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Overexpression of rice acyl-CoA-binding protein OsACBP5 protects *Brassica napus* against seedling infection by fungal phytopathogens

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ABSTRACT

Context. Class III acyl-CoA-binding proteins such as those from dicots (Arabidopsis and grapevine) play a role in defence against biotrophic pathogens. The overexpression of the monocot *Oryza sativa* (rice) OsACBP5 in Arabidopsis and rice has been demonstrated to enhance broad-spectrum disease resistance against selected phytopathogens in OsACBP5-overexpressing (OsACBP5-OE) lines. **Aims.** We aimed to develop transgenic rapid-cycling *Brassica napus* (*B. napus*-RC) and canola cv. Westar OsACBP5-OEs using tissue culture-based *Agrobacterium*-mediated transformation and to evaluate transgenic plants for resistance against Alternaria blight, blackleg and Sclerotinia rot diseases. **Methods.** Transgenic *B. napus*-RC and cv. Westar OsACBP5-OEs were generated through *Agrobacterium*-mediated transformation using *Agrobacterium* strain LBA4404 harbouring a plasmid with the rice complementary DNA encoding OsACBP5 driven by the cauliflower mosaic virus *35S* promoter. Alternaria blight and blackleg pathogen assays were based on infecting young cotyledons, while detached leaf assay was used to test the tolerance of *B. napus* plants toward *Sclerotinia sclerotiorum*. **Key results.** Average transformation efficiencies of 24.2% and 29.1% were obtained for *B. napus*-RC and *B. napus* cv. Westar cotyledons respectively. OsACBP5-OE plants exhibited resistance 5 days after inoculation with *Alternaria brassicae*, 12 days after inoculation with *Leptosphaeria maculans*, and 24 h after inoculation with *S. sclerotiorum*. **Conclusions.** Transformation of *B. napus*-RC was shown herein to be an effective trait testing platform for canola. This study also provides an insight into the usefulness of OsACBP5 in enhancing resistance to necrotrophic phytopathogens. **Implications.** OsACBP5 can be overexpressed in other crops to generate pathogen-resistant varieties.

Keywords: acyl-CoA esters, *Alternaria brassicae*, blackleg, hemibiotroph, *Leptosphaeria maculans*, necrotroph, oilseed, phytopathogens, *Sclerotinia sclerotiorum*.

Introduction

Oilseed rape (*Brassica napus* and its related species) is the second largest oilseed crop in the world, providing 13% of the world's food oil supply (Raymer 2002; Neik *et al.* 2017). These oil seeds consist of ~40% oil and generate animal feed with 35–40% protein (Raymer 2002). Apart from the production of edible oil (*B. napus* var. *oleifera* or *napus*; oilseed rape) and animal fodder (*B. napus* var. *napobrassica*; rutabagas, swedes), *B. napus* is cultivated worldwide for human consumption as a vegetable (*B. napus* var. *pabularia*; Siberian or rape kale) (Neik *et al.* 2017).

With an increase in *B. napus* cultivation worldwide, there is a corresponding threat in disease incidences arising from various phytopathogens (Neik *et al.* 2017). Fungal pathogens pose severe threats to the yield of *Brassica* crops, whereas bacterial and viral pathogens have very little effect (Abdel-Farid *et al.* 2009). The most significant fungal disease of *B. napus* affecting the global canola industry is blackleg caused by *Leptosphaeria maculans* and *L. biglobosa*, followed by Sclerotinia rot caused by

S. sclerotiorum, with Alternaria blight caused by *Alternaria brassicae* and *A. brassicicola* also of importance (Fitt *et al.* 2006; Li *et al.* 2009; Macioszek *et al.* 2020; Neik *et al.* 2020). Although *S. sclerotiorum* and *Alternaria* spp. are necrotrophic pathogens, *L. maculans* has an exceptionally complex interaction with *B. napus*, switching between asymptomatic, biotrophic, necrotrophic, and saprotrophic stages while being in contact with *B. napus* (Gay *et al.* 2021). The complex lifestyles of these pathogens make them very difficult to control with reliance on fungicides and resistant cultivars. This has led to the evolution of resistant strains of pathogens (Hahn 2014). To this end, transgenic *B. napus* lines overexpressing novel anti-fungal proteins would be desirable to the oilseed and vegetable industries.

Acyl-CoA-binding proteins (ACBPs) from a protein family associated with acyl-CoA ester transfer in eukaryotes (Xiao and Chye 2011*a*) have been identified to confer protection against microbial pathogens when overexpressed in transgenic plants (Xiao and Chye 2011*b*; Xia *et al.* 2012; Takato *et al.* 2013; Panthapulakkal Narayanan *et al.* 2019, 2020). Besides providing protection against phytopathogens, the Arabidopsis ACBPs have been demonstrated to bind to long-chain acyl-CoA esters and play important roles in plant growth, development and other stress responses (Liao *et al.* 2014; Du *et al.* 2016; Lung *et al.* 2017; Hu *et al.* 2018; Lung and Chye 2019; Panthapulakkal Narayanan *et al.* 2019; Lai and Chye 2021; Alahakoon *et al.* 2022). Although the expression of *AtACBP3* was induced by both a biotrophic bacterial pathogen, *Pseudomonas syringae* pv. *tomato* DC3000, and a necrotrophic fungal pathogen, *B. cinerea*, transgenic Arabidopsis AtACBP3-overexpressing (OE) lines were shown to confer protection only against *P. syringae* (Xiao and Chye 2011*b*).

