The role of pyriculol in the infection process of rice blast fungus-*Magnaporthe oryzae*

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Karlsruhe, im Oktober 2018

Junning Ma

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Abbreviations

PAMPs	pathogen associated molecular patterns
MAMPs	microbe associated molecular patterns
DAMPs	damage associated molecular patterns
PTI	PAMPs triggered immunity
ETI	effector triggered immunity
ETS	effector triggered susceptibility
HR	hypersensitive response
Avr-R	avirulence effectors - resistance protein
IH	invasive hyphae
PKS	polyketide synthase
BIC	biotrophic interfacial complex
CEBiP	chitin elicitor binding protein
NPRS	non-ribosomal peptide synthetase
DMATS	dimethylallyl diphosphate trytophan synthase
HST	host-specific toxin
ROS	reactive oxygen species
PCD	programmed cell death
JA	jasmonic acid
ET	ethylene
SA	salicylic acid
PAL	phenylalanin ammonium lyase
SAG	salicylic acid c-glucoside
NPR1	nonexpressor of pathogenesis related genes 1
PBZ	probenazole
BTH	benzothiadiazole

TDL	tiadinil
LOX	lipoxygenase
AOS	allene oxide synthase
AOC	allene oxide cyclase
OPDA	oxo-phytodienoic acid
OPR	oxo-phytodienoic reductase
JA-Ile	jasmonic acid isoleucine
JAR1	jasmonate resistant 1
PR	pathogenesis related
COI1	coronatine insensitive 1

Summary

Rice blast fungus, *Magnaporthe oryzae* (hemibiotrophic fungus) is the causal agent of the devastating rice blast disease on rice, causing annual yield loss sufficient to feed 60 million people. It starts infection by attaching conidia spores to leaf surface and then form a specialised structure called appressorium to penetrate leaf cuticle. After penetration, invasive hyphae develop to secrete and deliver effectors (proteinaceous and non-proteinaceous molecules) in the biotrophic stage to reprogram the host's defence signaling to facilitate rice blast fungus proliferation in the host plant, while fungal secondary metabolites (such as polyketides) are produced in the necrotrophic stage to kill the host and to feed on the dead tissue. In response to such attack, the host plants recruit defence phytohormones (such as jasmonic acid and salicylic acid etc.) which play central role in plants' immune responses. However, there exists evolutionarily conserved crosstalk between the two defence phytohormones to fine tune the output of plant defence responses. This crosstalk can be easily manipulated by plant pathogens in order to achieve a successful colonisation in the host plant.

In this study, an isolated polyketide phytotoxin, pyriculol, produced by rice blast fungus was used to evaluate its function in the infection process. In the results, firstly, it was shown that pyriculol could induce necrotic lesions in a light-dependent manner and the necrosis area induced by pyriculol could be reduced by salicylic acid and diphenylene-iodonium chloride treatment.

Secondly, both pyriculol and salicylic acid could inhibit the expression of wounding-induced jasmonic acid biosynthesis and signaling genes in the early wounding stage (0.5 h and 1 h after wounding treatment), but could up-regulate the expression of salicylic acid-responsive genes. In the late wounding response (24 h after

wounding treatment), both pyriculol and salicylic acid could either induce plant defence gene expression or enhance wounding-induced defence gene accumulation. Combining with the structural similarity between salicylic acid and pyriculol, this lead to the conclusion that pyriculol might function as mimicry of salicylic acid to down-regulate jasmonic acid-dependent gene expression. Since jasmonic acid was reported to be an important defence hormone against rice blast fungus, it was assumed that pyriculol might be manipulated by rice blast fungus to increase the fitness to live in the host.

However, exogenous application of pyriculol and its structural isomer pyriculariol in combination with the spores of rice blast fungus strain Gy11 (virulent strain) on rice resulted in enhanced resistance as compared to the rice plants with only Gy11 inoculation. In addition to this, pyriculol was shown to inhibit Gy11 spore germination when the applied concentration was above 160 μM. Taken together, this was inconsistent with the assumption that pyriculol was utilised to improve pathogens' fitness. Yet, there was still lack of evidence that pyriculol was produced in the infected plants by rice blast fungus.

