. Dichloromethane (1 ml) was added, the solution was shaken again for another 30 min, and centrifuged at $12,000 \times g$ for 10 min. After centrifugation, the bottom organic phase (approximately 1 ml) was transferred to a 10-ml tube and evaporated to dryness with a constant stream of nitrogen. Each sample was resolubilized in 80 % methanol (1 ml), vortexed for 5 min, and then extracted using a C18 SPE cartridge (CNWBOND HC-C18, 500 mg, 3 ml). The elutate (2 ml) was evaporated to dryness under vacuum in a water bath at 35 °C, and finally dissolved in 200 μL of methanol:0.05 % formic acid (1:1, v/v). The solution was filtered through a 0.45-μm microfilter before analysis.

The samples were separated by reverse-phase high performance liquid chromatography (HPLC) and scanned with electrospray ionization–tandem mass spectrometry (RP-HPLC/ESI–MS/MS) (ABI 4000 Q-Trap, Applied Biosystems, Foster City, CA, USA) in the MRM mode, with a 50 ms dwell time in the enhanced product ion scan mode; precursor ions were fragmented with a collision energy of −25 V, and the products were scanned in the m/z range of 80–355.

Identification of IAA-related sequences in *B. napus*

Coding sequences (CDS) of Arabidopsis IAA biosynthesis and regulatory genes were used to search for *B. napus* homologs obtained from the NCBI database (http://www.ncbi.nlm.nih.gov).Target sequences with the highest identity with the identified genes in Arabidopsis were counted as the same gene.

RNA isolation and RT–PCR

The seedlings were dug from the soil at 3, 7, and 10 DAI, and the roots were washed with sdH$_2$O, cut at the root collar, and immediately frozen in liquid nitrogen. The root and leaf samples were stored at −80 °C until RNA extraction. Approximately 30 seedlings were collected for each treatment. To check that the inoculation was successful, clubroot susceptibility was also evaluated on randomly selected samples from each treatment group at 35 DAI to confirm the presence or absence of clubroot disease.

Total RNA was isolated from control and infected roots and leaves of *B. napus* at different times after inoculation (3, 7, and 10 DAI) with TRIzol Reagent (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions. First-strand cDNA was generated from 3 μg total by using the Superscript first-strand synthesis system (Invitrogen, Foster City, CA, USA). The primers used for real-time PCR (RT–PCR) and quantitative real-time PCR (qPCR) were designed with Primer Premier 5.0 software and synthesized commercially (BGI, Shenzhen, China), as shown in Table 1. The standard PCR cycles for RT–PCR were as follows: one cycle at 94 °C for 5 min, followed by 32 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. The *Actin1* gene was used as the control. The PCR products were separated on 1.2 % agarose gel and stained with ethidium bromide.

For qPCR, 20 μL qPCR samples were run in three technical replicates on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster,CA, USA) using 5 μL first-strand cDNA and SYBR Green PCR Master Mix (Applied Biosystems). PCR cycles were as follows: 1 cycle of 1 min at 94 °C, followed by 40 cycles at 94 °C for 15 s and 58 °C for 45 s. Following amplification, all products were subjected to melt curve analysis. The *Actin1* gene was used as the reference gene to normalize the total amount of cDNA in each reaction. There were

Table 1 Sequence information and primers for RT–PCR and qPCR

Gene	Accession No. in GenBank	Primer sequence
BnACT1	AF1118122	F:TCTGGCATCACACTTTCTACAACGAGC
		R:CAGGGAACATGGTCGAACCACC
BnFMO5	EE426335	F:GTGTCTGATTGCGTGGCGAGTC
		R:GCAAATGGGTGGCTGCTGTTTT
BnAAO4	EV088287	F:ACTTGGAGGTGGCTTCGGTGG
		R:ATTCTTCGGCAGCATTGGACTT
BnCYP83B1	JX017302	F:CTTGACCCTAGCCGCCCTAAAC
		R:TGTCACCGACCACATTCCTCAC
BnNit4	EV099557	F:GTCCAGGCATGCACCATCTTCTAC
		R:CTCCGCCATACGTTCCACCTCA
BnGH3-1	GT085358	F:ACGACCATACGATCCGTACAACGT
		R:TCCTGCGAGCAAACCTCTACCAAG

three biological replicates for these study, and relative fold changes were calculated with $2^{-\Delta\Delta Ct}$ using the SDS software from the 7500 Real Time PCR System (Applied Biosystems) (Rieu and Powers 2009).