In contrast, the overexpression of a homologous Class III ACBP from *Vitis vinifera* (grape) in Arabidopsis displayed enhanced resistance to *P. syringae* and a hemibiotrophic fungal pathogen *Colletotrichum higginsianum* (Takato *et al.* 2013). ACBPs from monocotyledonous plants such as rice are less well studied compared to ACBPs from dicotyledonous plants (Xiao and Chye 2011*b*; Xia *et al.* 2012; Takato *et al.* 2013); nevertheless, there have been reports on the function of the rice ACBPs (OsACBPs) (Meng *et al.* 2011, 2020; Meng and Chye 2014; Du *et al.* 2016; Guo *et al.* 2017, 2019; Panthapulakkal Narayanan *et al.* 2019, 2020; Liao *et al.* 2020; Guo *et al.* 2021). OsACBP5, the sole rice Class III ACBP, resembles AtACBP3 in the location of the acyl-CoA-binding (ACB) domain at the carboxyl (*C*-)terminus, unlike the other three ACBP classes (Meng *et al.* 2011). Interestingly, among the six *OsACBPs*, only *OsACBP5* mRNA expression was induced upon infection with the hemibiotrophic rice blast fungal pathogen, *Magnaporthe oryzae* (Meng *et al.* 2011). Panthapulakkal Narayanan *et al.* (2019) demonstrated that the overexpression of OsACBP5 in transgenic Arabidopsis conferred broad-spectrum resistance to representative necrotrophic, hemibiotrophic and biotrophic phytopathogens through cell wall-mediated defence, as well as salicylic acid (SA)- and jasmonic acid (JA)-mediated defence pathways. Subsequently, when OsACBP5 was overexpressed in rice, the transgenic rice plants showed broad-spectrum resistance to representative necrotrophic, hemibiotrophic and biotrophic phytopathogens through SA- and JA-mediated defence pathways (Panthapulakkal Narayanan *et al.* 2020).

A recent study by Alahakoon *et al.* (2022) showed the effective regeneration and transformation of rapid-cycling (RC) *B. napus* from cotyledon explants, which has the potential to be an effective trait testing platform for canola. The short seed-to-seed cycle of these plants makes them an ideal model to evaluate the expression of novel genes in the *B. napus* genome. Moreover, the transgenic *B. napus*-RC overexpressing AtACBP6 displayed enhanced freezing tolerance compared to the wild type (Alahakoon *et al.* 2022). The current study reports on the enhanced protection of transgenic *B. napus* lines (RC and cv. Westar) overexpressing OsACBP5 against seedling infection by the fungal pathogens *L. maculans*, *S. sclerotiorum* and *A. brassicae*.

Materials and methods

Generation of OsACBP5-OE plants

A 1.7-kb *Xba*I-*Bam*HI full-length *OsACBP5* complementary DNA from plasmid pOS581 (Meng and Chye 2014) was cloned into the *Xba*I-*Bam*HI site of binary vector pCAMBIA1304 to generate pOS879, within which *OsACBP5* is driven by the cauliflower mosaic virus *35S* promoter (Panthapulakkal Narayanan *et al.* 2020). OsACBP5-OE Westar and RC lines were generated by *Agrobacterium tumefaciens*-mediated transformation according to Alahakoon *et al.* (2022) using *Agrobacterium* strain LBA4404 harbouring plasmid pOS879 which contains *OsACBP5* and *hpt* encoding hygromycin phosphotransferase for hygromycin resistance.

Two experiments were performed using RC *B. napus* explants and two separate experiments were conducted using *B. napus* cv. Westar explants. With RC *B. napus* explants, there were 11 culture plates in the first experiment and 12 in the second experiment, with 9–10 explants per plate. For cv. Westar, there were 17 individual culture plates in the first experiment and 22 in the second experiment, again with 9–10 explants per plate.

The initial antibiotic selection was applied 2 weeks after inoculation (Boszoradova *et al.* 2011) to facilitate transgenic cell proliferation and establishment. This ensured the transgenic plants developed adequately before undergoing two rounds of antibiotic selection (hygromycin B at 5 mg/L and 10 mg/L). After antibiotic selection, new shoot initials that survived both rounds of selection and remained green were counted in each of the four experiments (two with RC *B. napus* and two with cv. Westar) to calculate the efficiency of production of putatively transformed shoots. To avoid

using genetically identical shoots, only one healthy shoot was extracted per explant. The putatively transformed shoots were extracted and transferred into root induction medium according to the protocol of Alahakoon *et al.* (2022).

Established plants were confirmed to contain the *OsACBP5* cDNA by genotyping with gene-specific primers (Table 1). The same procedure was used to develop pCAMBIA1304 vector-only control RC and cv. Westar *B. napus* plants. The transformed shoots were selected on hygromycin B medium. The pCAMBIA transformation experiment with RC *B. napus* used 13 culture plates with 9–10 explants per plate. The transformation experiment with cv. Westar used 14 culture plates with 9–10 explants per plate.

Molecular characterisation of OsACBP5-OE transgenic plants

DNA was extracted from fully established putative T_o transgenic plants with 3–5 true leaves using Edward's crude extraction method (Edwards *et al.* 1991). Twenty microliters of total reaction volume [1X PCR buffer, 0.2 mM dNTPs (Bioline® 39044, Bioline Pty. Ltd., Australia), 0.2 μM of each primer (Sigma-Aldrich, USA) (Table 1), 2 mM $MgCl_2$ (Bioline® 37026, Bioline Pty. Ltd., Australia), 1-unit *Taq* polymerase (Bioline® 21083, Bioline Pty. Ltd., Australia)] and 50 ng of genomic DNA was used in each polymerase chain reaction (PCR). PCR products were separated in agarose 1.5% (w/v) gel electrophoresis and visualised in the Gel-Doc image system (Bio-Rad) with ethidium bromide (0.5 μg/mL) or 1X Sybr™ Safe DNA gel stain dye. The molecular weight of the amplicon was measured against a 1 Kb Plus Ladder (Invitrogen 10787018, Thermo Fisher Scientific®, U.S.A) by running it parallel to the samples.

Semi-quantitative reverse transcription PCR

RNA was extracted from the putatively transformed plants using TRIzol reagent (Invitrogen® 15596018, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. RNA was treated with DNase (TURBO DNA free kit Invitrogen® AM1907, Thermo Fisher Scientific, USA) to remove any traces of genomic DNA. One μg of RNA was reverse transcribed using iScript TM cDNA synthesis kits (Bio-Rad® 170-8891, Bio-Rad Laboratories, USA) according to the manufacturer's instructions. A 1.5-μL aliquot from each cDNA sample was used in conventional PCR with gene-specific primers, ML1111/ML1112 (Table 1).

