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TITLE PAGE

OVEREXPRESSION OF A MONOCOT ACYL-COA-BINDING PROTEIN CONFERS BROAD-SPECTRUM PATHOGEN PROTECTION IN A DICOT

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Author Contributions

S.P.N. performed most of the experiments. S.P.N and P.L. analysed proteomic data. S.P.N., P.L., P.T., C.L., and M.L.C. analysed data. S.P.N. and M.L.C. designed the research and wrote the manuscript with contribution from all authors.

Conflict of Interests

The authors of this manuscript declare that there was no conflict of interest.

Running title: Arabidopsis OsACBP5-overexpressors show enhanced fungal-protection

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ABSTRACT

Plants are continuously infected by various pathogens throughout their lifecycle. Previous studies have reported that the expression of Class III acyl-CoA-binding proteins (ACBPs) such as the Arabidopsis ACBP3 and rice ACBP5 were induced by pathogen infection. Transgenic Arabidopsis AtACBP3-overexpressors (AtACBP3-OEs) displayed enhanced protection against the bacterial biotroph, *Pseudomonas syringae*, although they became susceptible to the fungal necrotroph *Botrytis cinerea*. A Class III ACBP from a monocot, rice (*Oryza sativa*) OsACBP5 was overexpressed in the dicot Arabidopsis. The resultant transgenic Arabidopsis lines conferred resistance not only to the bacterial biotroph *P. syringae* but to fungal necrotrophs (*Rhizoctonia solani, B. cinerea*, *Alternaria brassicicola*) and a hemibiotroph, (*Colletotrichum siamense*)*.* Changes in protein expression in *R. solani*-infected Arabidopsis OsACBP5-overexpressors (OsACBP5-OEs) were demonstrated using proteomic analysis. Biotic stress-related proteins including cell wall-related proteins such as FASCILIN-LIKE ARABINOGALACTAN-PROTEIN10, LEUCINE-RICH REPEAT EXTENSIN-LIKE PROTEINS, XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE PROTEIN4 and PECTINESTERASE INHIBITOR18; proteins associated with glucosinolate degradation including GDSL-LIKE LIPASE23, EPITHIOSPECIFIER MODIFIER1, MYROSINASE1, MYROSINASE2 and NITRILASE1; as well as a protein involved in jasmonate biosynthesis, ALLENE OXIDE CYCLASE2, were induced in OsACBP5-OEs upon *R. solani* infection. These results indicated that upregulation of these proteins in OsACBP5-OEs conferred protection against various plant pathogens.

Significance of the study:

Earlier studies have shown that the overexpression of Arabidopsis ACYL-COA-BINDING PROTEIN3 displayed enhanced protection to the bacterial biotroph *Pseudomonas syringae,* whereas the same lines were susceptible to the fungal necrotroph *Botrytis cinerea.* Overexpression of the rice ACYL-COA-BINDING PROTEIN5 conferred resistance to necrotrophic, hemibiotrophic and biotrophic phytopathogens in transgenic Arabidopsis. Proteomic analysis indicated that the defence responses arising from OsACBP5 overexpression in transgenic Arabidopsis involved cell wall-mediated defence as well as salicylic acid (SA)- and jasmonic acid (JA)-mediated defence pathways.

1. INTRODUCTION

Fungal diseases are a significant concern in global agriculture and food production. To ensure a steady and uniform supply of food to a constantly mounting world population, there is a need to control fungal diseases which impose a bigger threat than bacteria and viruses, in crops. Over evolution, plants have developed sophisticated methods to overcome pathogen attack from phytopathogens to which they are susceptible throughout their life cycle. Plant-fungal pathogens are broadly classified into two groups, biotrophic pathogens and necrotrophic pathogens [1,2]. Biotrophic pathogens infect the host plant and utilize nutrients for growth from the plant cells without killing the host [1]. In contrast, necrotrophic pathogens kill the host cells after infection and utilize the resulting cell contents [3-5]. In addition to the biotrophs and necrotrophs, there are hemibiotrophs with characteristics of both biotrophs and necrotrophs. Hemibiotrophs invade the host cells, utilizing nutrients without killing the host, then become necrotrophic at the later stages of their life cycle [5].

Acyl-CoA binding proteins (ACBPs) have been reported to play a role in plant stress, including defence against pathogens. ACBPs facilitate the binding of medium- and long-chain acyl-CoA esters at the conserved acyl-CoA-binding domain [6-8]. Six ACBPs occur in the model plant *Arabidopsis thaliana* which function in plant growth, development and stress responses [9-23]. *AtACBP3* mRNA was induced by both bacterial (*Pseudomonas syringae* pv *tomato* DC3000) and fungal (*Botrytis cinerea*) pathogens and transgenic Arabidopsis AtACBP3-overexpressors (OEs) were protected against *P. syringae* but not *B. cinerea* [12]. Nevertheless, the T-DNA insertional mutant (*atacbp3*) was susceptible to *P. syringae,* hemibiotroph

Colletotrichum higginsianum and necrotroph *B. cinerea* [24], indicating that susceptibility to *B. cinerea* arises by both overexpression and loss of AtACBP3 [12,24].

Furthermore, the overexpression in Arabidopsis of a homologous Class III ACBP from the dicot *Vitis vinifera* (grapevine) was reported to improve resistance to *P. syringae* and *C. higginsianum* [25]. As ACBPs from monocotyledonous plants such as rice are least explored in comparison with ACBPs from dicotyledonous plants [12,24,25], rice ACBP5 (OsACBP5) which represents the homolog of AtACBP3 [26], was selected for investigations herein. Among the six *OsACBPs*, only *OsACBP5* expression was induced upon infection with the hemibiotrophic rice blast fungal pathogen, *Magnaporthe grisea* [26]. Given that the overexpression of dicot Class III ACBPs in Arabidopsis had conferred tolerance to pathogen infection [12,24,25], it would be pertinent to evaluate the response of a monocot ACBP such as OsACBP5 in transgenic Arabidopsis.