Inoculation using pyriculol-related transgenic strains (pyriculol-deletion strains, pyriculol-overproducing strains and wild type strains, based on the virulent strain 70-15) on 8 rice varieties revealed that there was no significant difference in terms of the virulence among these transgenic strains, except two negative transcription factor knock-out strains (pyriculol might not be the only substance affected by the transcription factor deletion). This experiment suggested that pyriculol was not involved in the infection process of rice blast fungus.

To sum up, pyriculol-induced necrosis was light-dependent and this necrosis could be

inhibited by application of salicylic acid and diphenylene-iodonium chloride. Secondly, both salicylic acid and pyriculol could inhibit the expression of jasmonic acid biosynthesis and signaling genes, but could up-regulate salicylic acid responsive gene expression. Thirdly, SA and jasmonic acid was antagonistic in terms of SA's repression JA biosynthesis and signaling gene expression at the early wounding stage, but synergistic in induction of some defense gene expression at the late wounding stage. Lastly, pyriculol inhibited spore germination and exogenous application of pyriculol enhanced rice plant defence against rice blast fungus infection, but pyriculol was not involved in the infection process of rice blast fungus.

Zusammenfassung

Der Reisbrandpilz Magnaporthe oryzae (hemibiotrophischer Pilz) ist der Erreger der verheerenden Reisbrandkrankheit, die einen jährlichen Ertragsverlust verursacht, der ausreichen würde, um 60 Millionen Menschen zu ernähren. Die Infektion beginnt mit der Anheftung von Konidiensporen an die Blattoberfläche, woraufhin eine spezialisierte Struktur gebildet wird, die Appressorium genannt wird, um in die Blattkutikula einzudringen. Nach der Penetration entwickeln sich invasive Hyphen, um in der biotrophen Phase Effektoren (proteinartige und nicht-proteinische Moleküle) zu sezernieren und abzugeben, welche die Abwehrsignale des Wirts umprogrammieren, um die Proliferation des Reisbrandpilzes in der Wirtspflanze zu erleichtern, während sekundäre Pilzmetaboliten (wie Polyketide) im nekrotrophen Stadium produziert werden, um den Wirt zu töten und sich vom toten Gewebe zu ernähren. Als Reaktion auf einen solchen Angriff rekrutieren die Wirtspflanzen Abwehrphytohormone (wie Jasmonsäure und Salicylsäure usw.), die eine zentrale Rolle bei der Immunantwort von Pflanzen spielen. Es gibt jedoch eine evolutionär konservierte Wechselwirkung zwischen den beiden Abwehrphytohormonen, um die Abwehrreaktionen der Pflanzen exakt abzustimmen. Diese Wechselwirkung kann durch Pflanzenpathogene leicht manipuliert werden, um eine erfolgreiche Kolonisierung in der Wirtspflanze zu erreichen.

In dieser Studie wurde ein isoliertes Polyketid-Phytotoxin, Pyriculol, das durch den Reisbrandpilz produziert wird, verwendet, um seine Funktion im Infektionsprozess zu bewerten. In den Ergebnissen wurde erstens gezeigt, dass Pyriculol lichtabhängige nekrotische Läsionen induzieren kann und die durch Pyriculol induzierte Nekrosefläche durch Behandlung mit Salicylsäure und Diphenylen-Iodoniumchlorid reduziert werden konnte.