Western blot analysis

Western blot analysis on transgenic T_o RC *B. napus* and T_o Westar OsACBP5-OE lines was conducted using total plant protein extracted from 3-week-old *B. napus* wild-type plants and OsACBP5-OE plants according to Fido *et al.* (1995). The concentration of the protein samples was measured using the Micro Lowry method with Peterson's modification (Sigma® TP0300, Sigma-Aldrich, USA) as per the manufacturer's instructions. Forty micrograms of protein were loaded per sample for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to Amersham Hybond-P Polyvinylidene Fluoride (PVDF)® membrane and the immunoblotting was performed with anti-recombinant $(His)_6$-OsACBP5 antibodies (Panthapulakkal Narayanan *et al.* 2020) to confirm protein expression in transgenic OE lines. These highly specific and sensitive rabbit polyclonal antibodies were generated by EzBiolab (http://www.ezbiolab.com/). The antibodies were raised against a synthetic peptide (VVDAGLEERVQAI) corresponding to amino acids 250–262 of OsACBP5 (Panthapulakkal Narayanan *et al.* 2020). Rice protein was used as the positive control in Western blot analysis with wild-type RC and *B. napus* cv. Westar canola as negative controls.

Plant material and plant growth conditions for bioassays

The pathogenicity assays were performed on T_3 plants for three independent *OsACBP5* transgenic *B. napus* lines (OE-6 (Westar), OE-7 (RC) and OE-10 (RC)) and on T_2 plants of one Westar line (OE-27). *B. napus* plants from these four OsACBP5-OE independent lines (OE-6, OE-7, OE-10 and OE-27) were maintained in the glasshouse at 23°C in 16/12 h light/dark. Transgenic seeds were selected at T_1 and T_2 by soaking in 100 mg/L hygromycin B for 36 h and directly sowing into soil (Li *et al.* 2010). Surviving plants, which were re-confirmed to be transgenic by PCR analysis, were maintained in the glasshouse to harvest T_3 seeds for the pathogenicity assays.

Table 1. Primer sequences and PCR conditions used in this study.

Primer pair	Primer sequence (5′–3′) and the base pair position within the cDNA in brackets	PCR condition
35SB ML2592	CAATCCCACTATCCTTCGCAAGACC (25–49) CTCCGTCTCTGCCAATGCCAC (928–948) Amplicon size – 1.1 kb	(95°C–30 s, 60°C–45 s, 72°C–1.5 min) 37 cycles
ML1111 ML1112 (Meng *et al.* 2011)	GAGGCTATTCCAGGATGGAT (1495–1514) CTGTCATGTTGGTTGATTGTAT (1592–1613) Amplicon size – 118 bp	(95°C–30 s, 60°C–45 s, 72°C–1.5 min) 37 cycles

Alternaria brassicae inoculation assay

A. brassicae (Mycology Lab, School of Biosciences, University of Melbourne) was cultured on potato-dextrose agar (PDA) plates for incubation at 25°C for 5 days. Fungal spores from *A. brassicae* culture were collected in sterile water, the suspension was filtered through sterile cheesecloth, and the spore concentration was adjusted to 1×10^6 spores/mL. Each of the four cotyledon lobes of a seedling (T_3 of OE-6, OE-7, OE-10 and T_2 of OE-27) was wounded using a sterile needle and inoculated with 10 μL of spore suspension. The inoculated seedlings were covered with a polyethylene bag for 24 h to maintain humidity. Disease severity was scored using a 0–6 scale 5 days post inoculation (dpi), where 0 = no disease, 1 = 10%, 2 = 20%, 3 = 40%, 4 = 60%, 5 = 80%, 6 = more than 80% chlorotic leaf area (O'Hara *et al.* 2016). The experiment was repeated three times ($n = 10$).

Leptosphaeria maculans cotyledon assay

The *L. maculans* cotyledon assay used Pathotype D5 (Sonah *et al.* 2016) sourced from the Mycology Lab, School of Biosciences, University of Melbourne. This is a key representative of the most aggressive pathotypes of *L. maculans*. The pathogen was inoculated onto 10% V8 medium (400 mL of V8 medium (pH 6.5) containing 40 mL of V8® juice (Campbell Australia Pty. Ltd), 1.5 g calcium carbonate ($CaCO_3$) and 8 g agar, supplemented with rifampicin at 10 mg/mL and chloramphenicol at 30 mg/mL) and incubated at 23°C for 12 days. The pycnidia on the V8 cultures were scraped and mixed with sterile distilled water, centrifuged at 2400*g* for 30 min, re-suspended in sterile distilled water and the spore concentration was adjusted to 1×10^6 spores/mL.

Brassica napus (T_3 of OE-6, OE-7, OE-10 and T_2 of OE-27) seeds were sown at a density of 10 seeds per 10 × 20 cm punnet, covered with plastic lids and kept in a plant growth cabinet at 23/18°C day/night in 16/8 h light/dark. After 12 days, the seedlings were gently pricked on each of the four cotyledon lobes using a sterile surgical needle and 10 μL of spore suspension was dispensed on the pinpricked point using a pipette. The plants were returned into the original plant growth cabinet and maintained for 2 weeks until symptoms developed. Twenty individual plants per genotype were tested with each plant having four inoculation points. On the 12th day after inoculation, the lesion diameters were recorded, and two measurements were made perpendicular to each other. Disease severity was recorded according to a modified version of the disease scoring system used by Mengistu *et al.* (1991).

To eliminate bias due to the non-linear nature of the 0–9 scoring system, the median disease scores were calculated by arranging the 4 observations per plant in numerical order and obtaining the mean of the second and third values.