Plants that have established various biotic stress-specific responses such as activation of defence-related genes and changes in the expression of plant proteins in response to different stresses can be studied by proteomics [27]. Proteomic analysis can help improve knowledge of various defence mechanisms triggered by invading pathogens [27,28] and the signalling pathways associated with stresses [28,29]. For instance, proteomic analysis of *Fusarium sporotrichioides*- and *F. graminearum*-infected Arabidopsis showed a significant difference in biotic stress-related proteins such as pathogenesis-related (PR) proteins and glutathione transferases [30,31]. Similarly, proteomic analysis of *F. moniliforme*-infected maize [32], *F. graminearum*-infected wheat and barley [33,34], and *F. oxysporum*-infected tomato [35] identified upregulated PR proteins. Proteomic analysis on rice leaves infected by *M. grisea* showed significant changes in defence-related proteins including a PR protein and a thaumatin-like protein [36,37].

The aim of this study was to address the efficiency of OsACBP5 in conferring resistance to root-infecting fungal necrotroph *Rhizoctonia solani,* leaf-infecting necrotrophs (*B. cinerea* and *Alternaria brassicicola*), the hemibiotrophic fungal pathogen *Colletotrichum siamense* and the bacterial biotroph *P. syringae* in transgenic Arabidopsis. *Pseudomonas syringae* is a leaf-infecting bacterial biotroph [38]. The other pathogens were selected because *Rhizoctonia solani, B. cinerea* and *A. brassicicola* are necrotrophic fungal pathogens which infect and cause production loss in many economically important crops [39-41]. *Botrytis cinerea* and *A. brassicicola* are foliar pathogens [39], whereas *R. solani* is a soil-borne pathogen [42]. The representative hemibiotrophic fungal pathogen selected was *C. siamense* because it has a broad host range and causes economically important anthracnose in a range of crops [43]. To explore the defence mechanism of OsACBP5-OEs against pathogen infection, proteomic analysis was carried out on *R. solani*-infected transgenic Arabidopsis OsACBP5-OE lines using vector-transformed (pBI-eGFP) lines as a control.

2. MATERIALS AND METHODS

2.1. Plant materials

Arabidopsis seeds from the wild type Col-0 (WT), vector-transformed control (pBI-eGFP), *35S::AtACBP3::GFP* lines (AtACBP3-OEs)[44] and *35S::OsACBP5::GFP* lines (OsACBP5-OEs)[45] were surface sterilised with 20% bleach containing 0.1%

Tween20 for 25 min, washed with distilled water and plated on Murashige Skoog (MS) [46] medium. The plates were chilled at 4°C for 2 days and transferred to a growth room under 16 h light /8 h dark at 21-23°C. Two-week-old seedlings were transferred to soil in a growth chamber under a 16 h light (24°C)/8 h dark (21°C) photoperiod. Supporting Information provides details on the generation of transgenic Arabidopsis (AtACBP3-OEs and OsACBP5-OEs), various pathogens used, pathogen assays, and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

2.2. Quantitative proteomic analysis

Sequential window acquisition of all theoretical mass spectra (SWATH-MS) proteomic analysis was carried out on necrotrophic fungal (*R. solani*)-infected transgenic Arabidopsis OsACBP5-OE line 7 using the vector-transformed line as a control. Proteins were extracted from Arabidopsis seedlings (500 mg fresh weight, consisting of ~25 seedlings each) by the trichloroacetic acid/acetone method [47]. The protein pellet was dissolved with 2 mL urea buffer (6 M urea and 4 mM calcium chloride in 200 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS), pH 8.0) [48]. An equal amount of protein (100 µg) was reduced using 10 mM dithiothreitol (DTT) and alkylated in 40 mM iodoacetamide (IAA) in the dark. After alkylation, the mixture was diluted with 4 mM $CaCl_2$ to reduce the concentration of urea to less than 2 M. Trypsin was added to digest the protein at a 1:20 ratio by incubation at 37°C overnight. Following trypsin digestion, the peptides were desalted utilising C18 SepPak reverse-phase cartridges and SWATH-MS analysis was performed [49]. All conditions for SWATH-MS measurement and analysis were exactly as described in Zhu et al

[49]. Supporting Information provides the protocol in detail. The data was analysed from five biological repeats. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD013031.

2.3. Statistical analysis

Significant differences in data between different samples were analysed by the Student's *t*-test.

3. RESULTS

3.1. Arabidopsis OsACBP5-OEs were protected against necrotrophs

Transgenic Arabidopsis OsACBP5-OEs were initially tested for protection against the root-infecting necrotroph, *R. solani,* and shoot-infecting necrotrophs, *B. cinerea* and *A. brassicicola*. When one-week-old Arabidopsis WT, vector (pBI-eGFP)-transformed control, AtACBP3-OEs (At3OE-1, At3OE-4 and At3OE-6) and OsACBP5-OEs (Os5OE-4, Os5OE-5 and Os5OE-7) were inoculated with fungal mycelium in the MS plate (Figure 1A) and soil assays (Figure 1B), OsACBP5-OEs were observed to be protected against *R. solani*. In MS plate assays, necrosis was visible 6-7 days post-inoculation (DPI) (Figure 1A). Arabidopsis OsACBP5-OEs displayed significantly lower disease incidence (26%) in comparison to the WT (64%) and vector-transformed plants (73%) (Figure 1C). The disease incidence of AtACBP3-OEs (60%) was not significantly different from the control lines (Figure 1C).