Zweitens konnten sowohl Pyriculol als auch Salicylsäure die Expression von verletzungsinduzierten Jasmonsäure-Biosynthese- und Signalgenen im frühen Verwundungsstadium hemmen (0,5 h und 1 h nach der Verwundung), konnten aber die Expression von auf Salicylsäure reagierenden Genen hochregulieren. Bei der späten Verwundungsreaktion (24 h nach der Verwundung) konnten sowohl Pyriculol als auch Salicylsäure entweder die Expression des Pflanzenabwehrgens induzieren oder die durch Verletzung verursachte Akkumulation von Abwehrgenen verstärken. Im Zusammenhang mit der strukturellen Ähnlichkeit zwischen Salicylsäure und Pyriculol führte dies zu der Schlussfolgerung, dass Pyriculol als Mimikry von Salicylsäure fungieren könnte, um die Jasmonsäure-abhängige Genexpression herabzuregulieren. Da von Jasmonsäure berichtet wurde, dass sie ein wichtiges Abwehrhormon gegen den Reisbrandpilz ist, wurde angenommen, dass Pyriculol durch den Reisbrandpilz manipuliert werden könnte, um die Lebensfähigkeit im Wirt zu erhöhen.

Jedoch führte die exogene Anwendung von Pyriculol und seines Strukturisomers Pyriculariol in Kombination mit den Sporen des Reisbrandpilzstammes Gy11 (virulenter Stamm) auf Reis zu einer erhöhten Resistenz im Vergleich zu den Reispflanzen mit ausschließlicher Gy11-Inokulation. Zusätzlich wurde gezeigt, dass Pyriculol die Gy11-Sporenkeimung hemmt, wenn die angewendete Konzentration über 160 µM lag. Zusammengefasst widersprach dies der Annahme, dass Pyriculol zur Verbesserung der Fitness des Pathogens eingesetzt wurde. Es gab jedoch immer noch keinen Beweis dafür, dass Pyriculol in den infizierten Pflanzen durch den Reisbrandpilz produziert wurde.

Die Inokulation mit Pyriculol-verwandten transgenen Stämmen (Pyriculoldeletionsstämme, Pyriculol-überproduzierende Stämme und

Wildtypstämme, basierend auf dem virulenten Stamm 70-15) von 8 Reissorten ergab, dass es keinen signifikanten Unterschied in Bezug auf die Virulenz unter diesen transgenen Stämmen gab, mit Ausnahme von zwei negativen Transkriptionsfaktor-Knockout-Stämmen (Pyriculol ist möglicherweise nicht die einzige Substanz, die von der Deletion des Transkriptionsfaktors betroffen ist). Dieses Experiment legte nahe, dass Pyriculol nicht an dem Infektionsprozess des Reisbrandpilzes beteiligt war.

Zusammengefasst war Pyriculol-induzierte Nekrose lichtabhängig und diese Nekrose konnte durch die Anwendung von Salicylsäure und Diphenylen-Iodoniumchlorid inhibiert werden. Zweitens könnten sowohl Salicylsäure als auch Pyriculol die Expression von Jasmonsäure-Biosynthese- und Signalgenen hemmen, könnten aber die auf Salicylsäure ansprechende Genexpression hochregulieren. Drittens waren SA und Jasmonsäure in Bezug auf die SA-Repressions-JA-Biosynthese und die Signalgenexpression im frühen Verwundungsstadium antagonistisch, aber synergistisch in der Induktion einer Verteidigungsgenexpression im späten Verwundungsstadium. Schließlich hemmte Pyriculol die Sporenkeimung und die exogene Anwendung von Pyriculol verbesserte die Abwehr von Reispflanzen gegen die Infektion mit Reis-Blastenpilz, aber Pyriculol war nicht an dem Infektionsprozess des Reis-Blastpilzes beteiligt.

1 Introduction

The rice Blast Fungus, *Magnaporthe oryzae*, is a hemibiotrophic and ascomycetous fungus. It can infect a variety of monocotyledonous plants, including rice, wheat, rye, or barley, either via spores that can enter the aerial organs, or via hyphae that can penetrate into the roots. This pathogen has turned into a major threat of global food security, since it can cause 10-30% yield loss of the total rice harvest worldwide, which is equivalent to feeding 60 million people (Talbot, 2003). Therefore, a deeper understanding of the rice blast disease is crucial to develop durable strategies for plant protection.