Sclerotinia sclerotiorum leaf assay

The tolerance or susceptibility of *OsACBP5* transgenic *B. napus* plants (T_3 of OE-6, OE-7, OE-10 and T_2 of OE-27) to *S. sclerotiorum* was tested using a detached leaf assay following Wang *et al.* (2009) with minor modifications. Three-millimetre diameter agar plugs were prepared from an actively growing 4-day-old *S. sclerotiorum* pathotype UQ1280 culture (Mycology Lab, School of Biosciences, University of Melbourne). Thirteen young but fully expanded leaves were collected from OsACBP5-OE lines and vector-control plants and arranged on Petri plates layered with moist filter paper. Agar plugs were placed on the adaxial surface of the leaves with hyphae facing down while avoiding major veins. The leaves were incubated at room temperature (23°C) in 10/14 h light/dark. Lesion development was measured at 24 and 48 h after inoculation (Solgi *et al.* 2015).

Statistical analysis

Differences in data between different samples were analysed by the Student's *t*-test and Fisher's least significant difference (protected) test.

Results

Transformation and characterisation of transgenic *B. napus* OsACBP5-OEs

Transgenic RC *Brassica napus* OsACBP5-overexpressor (OE) and *B. napus* cv. Westar lines generated through *Agrobacterium*-mediated transformation had an average transformation efficiency of 24.2% and 29.1% respectively. A total of 19 positive transgenic *B. napus 35Spro::OsACBP5* lines, consisting of 8 RC and 11 cv. Westar, harbouring the transgene from plasmid pOS879 (Panthapulakkal Narayanan *et al.* 2020) were identified by PCR analysis using the *35S* promoter-specific primer 35SB as the forward primer and the *OsACBP5* gene-specific primer ML2592 as the reverse primer (Fig. 1*a*). The PCR product showed an expected 1.7-kb band size in all 19 lines (Fig. 1*b*). Semi-quantitative reverse transcription PCR performed on the transgenic lines that were confirmed by PCR revealed that all the 19 lines showed the expected 118-bp band (Fig. 1*c*). Western blot analysis using anti-OsACBP5 antibodies to check OsACBP5 protein expression in transgenic *B. napus* OsACBP5-OE lines, with wild-type *B. napus* and the vector-transformed line (pCAMBIA1304) as negative controls and wild-type rice as a positive control, indicated that four (OE-6, OE-7, OE-10 and OE-27) of the 19 lines overexpressed OsACBP5 and produced a 61.2-kD cross-reacting OsACBP5 band (Fig. 1*d*). Of these lines, OE-6 and OE-27 were cv. Westar transgenic lines and OE-7 and OE-10 were RC transgenic lines.