When WT, vector-transformed plants, AtACBP3-OEs and OsACBP5-OEs were grown in *R. solani* pre-colonised soil in pots (Figure 1B), the WT and vector-transformed plants exhibited a high degree of susceptibility, with 71% and 69% of disease incidence, respectively (Figure 1C). In contrast, OsACBP5-OEs showed significantly lower disease incidence (48.6%) than the controls (Figure 1C). Results of soil-grown plants were consistent with those from MS plate assays, suggesting a role for OsACBP5 in reducing infection against the root-infecting necrotroph *R. solani*.

When transgenic Arabidopsis OsACBP5-OEs were subjected to infection by the leaf-infecting necrotrophs (*B. cinerea* and *A. brassicicola*) in detached leaf assays (drop inoculation), necrotic lesions appeared at the *B. cinerea* inoculated spots for the WT and vector-transformed plants at 7 DPI (Figures 2A and C) as well as in *A. brassicicola* inoculated leaves and plants (Figures 2B and D). Necrosis was more severe in the controls and AtACBP3-OEs than OsACBP5-OEs (Figure 2A, left panel), while whole plant assays using *B. cinerea* on four-week-old Arabidopsis plants revealed obvious signs of necrosis and chlorosis in the rosette leaves of the controls and AtACBP3-OEs, while OsACBP5-OE leaves remained relatively unscathed (Figure 2A, right panel). Results from detached leaf and whole plant assays consistently demonstrated that transgenic Arabidopsis OsACBP5-OEs were less susceptible to infection. Disease assessment of both assays at 7 DPI, by measuring disease severity in detached leaf assays and disease incidence in whole plant assays (Figure 2C), confirmed that infection was statistically lower in the transgenic Arabidopsis OsACBP5-OEs. Tests on both leaf and whole plant assays showed that protection was extended to *A. brassicicola* (Figure 2B) because significantly lower infection occurred in the transgenic Arabidopsis OsACBP5-OEs (Figure 2D).

3.2. Arabidopsis OsACBP5-OEs were conferred resistance to the hemibiotroph *C. siamense*

Whole plant assays on WT, vector-transformed control, AtACBP3-OEs and OsACBP5-OEs revealed necrotic lesions in rosette leaves of the controls and AtACBP3-OEs, but OsACBP5-OE leaves were relatively unaffected (Supporting Information Figure S1A), suggesting that OsACBP5-OEs were most resistant. Disease assessment by measuring disease incidence confirmed that infection was statistically lower in the OsACBP5-OEs than the controls (Figure S1B).

3.3. Arabidopsis OsACBP5-OEs were tolerant to biotroph *P. syringae*

When three-week-old Arabidopsis seedlings of WT, vector-transformed control, AtACBP3-OEs and OsACBP5-OEs were inoculated with the biotroph *P. syringae* (Figure S1C), severe chlorosis occurred in the leaves of the controls at 7 DPI, while those of AtACBP3-OEs and OsACBP5-OEs exhibited little yellowing (Figure S1C). When bacterial growth was measured from the inoculated plants, the controls displayed a 2.5-fold higher bacterial count than AtACBP3-OEs and OsACBP5-OEs (Figure S1D). Thus, transgenic Arabidopsis plants overexpressing OsACBP5 and AtACBP3 had become more tolerant to the biotrophic bacterial pathogen *P. syringae*, indicating that Arabidopsis and rice Class III ACBPs are conserved in defence against *P. syringae*.

3.4. Biotic stress-related proteins were induced by *R. solani* infection

SWATH-MS proteomic analysis was carried out to explore the effect of the OsACBP5 action on the necrotroph *R. solani* infection. A reference spectral library was constructed which contains 877 protein groups (1% critical false discovery rate) derived from 4,375 distinct peptides and 33,321 spectra. ProteinPilot 4.5 (Sciex) software identified 498 proteins, 1,614 peptides, and 9,739 spectra with 99% confidence and 1% global false discovery rate (FDR). Out of 498 identified proteins, 142 were significantly upregulated in Arabidopsis OsACBP5-OEs versus the vector-transformed control ($P<0.05$). Eleven biotic stress-related proteins that were upregulated in OsACBP5-OEs, not previously detected in *P. syringae*-infected Arabidopsis AtACBP3-OEs, include cell wall-related proteins such as FASCILIN-LIKE ARABINOGALACTAN-PROTEIN10 (FLA10), LEUCINE-RICH REPEAT EXTENSIN-LIKE PROTEINS (LRX4 and LRX5), XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE PROTEIN4 (XTH4), and PECTINESTERASE INHIBITOR18 (PME18); proteins involved in glucosinolate (GSL) degradation including GDSL-LIKE LIPASE23 (GLL23), EPITHIOSPECIFIER MODIFIER1 (ESM1), MYROSINASE1, MYROSINASE2, NITRILASE1 (NIT1) and a protein involved in jasmonic acid (JA) synthesis, ALLENE OXIDE CYCLASE2 (AOC2) (Table 1) suggesting their potential role in defence against *R. solani*. Supporting information File 2 shows all the 142 upregulated proteins.

When Arabidopsis OsACBP5-OE seedlings were further examined using GC-MS to test SA contents post-*R. solani* infection, the OsACBP5-OEs (Os5OE-4, Os5OE-5 and Os5OE-7) showed significantly higher SA contents (nitriles and isothiocyanate, Supporting Figure S3) than the WT and vector-transformed controls.

When qRT-PCR was performed on OsACBP5-OEs to confirm the results from proteomic analysis, increased expression of biotic-stress related genes (*FLA10, LRX4, LRX5, XTH4, PME18, GLL23, ESM1, MYROSINASE1* & *2, NIT1* and *AOC2*) in OsACBP5-OEs (Figure 3) supported the proteomic data. The expression level of *ACTIN* in *R. solani*-infected WT, VC and OsACBP5-OEs are shown in Supporting Information Figure S2. Taken together, these findings suggest that OsACBP5 plays a crucial role in the protection of plants against biotic stress. Figure 4 displays a model representing the plant defence pathways triggered in OsACBP5-OEs after *R. solani* infection.