Results

Effects of IAA and NPA on clubroot disease

To better understand the physiological effects of IAA in clubroot disease, IAA and its inhibitor NPA were applied to infected plants and examined at 35 DAI (Table 2 and Fig. 1). The data confirmed that the infection was successful, and control plants did not develop the disease. The typical symptoms of clubroot disease include the formation of differently sized galls on the primary root and/or lateral roots (Fig. 1). Higher concentrations of IAA did not alter the locations of the galls on the primary root but increased the plants' susceptibility to *P. brassicae*, increasing the number and size of galls, which increased the disease index. Conversely, treatment of *P. brassicae*-inoculated *B. napus* seedlings with NPA, an inhibitor of the polar transport of IAA, resulted in a reduced disease rate and disease index and altered locations and sizes of the root galls.

Determination of free IAA content

The concentrations of endogenous free IAA were determined in the control plants and plants infected with *P. brassicae* for 3, 7, and 10 DAI (Fig. 2). The production of IAA was significantly increased in the inoculated roots at 3 DAI. The IAA content then decreased to nearly that of the control at 10 DAI. The free IAA was higher in the leaves after *P. brassicae* inoculation than in the control. A higher content of IAA was detected in the leaves than in the roots.

Table 2 Investigation of clubroot disease after treatment with IAA or NPA

Treatment		Number of each scale				Number	Disease rate	Disease index
		0	1	2	3			
Control	——	22	0	0	0	22	0	0
Infected	——	0	12	35	37	84	100a	76.58b
	NPA(10^{-7}mol/L)	10	24	42	2	78	87.19ab	48.71a
	IAA(10^{-7} mol/L)	0	2	16	65	83	100a	91.97c
	IAA(10^{-10} mol/L)	0	5	47	29	81	100a	76.55b

Different letters above the bars indicate that the differences are significant ($P < 0.05$)

Fig. 1 Symptoms of clubroot disease in *B. napus* (35 DAI) after treatment with IAA or NPA

Control   Infected   Infected   Infected
                                   IAA (10⁻⁷ mol/L)   NPA (10⁻⁷ mol/L)
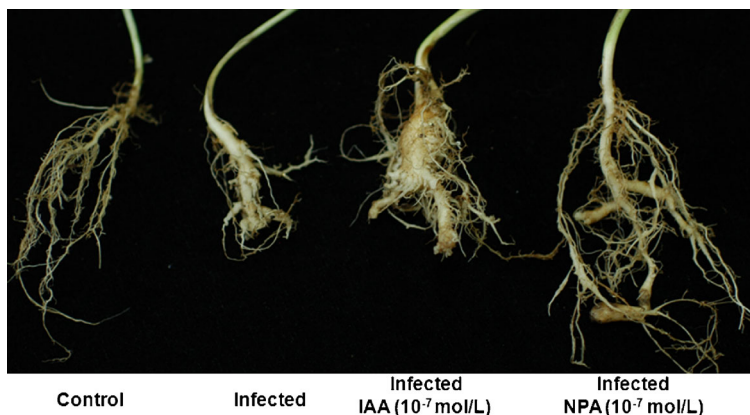
Effect of IAA treatment on the germination rate of resting spores

The germination of the resting spores started at 1 DAI and increased steadily from 3 to 9 DAI before reaching a constant rate (Fig. 3). At 11 DAI, the germination rate of the resting spores was 61 %. Treatment with different concentrations of IAA did not significantly alter the germination rate of the resting spores compared to control spores.