1.1 The rice Blast Fungus-*Magnaporthe oryzae*

1.1.1 The life cycle of *M. oryzae*

The infection process starts with the attachment of three-celled conidiospores to the leaf surface through mucilage, which is secreted from the tip of the spores (Hamer *et al.*, 1988). Subsequently, under favourable conditions, the spores will germinate and differentiate into a dome-shaped structure, called Appressorium (Fig. 1). This specialized structure can penetrate the hard leaf cuticle by accumulation of polyols, especially glycerol (de Jong *et al.*, 1997). To build up the necessary pressure, the appressorium tightens the cell wall by chitin and melanin, such that this wall becomes impermeable to solute efflux, thereby generating a strong pressure as high as 80 bar (de Jong *et al.*, 1997; Chumley and Valent, 1990). This huge turgor pressure will translate into mechanical force in the penetration peg which then allows to break through the cuticle (Howard and Valent, 1996). After penetration, the penetration peg swells further to form the primary infection hyphae, which then differentiate into bulbous invasive hyphae within the host cell (Heath *et al.*, 1992; Heath *et al.*, 1990; Koga, 1994). These invasive hyphae then colonise cell after cell by spreading through the plasmodesmata

(Kankanala *et al.*, 2007), absorbing water and nutrients and finally causing symptoms in 5-7 days. Under favourable conditions, the conidiophores will erupt and spread new spores to neighbouring plants, where they initiate a new infection cycle (Talbot, 2003). To what extent this infection cycle can be completed depends on signals exchanged between pathogen and host. This signaling is the product of a coevolutionary process and is therefore composed of several layers, as briefly summarised in the following paragraph.