4. DISCUSSION

4.1. OsACBP5 overexpression protected dicot Arabidopsis against fungal and bacterial pathogens

A monocot ACBP was shown to function in a transgenic dicot, Arabidopsis, to provide defence against a broad spectrum of phytopathogens. This investigation was achieved by phenotypic analyses of Arabidopsis OsACBP5-OEs in response to necrotrophic, hemibiotrophic and biotrophic pathogens. OsACBP5 was more effective than AtACBP3 because transgenic Arabidopsis OsACBP5-OEs were resistant not only to *P. syringae* but also to necrotrophs *R. solani, B. cinerea* and *A. brassicicola* as well as a hemibiotroph, *C. siamense*. Previous work on transgenic Arabidopsis overexpressing AtACBP3 showed that they conferred resistance only to a bacterial biotroph *P. syringae* accompanied by increased susceptibility to a fungal necrotroph *B. cinerea* [12]. Earlier reports illustrated that transgenic Arabidopsis overexpressing a grapevine (dicot) Class III ACBP, *Vitis vinifera ACBP*, protected against the biotroph *P. syringae* and a hemibiotroph *C. higginsianum* [25]*,* suggesting that both dicot and monocot Class III ACBPs are promising tools for application in disease protection of crop plants.

4.2. Upregulation of cell wall-related proteins in OsACBP5-OE*s* after *R. solani* infection

Results from proteomic analysis showing significantly higher expression of stress-related proteins provided an insight into the defence mechanism in transgenic Arabidopsis OsACBP5-OEs. A model representing the plant defence pathways triggered in OsACBP5-OEs after *R. solani* infection showed significant upregulation of five cell wall-related proteins (FLA10, LRX4, LRX5, XTH4 and PME18) (Figure 4). These were observed in proteomic analysis of *R. solani*-infected transgenic Arabidopsis OsACBP5-OEs in comparison to the vector-transformed control. Furthermore, qRT-PCR of plants sampled post-infection indicated significantly higher expression of these cell wall-related genes in transgenic Arabidopsis OsACBP5-OEs when compared to the vector-transformed control.

Previous reports had shown that the upregulation of cell wall-related proteins after *Fusarium oxysporum* infection in tomato by proteomics [50,51], *Phaseolus vulgaris* infection in bean by RNA sequencing [51,52], *B. cinerea* infection in raspberry by transcriptomic assays [53], as well as *P. syringae, A. brassicicola* and *B. cinerea* infection in Arabidopsis by transcriptomic analysis [54,55], led to cell wall remodelling, inhibition of cell wall degrading enzymes, improvement of cell wall strength and extensibility and increase in cell wall resistance to microbial endopolygalacturonases. Previous investigations on Arabidopsis defence responses against the necrotrophic fungus *Plectosphaerella cucumerina* using recombinant inbred line (RIL) analysis and quantitative trait loci (QTL) mapping revealed that plants can detect widespread degradation of cell wall polysaccharides from the necrotrophs, and thus retaliate by secreting proteins involved in cell wall remodelling such as LEUCINE-RICH REPEAT RECEPTOR-LIKE KINASE (LRR-RLK) and heterotrimeric G-protein [56,57]. These proteins recognise invading elicitors, and mediated plant innate immune responses, and R-gene mediated pathogen-specific responses resulting in plant protection [56]. Arabidopsis LRR-RLKs such as BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1/SOMATIC-EMBRYOGENESIS RECEPTOR-LIKE KINASE3 (BAK1/SERK3) and BAK1-LIKE1/SERK4 (BKK1/SERK4) play an important role in innate immunity to hemibiotrophic and biotrophic pathogens [58].

The FLA10 proteins observed to be upregulated in proteomic assay on *R. solani*-infected transgenic Arabidopsis OsACBP5-OEs were a subclass of arabinogalactan proteins (AGPs) containing putative cell adhesion domains, designated the fascilin domains, in addition to the AGP-like glycosylated regions [59]. FLAs function in plant growth, development, and responses to various biotic and abiotic stresses [60-64]. These results support a potential role for FLA10 in *R. solani*-infected transgenic Arabidopsis OsACBP5-OEs, indicating the significance of FLA in plant resistance against necrotrophic fungal pathogens and in biotic stress response (Figure 4).

Cell wall LRR proteins, LRX4 and LRX5, also upregulated in fungus-infected transgenic Arabidopsis OsACBP5-OEs upon pathogen invasion, which may participate in the plant defence response similar to the endopolygalacturonase-inhibiting proteins (PGIPs). Previous reports using trangenic tomato transformed with a *35S::PvPGIP1* (PGIP isoform from *Phaseolus vulgaris*) construct demonstrated that PGIPs are involved in plant development and pathogen defence [51,65]. The PGIPs were shown to inhibit cell wall degrading enzymes [51,65] and their LRR domain contained 11 repeats, very similar to the ten and a half repeats of LRX proteins [66].

XTH4 was also upregulated herein in proteomic analysis of pathogen-infected transgenic Arabidopsis OsACBP5-OEs. The XTH family of enzymes catalyzes modification of the cell wall network [67]. Enzymologically, XTH proteins provide xyloglucan endotransglucosylase (XET) and xyloglucan endohydrolase (XEH) activities [68] through cell wall extension either by XET action, which cuts and rejoins xyloglucan chains [69], or XEH activity which catalyzes the hydrolysis of xyloglucan [70]. XTH via XET activity contributes to cell wall remodeling, architecture and its reconstruction, as well as to cell wall strength and extensibility, and the cell wall provides an important barrier against pathogen invasion [71-73]. Past transcriptomic studies in tomato revealed that a reduction in XTH expression during pathogen infection led to cell wall disassembly and fruit softening which enabled fungal colonization and promoted infection [74]. Similarly, an upregulation of pectinesterase inhibitors such as PME18 upon fungal infection in transgenic Arabidopsis OsACBP5-OEs could enhance plant defence because specific pectin methylesterase inhibitors in Arabidopsis affect pectinesterase activity and are involved not only in plant development but also in defence by influencing the susceptibility of the wall to microbial endopolygalacturonases [55,75].