Effect of IAA treatment on root hair infection

An improved solution culture technique was employed to study the role of IAA on root hair infection. To better understand the role of IAA on root hair infection, the number of root hairs, including infected and uninfected

hairs, was calculated every 2 days after treatment. In this study, infection was first observed in root hairs at 3 DAI, and maximal root hair infection was observed at 9 DAI, which corresponds to the germination timeline of resting spores. Considering that the IAA content increased from 3 to 7 DAI, we focused on the 7 DAI time point (Fig. 4a–d). Pathogen inoculation enhanced the number of total root hairs compared to the control, but there was no significant difference between control and inoculated plants under IAA treatment. Furthermore, IAA treatment resulted in an increased number of total root hairs and infected root hairs, which subsequently exhibited a higher infection rate at 7 DAI compared to infection without IAA (57.69 % versus 43.24 %) (Fig. 4e).

Expression patterns of IAA-related genes during the early response of *B. napus* L. to *P. brassicae*

Fig. 2 Free IAA levels of control plants and plants inoculated with *P. brassicae*. *Error bars* represent the SD of three independent experiments. Different letters above the bars indicate that the differences are significant ($P < 0.05$)
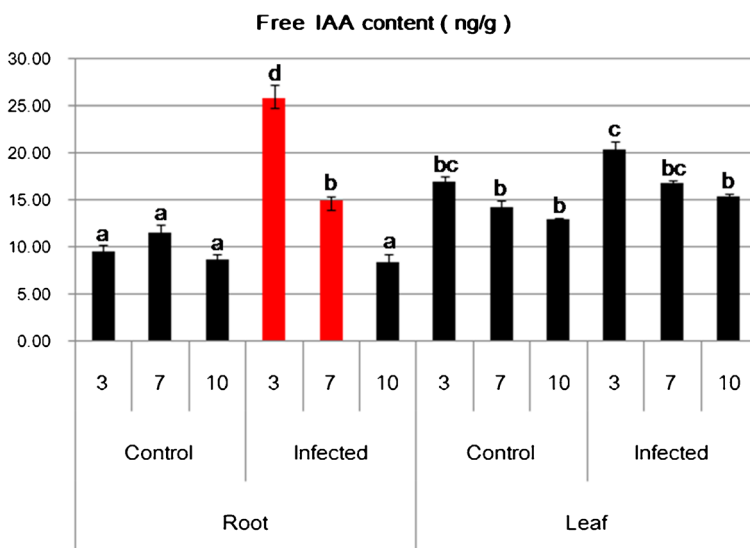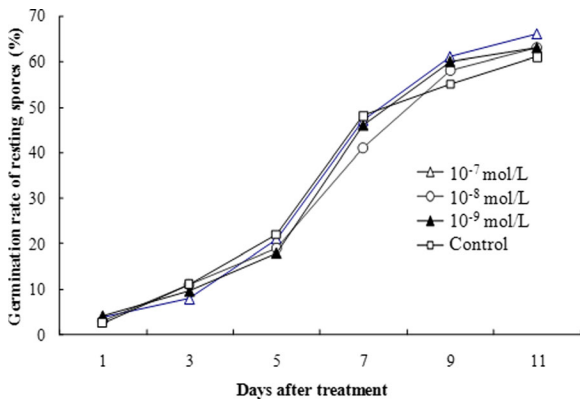
Fig. 3 Effect of different concentrations of IAA on the germination rates of resting spores assessed on different days

In this study, we looked for IAA-related gene sequences of *B. napus* in the GenBank database to identify sequences with homology to *Arabidopsis* genes. Eighteen sequences with high homology to IAA synthesis-related genes in *Arabidopsis* were identified,

which encoded proteins involved in the major pathways of IAA synthesis, including the indole-3-acetaldoxime (IAOx), indole-3-pyruvate (IPyA), and tryptamine pathways (Fig. 5a). No genes in the indole-3-acetamide (IAM) pathway were identified.