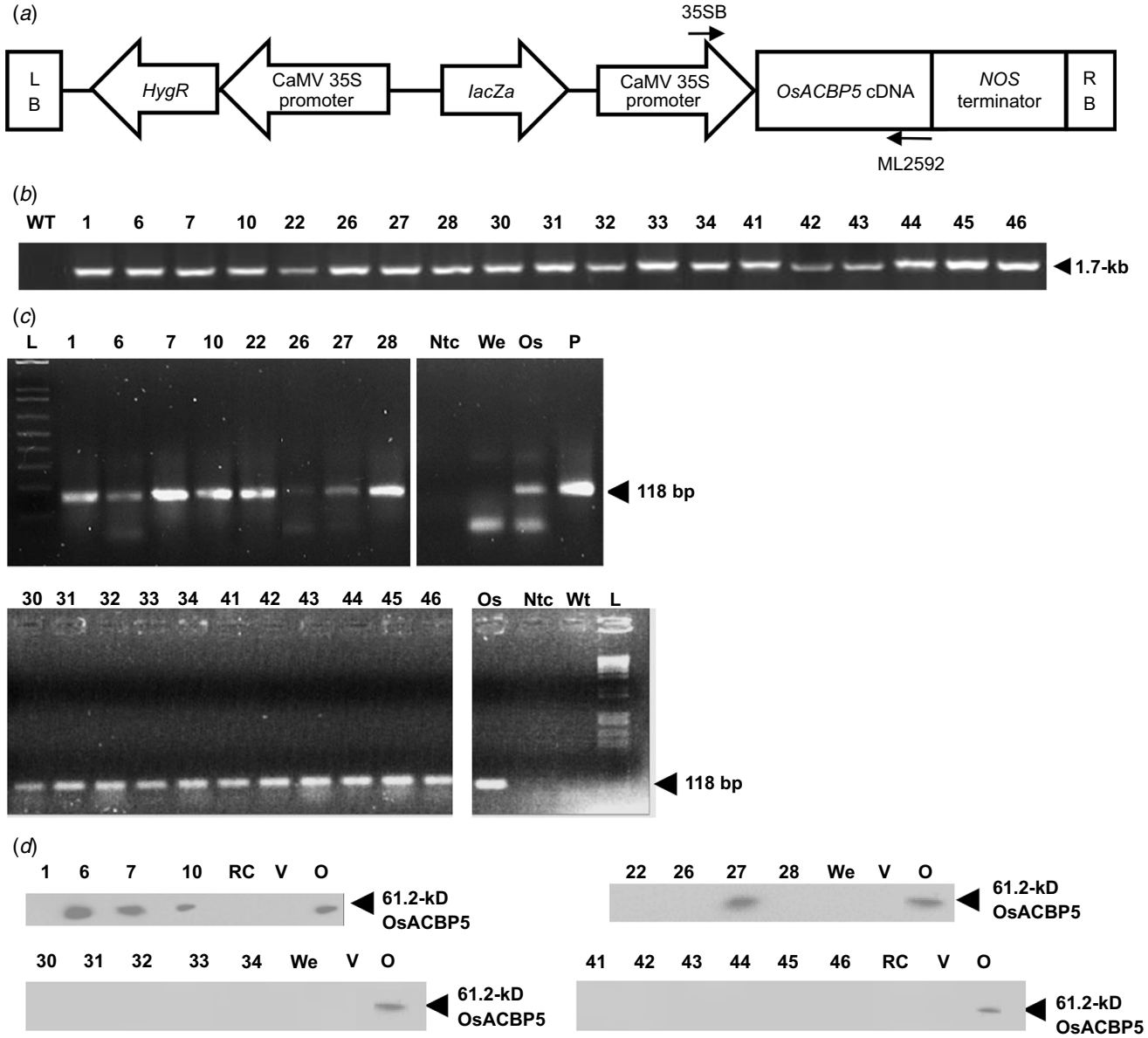

Fig. 1. Identification of transgenic *Brassica napus* OsACBP5-OE lines. (*a*) Schematic diagram showing the *35Spro::OsACBP5* construct (pOS879) used to generate transgenic *B. napus* OsACBP5-OE lines CaMV, cauliflower mosaic virus, *lacZα*, β-galactosidase α, *NOS* terminator, nopaline synthase terminator, *HygR*, hygromycin resistant gene, LB and RB, left border and right border of transfer (T-) DNA, respectively. (*b*) PCR genotyping on DNA from transgenic *B. napus* OsACBP5-OE leaves using a *35S* promoter-specific forward primer (35SB) and *OsACBP5*-specific reverse primer (ML2592) targeting a 1.7-kb band (arrowed). WT, wild-type RC as a negative control. (*c*) Reverse transcription PCR using ML1111/ML1112 primer pair on cDNA synthesised from RNA extracted from PCR-positive transgenic T_o lines. Expected band (118 bp) is indicated with an arrowhead. L, 1 kb plus ladder; Wt, wild type; Ntc, control without template; We, wild-type Westar; Os, rice positive control; P, plasmid (pOS879) control. (*d*) Western blot analysis of total protein extracted from transgenic *B. napus* OsACBP5-OE lines using antibodies specific against OsACBP5. Twenty μg of total leaf protein per well were separated on a 10% SDS-PAGE. Lanes labelled in numbers above indicate transgenic *B. napus* OsACBP5-OE lines (1, 6, 22, 26–28, 30–34 are Westar *B. napus;* 7, 10, 41–46 are RC *B. napus*); lane RC, *B. napus* wild-type RC; lane We, *B. napus* wild-type Westar; lane V, vector-transformed control; lane O, wild-type rice. Arrowhead indicates the cross-reacting 61.2-kD OsACBP5 protein. Among the positive transformants, lines 6 and 27 are Westar *B. napus*; and lines 7 and 10 are RC *B. napus*.

Under normal growth conditions, no obvious differences in growth and development were observed between the *B. napus* vector control and the OsACBP5-OE lines. Hence, measurements of phenotypic differences between transgenic *B. napus* OsACBP5-OEs and the vector control were not pursued.

B. napus OsACBP5-OEs are protected against *A. brassicae*

The RC vector control and Westar vector control at 5 days post-infection displayed 3–4 average disease scores. The average disease score on RC transgenic *B. napus* OsACBP5-OEs and Westar transgenic *B. napus* OsACBP5-OEs was around 1, an approximately two-fold reduction in chlorotic leaf area when compared to their respective control lines (Fig. 2*a*, *b*).

Transgenic *B. napus* OsACBP5-OEs are better protected against *L. maculans*

The average median disease scores were significantly lower in the OsACBP5-OE lines compared to their respective vector controls (Fig. 3*a*). RC OE-7 displayed the lowest disease score at 3.6 followed by RC OE-10 at 4.4. VC RC plants displayed an average disease score of 5.7. The average disease score was the highest for VC Westar plants at 6.9