4.3. Proteins which degrade GSLs were upregulated in OsACBP5-OEs after *R. solani* infection

SWATH-MS proteomic analysis showed that the upregulation of proteins that degrade GSLs (such as GLL23, ESM1, MYROSINASE1, MYROSINASE2 and NITRILASE1) [76-82] in *R. solani*-infected transgenic Arabidopsis OsACBP5-OEs were significantly higher than the vector-transformed control. To infect a plant, the invading fungus has to overcome different defence responses imposed by the plant. GSLs are sulphur- and nitrogen-containing secondary metabolites largely found in cruciferous plants, including Arabidopsis, and they are known to play a role in defence against pathogens [76]. Myrosinase causes the degradation of GSLs to isothiocyanates and indoles, and the enzyme only comes into contact with its GSL substrates if the plant tissues are injured through wounding, insect or pathogen attack [77].

After infection of Arabidopsis by *A. brassicicola*, *B. cinerea*, *Erwinia carotovora*, *F. oxysporum, Peronospora parasitica*, *Plectosphaerella cucumerina* and *P. syringae*, myrosinase-associated proteins can catalyse GSL degradation culminating in the production of isothiocyanates, thiocyanates and nitriles [78,79] that possess broad biocidal and fungicidal properties [79-81]. The GSL degradation products such as isothiocyanates, nitriles, and thiocyanates facilitate peroxidase-mediated reactive oxygen species (ROS) production in Arabidopsis [82]. As the activation of SA signaling in stressed plants is preceded by oxidative bursts originating in different cellular compartments [83], GSL degradation by myrosinase upon pathogen infection in the transgenic Arabidopsis OsACBP5-OEs is expected to trigger an SA-mediated defence response. When Arabidopsis OsACBP5-OE seedlings were further examined using GC-MS to measure SA content post-*R. solani* infection, the

OsACBP5-OEs (Os5OE-4, Os5OE-5 and Os5OE-7) showed significantly higher SA content than the wild-type and vector-transformed controls (Supporting Figure S3) in agreement with proteomic data (Figure 4).

The upregulation of ESM1 herein in proteomic analysis upon pathogen infection in the transgenic Arabidopsis OsACBP5-OEs further suggests the facilitation of an SA-mediated fungal defence response. Earlier studies using RIL QTL mapping had shown that myrosinase *per se* was not sufficient for indol-3-acetonitrile production from indol-3-ylmethyl glucosinolate and the presence of a functional epithiospecifier protein such as ESM1, which is crucial in the production of significant levels of indol-3-acetonitrile, is essential [84].

4.4. JA-mediated defence in OsACBP5-OEs following *R. solani* infection.

Proteomic analysis revealed that upregulation of AOC2 (a protein involved in JA biosynthesis) in *R. solani*-infected transgenic Arabidopsis OsACBP5-OEs is significantly higher than the vector-transformed control. JA is synthesized from α-linolenic acid which is converted by the enzyme C13-lipooxygenase to produce 13-hydroperoxylinolenic acid (13-HPOT) [85]. AOC2 then catalyses the conversion of 13-HPOT to 12-oxo-phytodienoic acid (OPDA), which is a precursor of JA [85]. Hence, an upregulation of AOC2 is expected to result in JA accumulation which would lead to JA-induced defence responses.

Based on the results from proteomic analysis and qRT-PCR, the underlying mechanism of defence in *R. solani*-infected transgenic Arabidopsis OsACBP5-OEs appear to be related to cell wall-mediated defence and SA- and JA-induced defence pathways (Figure 4).

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Figure Legends

Figure 1. Transgenic Arabidopsis OsACBP5-OEs show enhanced resistance to root-infecting fungal necrotroph *R. solani*. **A**, Symptoms of *R. solani* infection on one-week-old Arabidopsis WT, vector (pBI-eGFP)-transformed control, AtACBP3-OEs (At3OE-1, At3OE-4, At3OE-6) and OsACBP5-OEs (Os5OE-4, Os5OE-5, Os5OE-7) in MS medium. The plants were photographed 7 days post-inoculation (DPI). Scale = 1 cm. **B**, Symptoms on one-week-old Arabidopsis WT, vector-transformed control, AtACBP3-OEs (At3OE-1, At3OE-4, At3OE-6) and OsACBP5-OEs (Os5OE-4, Os5OE-5, Os5OE-7) inoculated with *R. solani* agar plugs in the soil. The plants were photographed 14 DPI. Scale = 1 cm. **C**, Disease incidence following *R. solani* infection on the WT, vector-transformed control, AtACBP3-OEs, and OsACBP5-OEs. Data points represent means ± SD from three independent experiments. Asterisks indicate significant difference ($P<0.05$) in comparison to the controls by Student's *t*-test.