To determine the role of IAA in the regulation of the early infection stage of clubroot disease, the expression patterns of IAA-related genes in *B. napus* at early time points (3, 7, and 10 DAI) after inoculation were analyzed by RT–PCR (Fig. 5b). Five genes were identified after *P. brassicae* inoculation by semi-quantitative RT–PCR. Considering that RT-PCR cannot detect gene expression levels quantitatively, further study was conducted using qPCR (Fig. 5c). Through qPCR analysis, detailed relative gene expression levels were determined, especially for genes with low expression levels such as *BnNit4*. In general, the qPCR measurements were well correlated with the RT–PCR results. The expression of *BnAAO4* (aldehyde oxidase) showed a transient increase at 3 DAI in the inoculated roots. The

Fig. 4 Root hair infection of *B. napus* roots at 9 DAI with IAA treatment. Root segments were stained by Phloxine B. Staining in root hair indicated the presence of root hair infection. Control (**a**) and inoculated (**b**) roots not treated with IAA. Control (**c**) and inoculated (**d**) roots treated with IAA. Numbers of infected and uninfected root hairs are shown in (**e**). Different letters above the bars indicate that the differences are significant ($P < 0.05$)
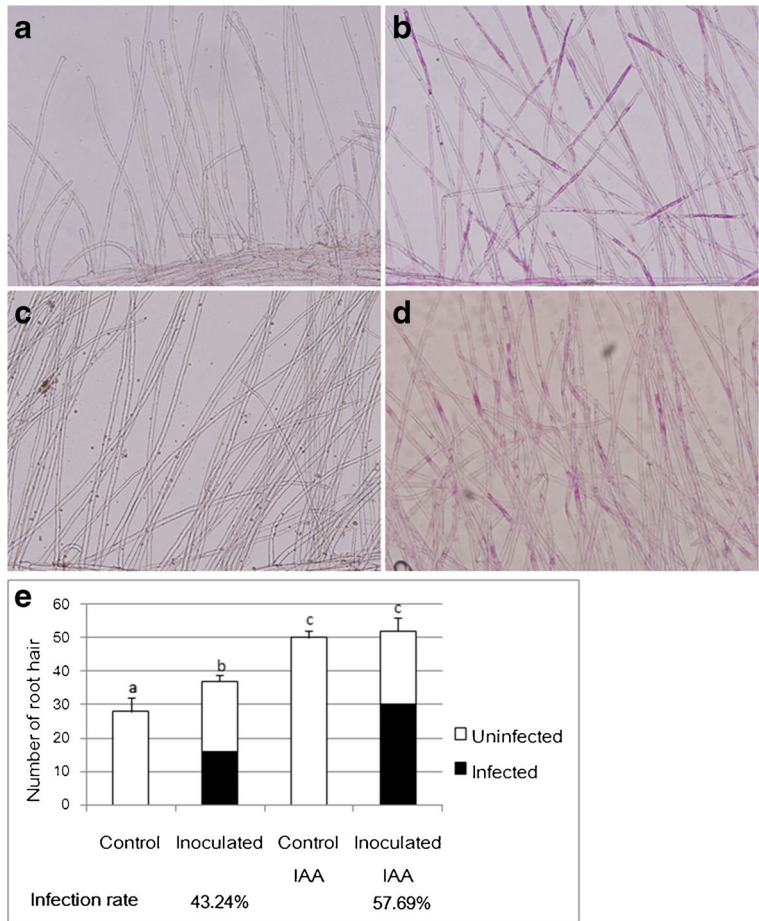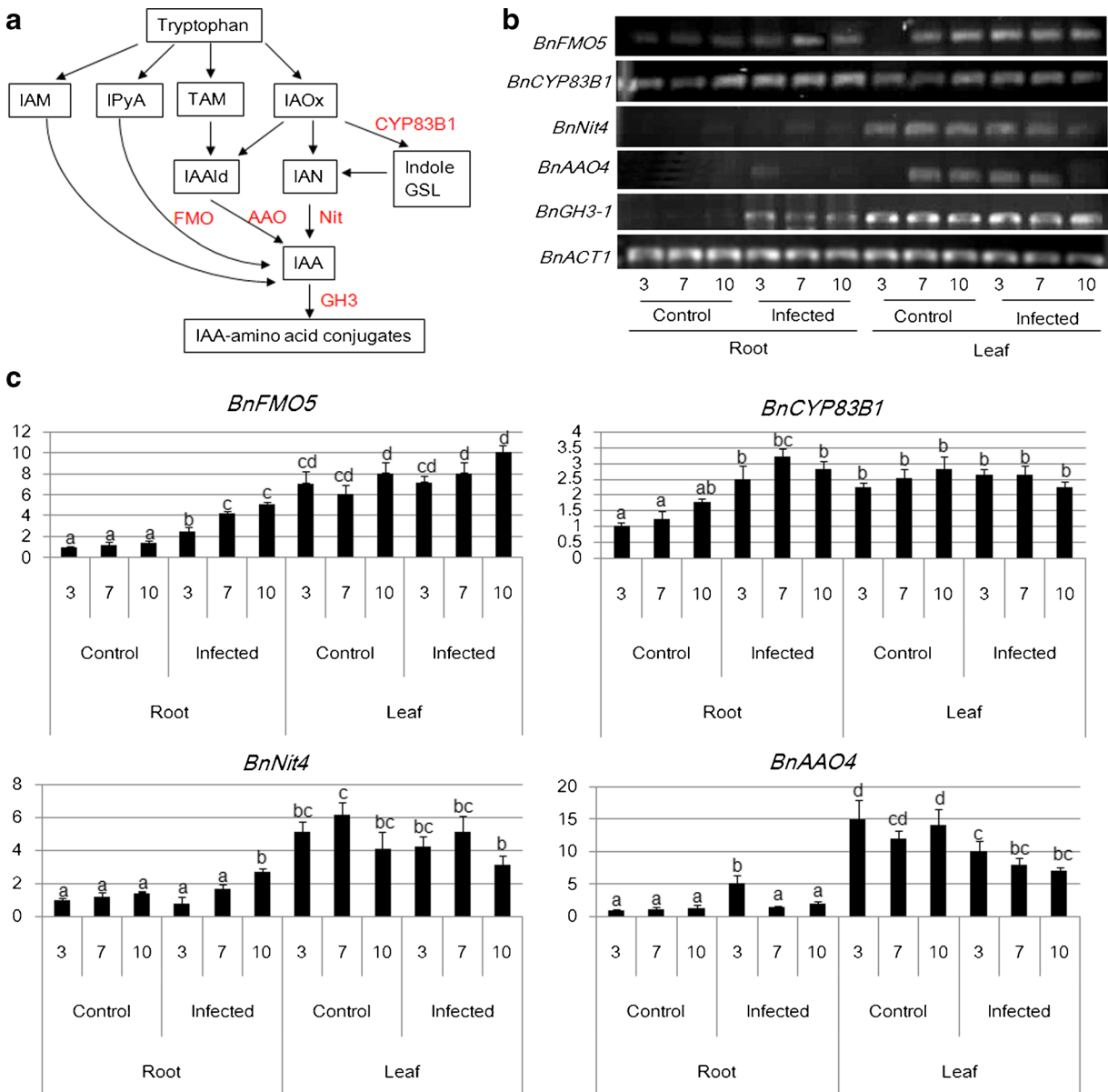