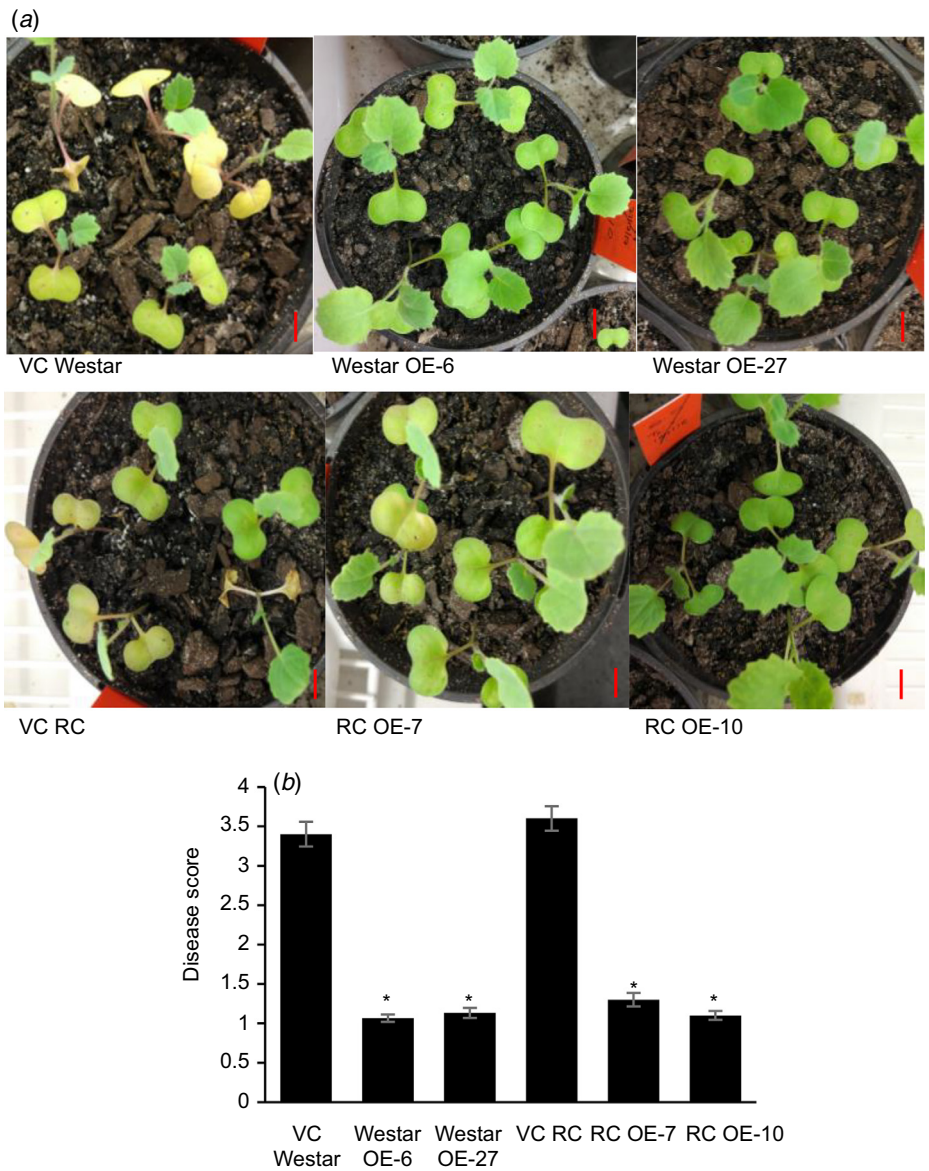

Fig. 2. Transgenic *Brassica napus* OsACBP5-OEs are better protected against the fungal necrotroph *Alternaria brassicae*. (*a*) Disease symptoms on the 2-week-old vector-transformed control (VC) of Westar and transgenic Westar *B. napus* OsACBP5-OEs (OE-6, OE-27) (upper panel) and rapid-cycling (RC) VC and transgenic RC *B. napus* OsACBP5-OEs (OE-7, OE-10) (bottom panel) inoculated with *A. brassicae* photographed 5 days post-infection. Scale = 1 cm. (*b*) Disease scores from *A. brassicae*-infected cotyledons. Data points represent means ± s.e. from three independent experiments. Asterisks indicate significant difference ($P < 0.05$) in comparison to respective controls by Student's *t*-test.

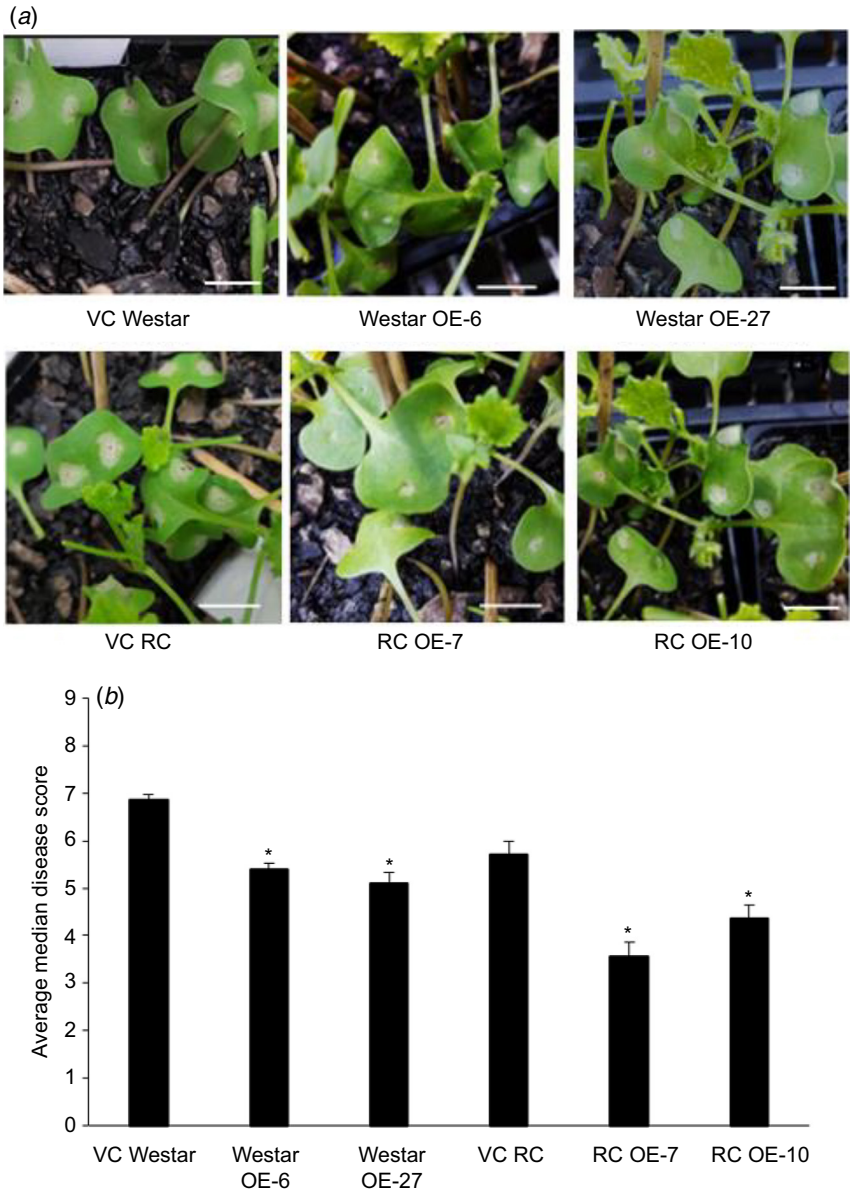

Fig. 3. Disease resistance of transgenic *Brassica napus* OsACBP5-OEs against fungal hemibiotroph *Leptosphaeria maculans*. (*a*) Disease symptoms on 2-week-old Westar *B. napus* vector-transformed control (VC Westar), rapid-cycling *B. napus* vector-transformed control (VC RC) and OsACBP5-OE *B. napus* plants (Westar OE-6, Westar OE-27, RC OE-7, and RC OE-10) showing variable but comparatively smaller lesions 12 days post-infection. Scale = 1 cm. (*b*) Average median disease scores of *L. maculans*-infected cotyledons. Data points represent means ± s.e. Asterisks indicate significant difference ($P < 0.05$) in comparison to respective controls by Fisher's least significant difference (protected) test ($n = 20$).