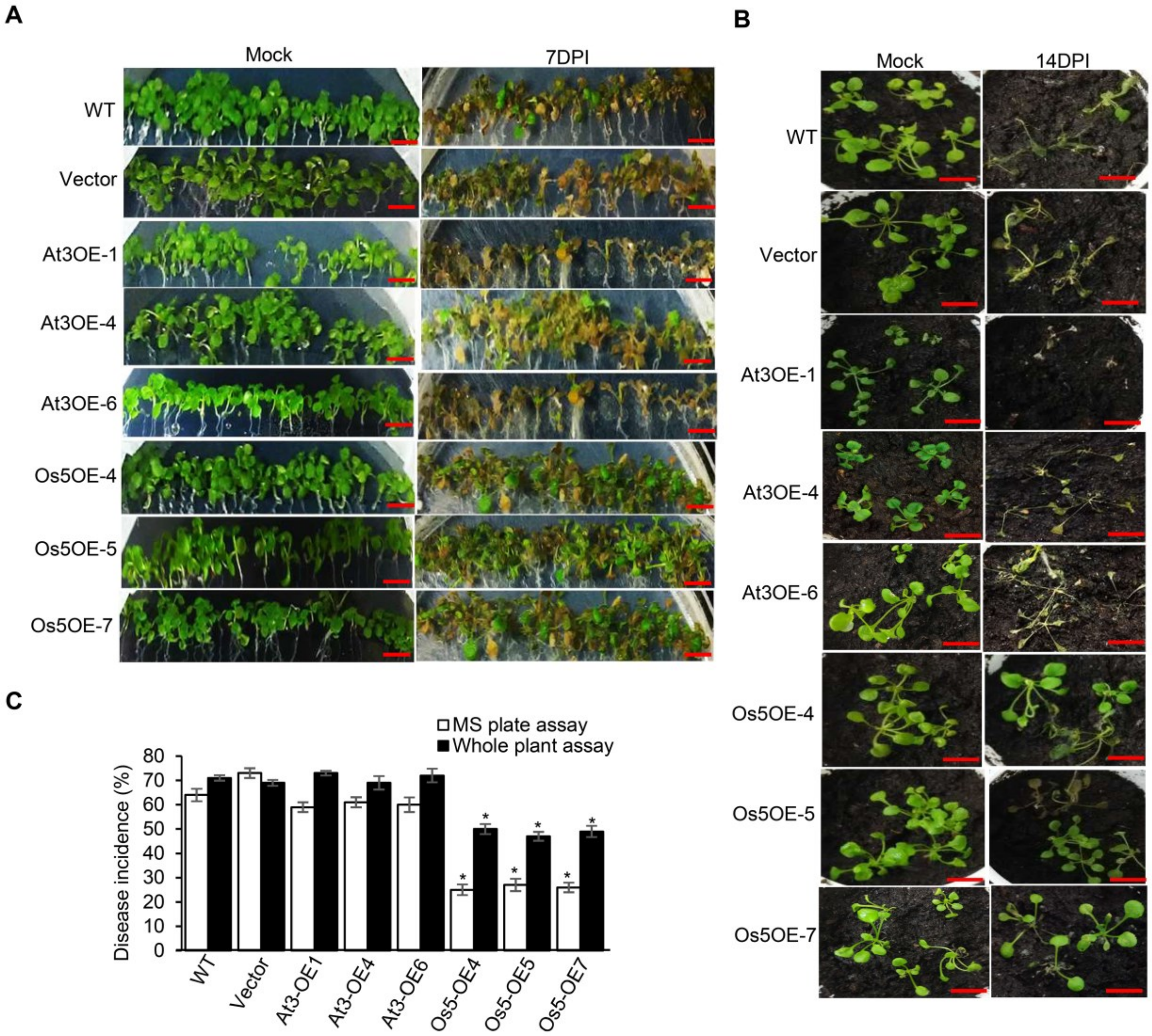
A
Mock
7DPI
WT
Vector
At3OE-1
At3OE-4
At3OE-6
Os5OE-4
Os5OE-5
Os5OE-7
B
Mock
14DPI
WT
Vector
At3OE-1
At3OE-4
At3OE-6
Os5OE-4
Os5OE-5
Os5OE-7
C
□ MS plate assay
■ Whole plant assay
Disease incidence (%)
0
10
20
30
40
50
60
70
80
*
WT
Vector
At3-OE1
At3-OE4
At3-OE6
Os5-OE4
Os5-OE5
Os5-OE7

Figure 1.

Figure 2. Transgenic Arabidopsis OsACBP5-OEs show improved tolerance to shoot-infecting fungal necrotrophs *B. cinerea* and *A. brassicicola*. **A**, Detached leaf assays (left), representative leaves of four-week-old Arabidopsis WT, vector (pBI-eGFP)-transformed control, AtACBP3-OEs (At3OE-1, At3OE-4, At3OE-6) and OsACBP5-OEs (Os5OE-4, Os5OE-5, Os5OE-7) showing disease manifestation after inoculation with *B. cinerea*. Whole plant assays (right), phenotypes of four-week-old Arabidopsis WT, vector-transformed control, AtACBP3-OEs, and OsACBP5-OEs sprayed with *B. cinerea.* The plants were photographed 7 days post-inoculation (DPI). Scale = 1 cm. **B**, Detached leaf assays (left), representative leaves of six-week-old Arabidopsis WT*,* vector-transformed control, AtACBP3-OEs and OsACBP5-OEs showing disease appearance following infection with the fungus *A. brassicicola*. Whole plant assays (right), phenotypes of four-week-old Arabidopsis WT*,* vector-transformed control, AtACBP3-OEs and OsACBP5-OEs sprayed with the pathogen *A. brassicicola.* The plants were photographed at 7 DPI. Scale = 1 cm. Percentage of disease by **(C)** *B. cinerea* and **(D)** *A. brassicicola,* on the WT*,* vector-transformed control, AtACBP3-OEs, and OsACBP5-OEs. Data points represent means ± SD from three independent experiments. Asterisks indicate significant difference (*P*<0.05) in comparison to the controls by Student's *t*-test.