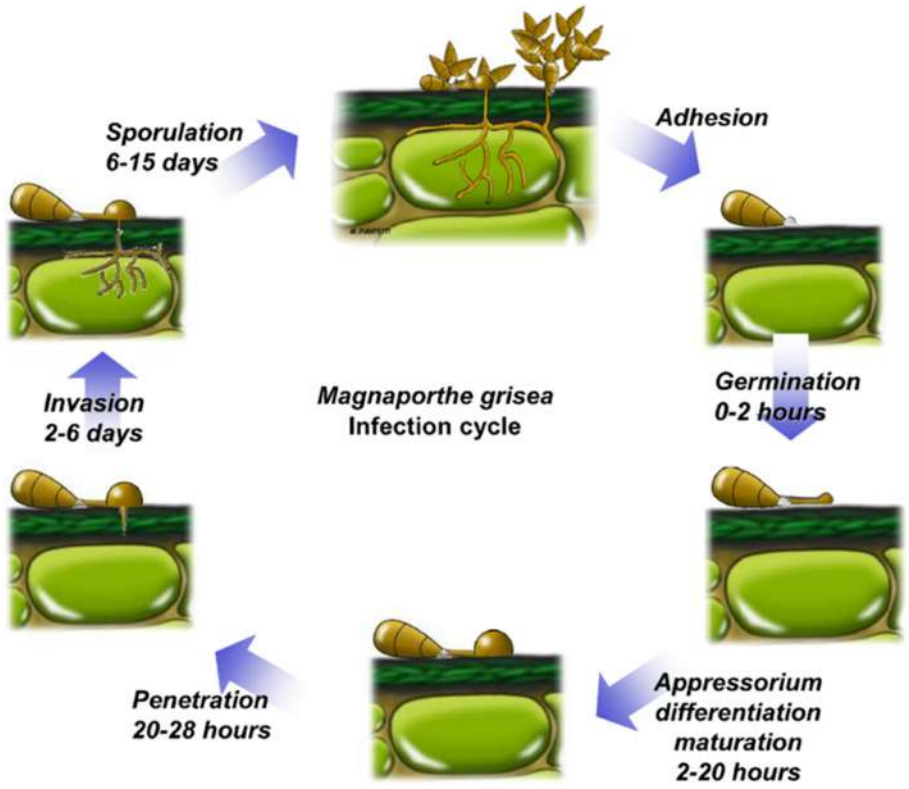


Fig. 1 Infection cycle of the Rice Blast Fungus, *M. oryzae* on rice (Ribot *et al.*, 2008). The conidiospore attaches to the leaf surface, germinates and differentiates into an appressorium within 20 hours. This appressorium then penetrates through the cuticle and colonises the first host cell. Then the fungus develops infectious hyphae to obtain nutrients. After approximately 4-5 days of biotrophic colonisation, the fungus starts to kill the host and to sporulate, such that the pathogen spread to neighbouring plants.

1.1.2 The layers of the plant immune system

Plants are sessile organisms, thus, they cannot walk away when attacked by pathogens like animals do. Instead, they have evolved a variety of strategies to protect themselves from invasion by pathogens. Firstly, they have developed passive defence (also termed preformed defence). These are constitutive physical or chemical barreers, such as cuticles (Serrano *et al.*, 2014), constitutively produced phytoalexins (sometimes called phytoanticipins) (Osbourn, 1996; Piasecka *et al.*, 2015), or constitutively expressed defence genes (Vergne *et al.*, 2010). Generally speaking, these preformed defences are not specific for a certain pathogen, but rather confer a broad and durable resistance to a variety of pathogens (Vergne *et al.*, 2010). In addition to this passive defence, plants also are endowed with active defence (also termed induced defence). The first layer of induced defence is so called pathogen-associated molecular pattern triggered immunity (PTI), utilised to recognise conserved molecular features of microbial pathogens, the pathogen or microbe-associated molecular patterns (PAMPs or MAMPs). For instance, detection of flagellin allows to sense most bacterial invaders, while detection of chitin allows to recognise a fungal attack (Bigeard *et al.*, 2015). Likewise, mechanical wounding or cellular damage can trigger defence responses through damage-associated molecular patterns (DAMPs), such as oligogalacturonides, a breakdown product of fragmented plant cell walls (Ferrari *et al.*, 2013). The basal layer of plant immunity (PTI) is often complemented by a second layer of induced defence called effector-triggered immunity (ETI), resulting from co-evolution between host and pathogen summarised in a zig-zag model of plant immunity (Fig. 2 (Jones and Dangl, 2006)):

The recognition of PAMPs or MAMPs by host receptors (usually localised in the plasma membrane) is expected to impose a selective pressure upon the pathogen to get rid of the triggering PAMP. However, these molecules are essential – a bacterial cell without flagellin will not be able to swim, a fungus without chitin will not be able

to grow. Therefore, as consequence of interaction with the host, pathogenic microbes have evolved signals (termed effectors) able to interfere with the PTI of the host, such that host defence is silenced (so called effector-triggered susceptibility, ETS). In a next round of evolutionary interaction with specific pathogens, some host plants have evolved receptors that are able to bind the effector and initiate a second round of so called effector-triggered immunity (ETI). Since effectors are usually injected into the cytoplasm, these receptors are usually cytoplasmic, since effectors act at specific targets of PTI, the host receptors are specific as well. This specificity leads to a situation, where a gene at the side of the pathogen (the classical avirulence gene of phytopathology) corresponds to a specific gene on side of the host (the classical resistance or R-gene) in a gene-for-gene interaction. The successful recognition of the effector by the product of the R-gene typically initiates a hypersensitive response culminating in programmed cell death (Cui *et al.*, 2015). However, this dichotomy between PTI and ETI is meanwhile seen as "blurred" rather than absolutely strict (Thomma *et al.*, 2011).