(close to 7 on the disease scale) and most of the cotyledons developed grey-green lesions that were larger than 5 mm. Westar OE-6 and Westar OE-27 displayed an average median disease score of 5.4 and 5.1 respectively (Fig. 3*b*).

B. napus OsACBP5-OEs show better protection against *S. sclerotiorum*

After young, fully expanded detached leaves from 4-week-old plants were inoculated with *S. sclerotiorum*, light brown necrotic lesions started to develop around the agar plug by 24 h after inoculation (Fig. 4*a*). The smallest mean lesion size ($P < 0.05$) among the RC *B. napus* plants occurred in transgenic line OE-7 at 8.8 mm. Line OE-10 (RC) also showed a low mean lesion size of 10.3 mm, but this was not significantly different when compared to corresponding vector-control plants (Fig. 4*b*). Of the cv. Westar plants, transgenic lines OE-6 and OE-27 showed significantly smaller ($P < 0.05$) lesion sizes of 8.5 mm and 9.2 mm, respectively, when compared to the vector-control plants (12.1 mm) (Fig. 4*b*).

Discussion

Rapid cycling plants: an effective trait testing platform for *B. napus*

The transformation efficiencies for *Brassica napus* cv. Westar plants (24.2%) and RC plants expressing OsACBP5 (29.1%)

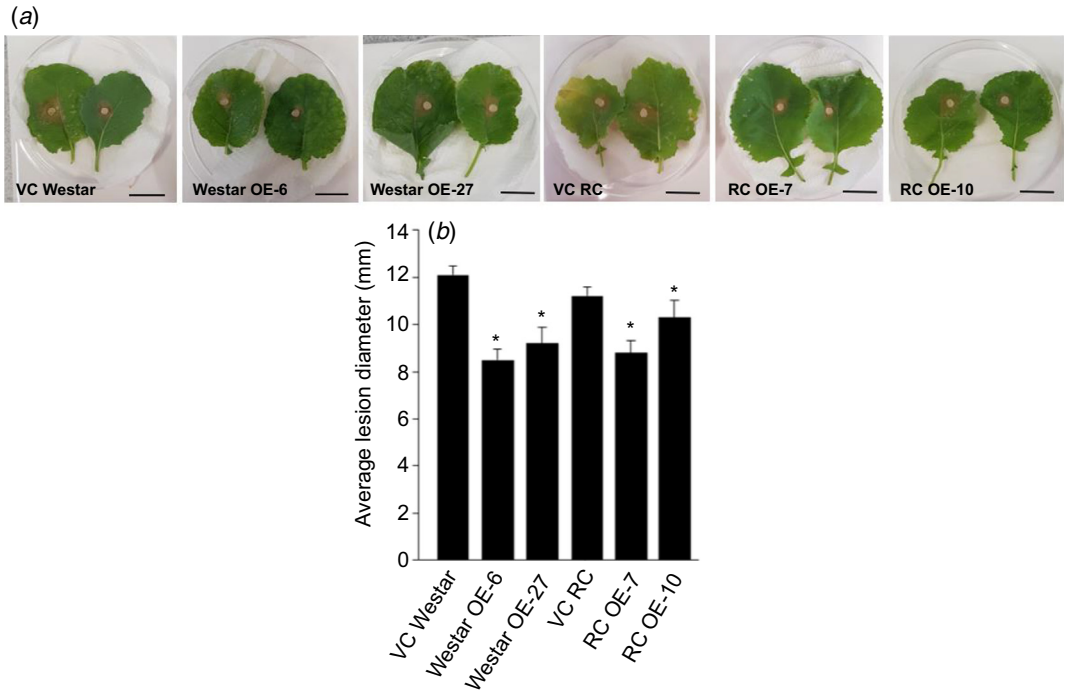

Fig. 4. Disease resistance of transgenic *Brassica napus* OsACBP5-OEs against fungal necrotroph *Sclerotinia sclerotiorum*. (*a*) Disease symptoms on fully expanded Westar vector-transformed control (VC Westar) compared to OsACBP5-OE transgenic line Westar OE-6, OE-27, and rapid-cycling *B. napus* vector-transformed control (VC RC) compared to OsACBP5-OE *B. napus* transgenic line RC OE-7, RC OE-10. Scale = 2 cm. (*b*) Average lesion diameters of *S. sclerotiorum*-infected leaves 24 h post-infection. Data points represent means ± s.e. Asterisks indicate significant difference ($P < 0.05$) in comparison to respective controls by Fisher's least significant difference (protected) test ($n = 13$).

were higher than the transformation efficiency reported for *B. napus*-RC cotyledons (16.4%) containing the *Arabidopsis thaliana* cDNA for ACYL-COA-BINDING PROTEIN6 (AtACBP6) reported by Alahakoon *et al.* (2022). The improved transformation efficiencies were most likely related to the use of *Agrobacterium* strain LBA4404 compared to the strain GV3101 used by Alahakoon *et al.* (2022). The choice in the *Agrobacterium* strain for a specific plant species is an important factor in the success of plant genetic transformation. Previous studies have reported the use of different *Agrobacterium* strains to optimise transformation of a specific plant. When *Agrobacterium* strains LBA4404, EHA101 and EHA105 were used for banana transformation, EHA105 was found to be more efficient than the others (Subramanyam *et al.* 2011). Kim *et al.* (2013) obtained higher transformation efficiencies in reed plants using *Agrobacterium* strain EHA105 compared to LBA4404 and GV3101. Similarly, Kuvshinov *et al.* (1999) found higher transformation efficiency using LBA4404 in transformation of *Brassica rapa* than with C58C1 and EHA105 *Agrobacterium strains*. Higher transformation efficiencies in *Bacopa monnieri* were obtained using *Agrobacterium* strain GV3101 compared to LBA4404 and EHA105 (Yadav *et al.* 2014). Hence, selection of *Agrobacterium* strain appears to be vital in transformation experiments as it can considerably alter the transformation efficiency.