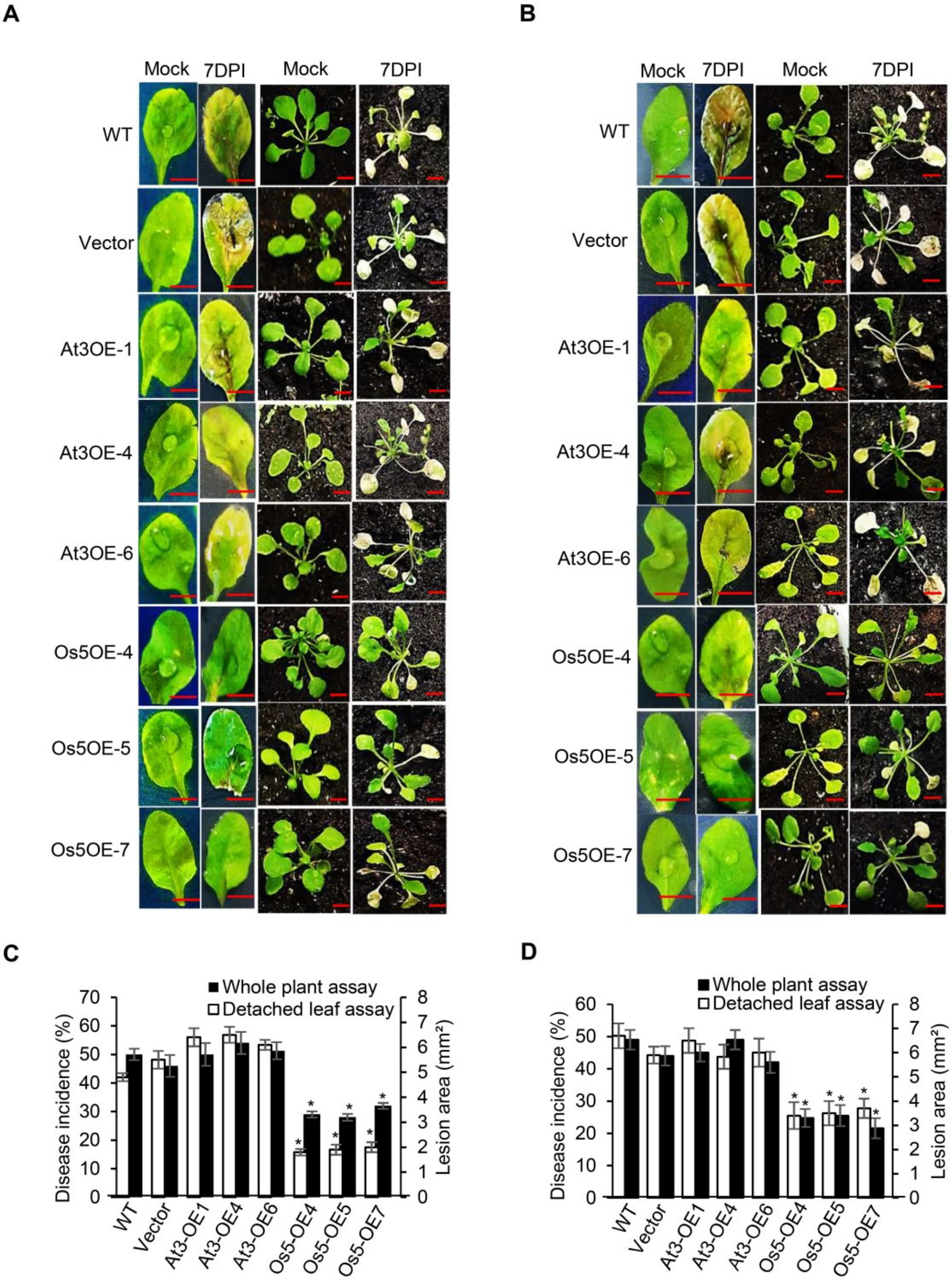
A
B
Mock
7DPI
Mock
7DPI
WT
Vector
At3OE-1
At3OE-4
At3OE-6
Os5OE-4
Os5OE-5
Os5OE-7
C
D
Whole plant assay
Detached leaf assay
Disease incidence (%)
Lesion area (mm²)
0
10
20
30
40
50
60
70
0
1
2
3
4
5
6
7
8
*
WT
Vector
At3-OE1
At3-OE4
At3-OE6
Os5-OE4
Os5-OE5
Os5-OE7

Figure 2.

Figure 3. qRT-PCR analyses verify the relative expression of upregulated genes in OsACBP5-OEs in comparison to the wild type and vector-transformed control. The genes analysed were At3g60900 (*FLA10*), At3g24480 (*LRX4*), At4g18670 (*LRX5*), At2g06850 (*XTH4*), At1g11580 (*PME18*), At1g54010 (*GLL23*), At3g14210 (*ESM1*), At5g25980 (*MYROSINASE1*), At5g26000 (*MYROSINASE2*), At3g44310 (*NIT1*) and At3g25770 (*AOC2*). The bar graph shows gene expression changes in *R. solani* infected Arabidopsis OsACBP5-OE (shown in striped bar) compared to wild type (WT) (shown in black bar) and vector-transformed control (VC) (shown in white bar). The expression levels were normalized to that of *ACTIN*. Error bars represent SE (n = 3). Asterisks indicate significant difference (P<0.05) in comparison to the controls by Student's *t*-test.

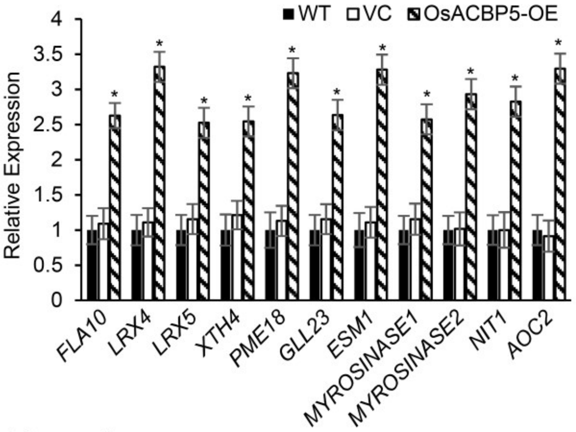

Figure 3.