Fig. 5 Summary of the proposed IAA biosynthetic pathway in plants (**a**) and the expression patterns of genes involved in this pathway after infection with *P. brassicae* by RT–PCR (**b**) and qPCR (**c**). Enzyme names shown in bold indicate genes induced by *P. brassicae* infection. Different letters above the bars indicate that the differences are significant (*P* < 0.05). *IAM* indole-3-acetamide, *IPyA* indole-3-pyruvic acid, *TAM* tryptamine, *IAOx* indole-3-acetaldoxime, *IAAld* indole-3-acetaldehyde, *IAN* indole-3-acetonitrile, *GSL* glucosinolates, *CYP83B* cytochrome P450 mono-oxygenase, *FMO* flavin monooxygenase-like, *AAO* aldehyde oxidase, *Nit* nitrilase, *GH3* Gretchen Hagen 3

expression of *BnFMO5* (flavin monooxygenase-like) and *BnGH3-1* (Gretchen Hagen 3) increased consistently in inoculated roots compared with the control roots during the first stage of infection (3–10 DAI). The expression of *BnCYP83B1* (cytochrome P450 mono-oxygenase) increased significantly at 3 and 7 DAI in the infected roots. *BnNit4* (nitrilase) expression, which was extremely low in the roots, increased significantly at 10 DAI in the roots. All the genes investigated displayed higher expression in the leaves than in the roots and did not show significant changes in leaves compared with the control.