Moreover, the high transformation efficiencies for *B. napus*-RC plants is a substantial improvement compared to recent studies, which reported transformation efficiencies of 1.4–3% (Min *et al.* 2007), 2.89–5% (Lee *et al.* 2004), 1.2% (Konagaya *et al.* 2013), 15% (Baskar *et al.* 2016) from hypocotyl explants and 10.83% (Li *et al.* 2021) and 14% (Sivanandhan *et al.* 2021) from explants from cotyledons of *Brassica rapa* L. ssp. *pekinensis*. Hence, the current transformation protocol demonstrates the usefulness of the RC plants in being an effective trait testing platform for *B. napus*.

Rice Class III OsACBP5 does not confer a change in development when overexpressed in *Brassica*

The lack of obvious differences in growth and development under normal growth conditions between the *B. napus* vector control and the OsACBP5-OE lines resembled transgenic Arabidopsis and rice overexpressing OsACBP5. When 4-week-old transgenic Arabidopsis OsACBP5-OEs were grown for pathogen (*Botrytis cinerea* and *A. brassicicola*) treatments, the mock-treated plants did not show any

phenotypic differences from the vector control (Panthapulakkal Narayanan *et al.* 2019). Likewise, no phenotypic changes in growth were seen between 4-week-old vector control and transgenic rice OsACBP5-OEs (Panthapulakkal Narayanan 2018). All plants exhibited normal growth and development and adverse effects were not evident (Panthapulakkal Narayanan 2018). Also, when 3-week-old transgenic Arabidopsis AtACBP3-OEs and WT were grown for dark treatment, no phenotypic differences were observed in these plants before treatment (Xiao *et al.* 2010), suggesting that under normal growth conditions, Class III AtACBP3 and OsACBP5 did not appear to affect plant development.

Rice Class III OsACBP5 protects against seedling infection of canola by phytopathogens when overexpressed in *Brassica*

This study extends the application of OsACBP5 in disease protection to *B. napus*. In pathogen assays against the fungal pathogens *L. maculans, S. sclerotiorum* and *A. brassicae*, the transgenic *B. napus* Westar and RC OsACBP5-OEs lines were better protected than the vector-transformed controls (Figs 2, 3, 4), suggesting the usefulness of OsACBP5 in seedling protection against initial infection. These results are consistent with those observed for enhanced resistance of transgenic Arabidopsis OsACBP5-OEs following infection with various phytopathogens including representative necrotrophic fungal pathogens (*Rhizoctonia solani*, *B. cinerea* and *A. brassicicola*), hemibiotrophic fungal pathogen *Colletotrichum siamense* and a biotrophic bacterial pathogen *P. syringae* (Panthapulakkal Narayanan *et al.* 2019). Similar results were observed in transgenic rice OsACBP5-OEs when infected with various phytopathogens; rice OsACBP5-OEs improved tolerance to necrotrophic fungal pathogens *R. solani* and *Cercospora oryzae*, hemibiotrophic fungal pathogens *Magnaporthe grisea* and *Fusarium graminearum*, and a biotrophic bacterial pathogen *Xanthomonnas oryzae* (Panthapulakkal Narayanan *et al.* 2020). Thus, it appears that this rice Class III ACBP is a promising tool for application in disease protection in both dicot and monocot seedlings.

The plant–pathogen interaction takes place at an early stage after the initial contact of the fungal pathogen with the host. Studies have demonstrated that various biochemical responses occurred as soon as within 5 min of plant–pathogen interaction (Dodds and Rathjen 2010; Kabbage *et al.* 2015; Shen *et al.* 2017; Neik *et al.* 2020). Protein phosphorylation, ion fluxes, reactive oxygen species and other signal transduction processes are considered responsible for initial plant–pathogen interactions (Boller and Felix 2009; Dodds and Rathjen 2010; Robatzek 2014; Bolouri Moghaddam *et al.* 2016). Further investigations into molecular mechanism of initial protection against various phytopathogens are important for pathogenesis study as well as generation of resistant crop varieties. Moreover, interpretation of molecular responses responsible for initial protection would be vital for understanding the primary mechanism of plant–pathogen interaction in various essential agricultural disease as well as medical pathogen systems.

Transgenic *B. napus* overexpressing genes involved in the glucosinolate (GSL)-biosynthesis pathway resulted in a reduction in disease symptoms and tissue damage upon infection with the necrotrophic foliar pathogens, *S. sclerotiorum* and *B. cinerea*, when compared with the wild-type control (Zhang *et al.* 2015). An increased level of GSL content was observed upon *A. brassicae* infection in *B. napus* (Doughty *et al.* 1991). The GSL-degradation products such as isothiocyanates have the potential to inhibit *A. brassicae* growth in *B. napus* as they have antibiotic effects on the *Brassica* pathogens, *A. brassicae*, *Sclerotinia scletoriorum, Xanthomonas campestris* pv. *campestris* and *P. syringae* pv. *maculicola* (Sotelo *et al.* 2015). Hence, similar to transgenic Arabidopsis OsACBP5-OEs, upregulation of proteins associated with GSL-degradation could likely account for the necrotrophic resistance in transgenic *B. napus* OsACBP5-OEs as observed in this study and this should be confirmed by future proteomics, transcriptomics and GSL analyses on the *A. brassicae*-infected *B. napus* OsACBP5-OE lines.

Conclusion

In conclusion, this study applied an *Agrobacterium*-mediated transformation protocol using the *Agrobacterium* strain LBA4404 to efficiently generate *OsACBP5*-overexpressing rapid-cycling transgenic plants. This study highlights the use of *Agrobacterium* strain LBA4404 for achieving a higher transformation efficiency in *B. napus*-RC plants as compared to previous studies. This study also provides an insight into the usefulness of OsACBP5 in enhancing resistance to necrotrophic phytopathogens *S. sclerotiorum*, *A. brassicae* and *L. maculans*. Pathogen assays using OsACBP5-overexpressing seedlings indicated that the *OsACBP5*-overexpressing *B. napus*-RC were more tolerant to early infection than the wild type.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflicts of interest.

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