Figure 4. Model illustrating defence response pathways in OsACBP5-OE plants upon pathogen infection. This model is based on results from Table 1 and Figure 3. Upregulation of proteins (denoted by red arrows) associated with cell wall such as LRR-RLK, heteromeric G-protein, PGIP, XTH and PME (Table 1, Figure 3) leads to the first line of defence upon pathogen attack involves cell wall remodelling, inhibiting cell wall degrading enzymes, improving cell wall strength and extensibility, and increasing cell wall resistance to microbial endopolygalacturonases [51,65,66,74,75]. Upregulation of GSL-degrading enzymes such as myrosinases and nitrilase (Table 1, Figure 3) results in the production of isothiocyanate and nitriles which activates ROS production culminating in SA accumulation and SA-induced defence [77-81]. Upregulation of AOC in JA biosynthesis (Table 1, Figure 3) leads to the production of OPDA from 13-HPOT. Accumulation of OPDA, the precursor of JA, leads to JA-induced defence response [85]. The red arrow indicates upregulation. AOC, allene oxide cyclase; GSL, glucosinolate; 13-HPOT, 13-hydroperoxylinolenic acid; JA, jasmonic acid; LRR-RLK, leucine-rich repeat receptor-like kinase; OPDA, 12-oxo-phytodienoic acid; PGIP, endopolygalacturonase-inhibiting proteins; PME, pectinesterase inhibitor; ROS, reactive oxygen species; SA, salicylic acid; XTH, xyloglucan endotransglucosylase/ hydrolase protein.

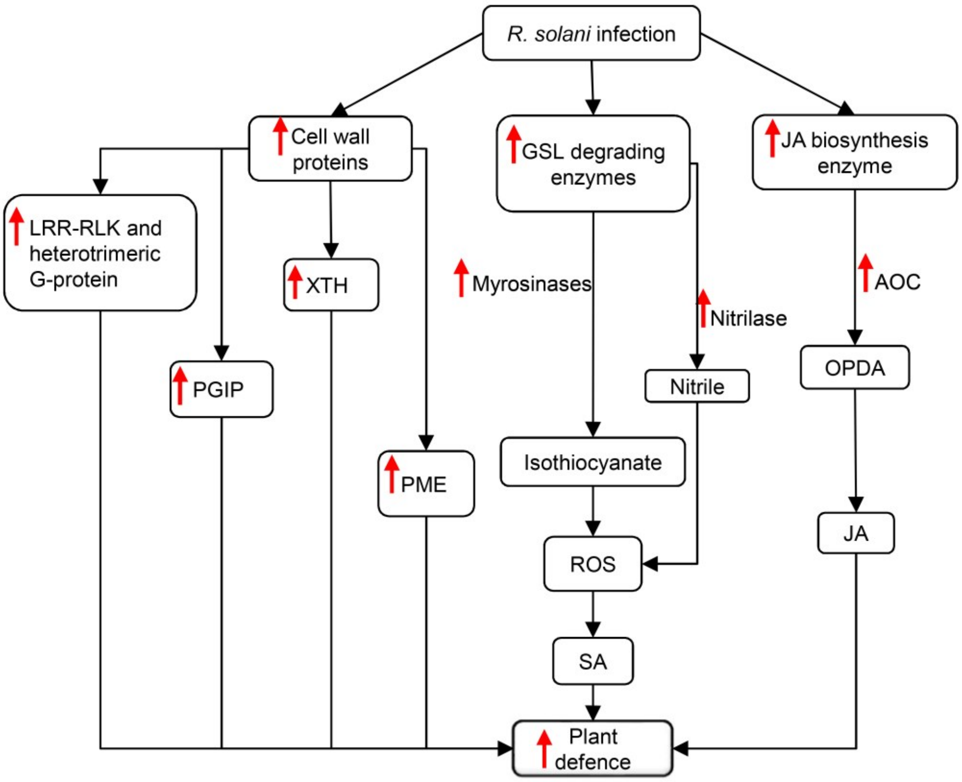
R. solani infection
Cell wall proteins
GSL degrading enzymes
JA biosynthesis enzyme
LRR-RLK and heterotrimeric G-protein
XTH
Myrosinases
Nitrilase
AOC
PGIP
Nitrile
OPDA
PME
Isothiocyanate
JA
ROS
SA
Plant defence

Figure 4.

Table 1. Biotic stress-related proteins with increased abundance in response to *R. solani* infection in transgenic Arabidopsis OsACBP5-OEs. Proteins with fold change >2.0 is considered upregulated.

	Accession	**Protein name**	**Fold-change**	***p*-value**
Cell wall proteins	At3g60900	FASCICLIN-LIKE ARABINOGALACTAN-PROTEIN10 (FLA10)	2.40	0.022
	At3g24480	LEUCINE-RICH REPEAT EXTENSIN-LIKE PROTEIN4 (LRX4)	2.97	0.005
	At4g18670	LEUCINE-RICH REPEAT EXTENSIN-LIKE PROTEIN5 (LRX5)	2.50	0.017
	At2g06850	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE PROTEIN4 (XTH4)	2.83	0.007
	At1g11580	PECTINESTERASE INHIBITOR18 (PME18)	2.08	0.046
Proteins involved in GSL degradation	At1g54010	GDSL-LIKE LIPASE23 (GLL23)	2.72	0.009
	At3g14210	EPITHIOSPECIFIER MODIFIER1 (ESM1)	3.35	0.001
	At5g26000	MYROSINASE1	3.39	0.001
	At5g25980	MYROSINASE2	2.59	0.013
	At3g44310	NITRILASE1 (NIT1)	3.10	0.003
JA synthesis protein	At3g25770	ALLENE OXIDE CYCLASE2 (AOC2)	3.18	0.002

Fold-change was calculated from five biological replicates.