Discussion

Recent research has addressed the role of IAA in gall formation during the late stage of clubroot disease (Devos and Prinsen 2006). The initiation of cell division and cell enlargement, which are responsible for gall formation, are possibly regulated by changes in IAA balance (Ludwig-Muller and Schuller 2008). The gall formation observed in the late infection stage can be considered a secondary effect of the altered morphology of the host; disease development is triggered in the early stage. A rapid increase in free IAA levels is detected after *P. brassicae* inoculation in *B. napus*. Therefore, we focus on the primary stage of the infection process (3–10 DAI) to determine the regulatory role of IAA in clubroot disease.

IAA promotes the development of clubroot disease in *B. napus* L

Treatment with a higher concentration of IAA results in an increased disease index, including the number of galls on the lateral roots and the size of the galls on the primary roots, suggesting that early treatment with IAA promotes the development of clubroot disease in *B. napus*.

Auxin is the only plant hormone with polar transport characteristics, and its polar transport allows its broad distribution to control differences in plant growth and development (Hayashi 2012). This fact suggests that the transport of IAA from the stem apex to the root is required for gall development. It is also demonstrated in a study of the Arabidopsis mutant *alh*1, which showed inhibited auxin transport and had a defect in the crosstalk between ethylene and auxin (Vandenbussche et al. 2003). The mutant shows attenuated disease symptoms after *P. brassicae* inoculation, whereas the addition of 1-naphthaleneacetic acid (NAA) to the infected mutant does not rescue the mutant phenotype (Devos et al. 2006). However, infected wild-type plants show an increased infection rate when treated with NAA. These results also suggest that IAA transport plays a central role in gall development.

In summary, treatment with IAA resulted in enhanced disease severity and inhibited IAA transport, causing attenuated disease symptoms. These results emphasize the significance of IAA in clubroot disease in *B. napus*.

Rapid increase in free IAA after inoculation with *P. brassicae*

Reports have shown that the IAA content of the cruciferous plants *A. thaliana* and *B. rapa* is dramatically altered during clubroot disease (Grsic et al. 1999; Ludwig-Muller et al. 2009). However, it is generally accepted that increased levels of IAA during the later infection stage play an important role in clubroot disease (Devos et al. 2006; Devos and Prinsen 2006; Ludwig-Muller and Schuller 2008; Ludwig-Muller 2009a).

In this study, we detect a rapid increase in IAA production in the root at 3 and 7 DAI with *P. brassicae*. The result is supported by the rapidly induced expression of IAA biosynthesis-related genes, which shows that de novo IAA synthesis is elevated quickly after *P. brassicae* inoculation. Devos et al. (2005) detect a rapid increase in IAAconjugate levels but not in IAA synthesis during primary infection at 6 DAI. Further study suggests that the induction of IAA correlates with an increase in seedling growth and xyloglucan endotransferase/hydrolase, leading to wall loosening. There has been little research that focused on early infection after inoculation, especially at 3 DAI. Complex perception, transduction, and exchange of signals happen at this time point. In light of these phenomena, IAA is proposed to be a signal molecule or involved in the induction of plant development involved in the early response of *B. napus* to *P. brassicae*. Taken together, these results are interesting and prompt the study of the exact role of IAA during early infection.

Treatment with IAA promoted root hair infection

In the life cycle of *P. brassicae*, the primary infection stage occurs in root hair. The resting spores germinate and then release primary zoospores, which can penetrate through the cell walls of root hairs (Kageyama and Asano 2009). Resting spores germinate not only in the presence of a host plant but also spontaneously (Friberg et al. 2005; Rashid et al. 2013). Spontaneous germination is affected by different environmental conditions. The production of IAA is significantly increased at 3–7 DAI, which coincide with the germination of most resting spores and infection of zoospores. In light of this fact, different concentrations of IAA are added to suspensions of resting spores under optimum germination conditions. However, treatment with different

concentrations of IAA does not significantly alter the germination rate of the resting spores.

Then, root hair infection is investigated by dynamic observation of infected roots. It has been suggested that treatment with IAA results in increased numbers of both total root hairs and infected root hairs. It is well known that a low concentration of exogenous IAA triggers an increased density of lateral roots and root hairs (Saini et al. 2013). Therefore, it is speculated that the increased total number of root hairs is due to the increased IAA content. A previous study suggests that IAA increases plant disease susceptibility to plant–pathogen interactions, leading to the loosening of the plant cell wall, thus weakening its role in the plant defense against pathogens (Bari and Jones 2009). The rapid induction of IAA likely facilitates the primary infection phase by altering the cell wall structure and allowing penetration by zoospores.

It can be deduced from these data that exogenous IAA treatment resulted in higher root hair infection. However, determining whether it directly correlated to the increased disease index and disease rate requires more study. Further research is required to establish the cytoarchitecture and cytochemistry of *P. brassicae*-inoculated roots and to determine the exact role of IAA during the early infection stage of clubroot disease.

Regulation of IAA homeostasis during the early stage of disease development

Nitrilase is known to play a key role in several biosynthetic pathways in the Brassicaceae family because it catalyzes the last step in the IAOx pathway of IAA biosynthesis (Mashiguchi et al. 2011). The expression of *NIT1* is induced during the later stage of infection (14, 28, and 35 DAI) in *A. thaliana* and *B. rapa*, which indicates a major role for nitrilase in gall formation (Devos et al. 2005; Devos et al. 2006; Grsic-Rausch et al. 2000; Siemens et al. 2006). A *NIT1* mutant (*nit1*) displays smaller root galls and lower free IAA levels in the clubbed roots of infected plants, emphasizing the relevance of enhanced auxin synthesis in pathogenicity (Neuhaus et al. 2000). In this study, the expression of *BnNit4* is extremely low at the earlier time points and is significantly induced at 10 DAI, when cortex infection is initiated. These findings correlate with the results of the present study, in that the increased production of IAA *via* the nitrilase pathway likely affects gall formation during the later stage of infection. Taken together,

these studies show that *BnNit4* may not be directly responsible for the enhanced content of IAA at 3–7 DAI.

Indole glucosinolates are precursors for IAA biosynthesis, and they have been found to correlate with disease severity and indole glucosinolate content (Ludwig-Muller 2009a; Ludwig-Muller et al. 1999). CYP83B1, which converts IAOx to indole glucosinolates, is upregulated at 10 DAI and then downregulated at 23 DAI in *Arabidopsis*, according to a microarray analysis (Siemens et al. 2006). Similarly, the expression of *BnCYP83B1* is slightly increased at 3 and 7 DAI but is not induced at 10 DAI. Despite the increased expression of *BnCYP83B1* at the earlier time points, *BnNit4*, which is directly involved in IAA synthesis, was not induced at those times. Therefore, *BnCYP83B1* may not be a direct regulator of the increase in IAA in response to *P. brassicae* infection, and indole glucosinolates may not be the primary source of IAA.

Aldehyde oxidase (AAO) is presumed to be responsible for the conversion of indole-3-acetaldehyde (IAAld) to IAA, and it is involved in IAA biosynthesis *via* the IAOx and TAM pathways (Mashiguchi et al. 2011). In this study, the rapidly induced expression of *BnAAO4* at 3 DAI may be responsible for the overproduction of IAA at 3 and 7 DAI during the first stage of infection. Of the two *AAO* genes identified in *B. rapa* (*BrAAO1* and *BrAAO2*), the expression of *BrAAO1* is enhanced in the infected roots from 15 DAI, when clubroot is still undetectable, and expression of *BrAAO2* is repressed (Ando et al. 2006). These different expression patterns also indicate the diverse roles of the *AAO* gene family in the plant response to clubroot disease. However, *AAO* has also been reported to be involved in the biosynthesis of abscisic acid (ABA) (Seo and Koshiba 2002). AAO3 plays an important role in ABA biosynthesis in the leaves and seeds of Arabidopsis (Seo et al. 2004). Whether the early increase in expression of *BnAAO4* is involved in the biosynthesis of ABA has not been determined. To date, little attention has been paid to the involvement of the AAO proteins in clubroot disease. However, these results suggest a promising research direction for clubroot disease.

Yucca-encoded flavin monooxygenase-like proteins (FMOs) have been implicated in the conversion of IPyA to IAA in *Arabidopsis*, which is the main IAA biosynthetic pathway in *Arabidopsis* (Mashiguchi et al. 2011). The expression of *BnFMO5* is induced at 3–10 DAI in

pathogen-inoculated roots, which suggests the involvement of *BnFMO5* in the response of *B. napus* to *P. brassicae*. However, due to the constitutively induced expression of *BnFMO5* in the early infection stage, *BnFMO5* cannot be directly linked to the increase in IAA levels at 3 to 7 DAI. Although the *YUC* genes participate in plant development and different stress responses, there is limited information about their involvement in disease responses (Kim et al. 2011). Because it is involved in the major IAA biosynthetic pathway, a detailed investigation of *BnFMO5* will be key to understanding the regulation of IPyA-dependent IAA biosynthesis in the development of clubroot disease.

The *GH3* genes encode IAA-amido synthetases, which are involved in the regulation of IAA homeostasis by conjugating IAA to amino acids by adenylation (Park et al. 2007). Most of the total auxin in plants is found in the conjugated form, and the formation of auxin conjugates is one of the important mechanisms regulating the activation or inactivation of IAA. GH3 proteins play roles in the plant defense responses of *Arabidopsis* and rice (*Oryza sativa*). Overexpression of GH3-8 in rice resulted in the increased accumulation of conjugated IAA (IAA–Asp) and reduced levels of free IAA compared to wild-type plants, which enhanced the plants' resistance to *Xanthomonas oryzae* pv. *Oryzae* (Ding et al. 2008). The increased expression of several members of the *GH3* gene family is observed at 10 DAI in *Arabidopsis* with an ATH1 genome array, whereas *BnGH3-1* shows transiently increased expression at 3 DAI, but not at 10 DAI, in *B. napus* (Siemens et al. 2006). The different expression patterns in Arabidopsis and Brassica species, in terms of gene regulation or enzymatic activity, must be considered. The expression of *BnGH3-1* increases from 3 to 10 DAI in the study. Because the GH3 family may markedly contribute to maintaining the level of IAA when excess amounts of IAA are produced, the induced expression of *BnGH3-1* is consistent with the rapidly elevated production of free IAA content at 3–7 DAI. It has been speculated that not only the level of free IAA but also the level of total auxin are involved in the regulation of the early response of *B. napus* to *P. brassicae*.

In summary, the detailed relative expression levels, which were determined by qPCR, provide a better understanding of the role of IAA during early infection of *P. brassicae* in *B. napus*. The genes that are differentially expressed after pathogen infection were considered to respond directly to this infection. However, taking the IAA synthesis pathway into consideration, *BnAAO4* may be directly responsible for the overproduction of IAA at 3 and 7 DAI during the first stage of infection.

Induced IAA signaling in the early response to *P. brassicae* in roots but not leaves

The genes investigated show higher expression in the leaves than in the roots, which confirms that the majority of IAA was generated in leaves. Despite this finding, most of the genes are induced by *P. brassicae* infection in the roots but not in the leaves. The slightly and quickly induced expression of IAA-biosynthesis-related genes in the root suggests that IAA acts as a signal molecule, regulating the early response to *P. brassicae* infection.

In conclusion, the IAA response is likely rapidly activated by pathogen inoculation and is involved in regulating the early response of *B. napus* to *P. brassicae*. The induced production of IAA results in an increased number of root hairs, which likely induces infection and disease development. However, the local changes induced at the cytochemical level early after infection must be examined to determine the precise influence of IAA on the early response. It is also suggested that *BnAAO4* plays a crucial role in the early response of *B. napus* to *P. brassicae*, and further functional characterization is required to clarify the regulatory role of IAA in the development of this disease.

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