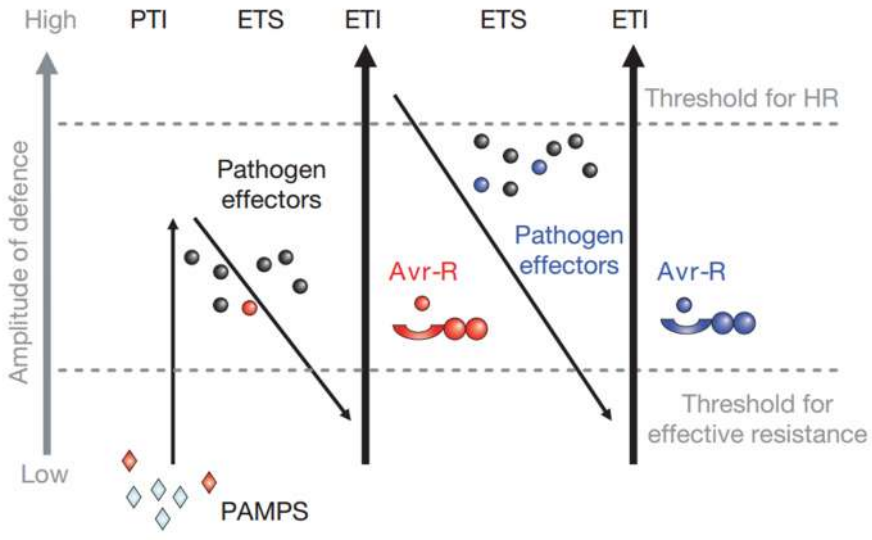


Fig. 2 Typical zig-zag model showing the PTI and ETI output in plant defence system (Jones and Dangl, 2006). PAMPS: pathogen associated molecular patterns; PTI: PAMPs triggered immunity; ETS: effector triggered susceptibility; ETI: effector triggered immunity; Avr-R: avirulence effector-resistance protein; HR: hypersensitive response.

1.1.3 Effectors of the Rice Blast Fungus

M. oryzae is a hemibiotrophic fungus with a initial biotrophic phase relying on living host tissue for water and carbohydrates enabling a successful colonisation, followed by a switch, where the host is killed actively as transition to the subsequent necrotrophic phase (Ebbole, 2007). During the establishment of biotrophic interaction, the invasive hyphae from *M. oryzae* absorb nutrients from the first infected cells, which requires that the immunity of the host cell is reprogrammed by production of effector proteins.

1.1.3.1 Avirulence Effectors of the Rice Blast Fungus

Avirulence (AVR) effectors are those proteins secreted by the pathogen that are recognized by the corresponding resistance (R) proteins of the host to induce a strong and race or variety specific resistance response, typically comprising programmed cell death (De Wit *et al.*, 2009). In *M. oryzae*, more than 40 AVR effectors have been identified that are functional in the interaction with rice (Zhang and Xu, 2014). Among these AVR effectors, ACE1 is distinct, because it is not secreted by the invasive hyphae (IH), but specifically produced in the appressorium able to induce resistance in hosts harbouring a respective R-gene (Böhnert *et al.*, 2004). The *ACE1* locus encodes a enzyme of the polyketide synthase (PKS) family. As corresponding R gene of rice the *Pi33* has been identified (Böhnert *et al.*, 2004) (Fudal *et al.*, 2007). All the other AVR effectors identified to date are secreted by the invasive hyphae. Among those AVR effector families, one group consisting of the loci *PWL1*, *PWL2*, *PWL3* and *PWL4* have been identified from the interaction between *M. oryzae* and the hosts finger millet and weeping lovegrass (Kang *et al.*, 1995). Another family comprises the zinc metalloproteases AVR-Pita-1 and AVR-Pita2 recognised by the rice R protein Pita (Khang *et al.*, 2008). The *M. oryzae* effector Avr-Piz-t can suppress PTI by inhibiting the RING E3 ubiquitin ligase APIP6 of the host leading to susceptibility (Park *et al.*, 2012). Three new AVR effectors *AVR-Pia, AVR-Pii* and *AVR-Pik/km/kp*, were predicted

from the genome of the *M. oryzae* strain Ina168 (Yoshida *et al.*, 2009), and the effector *AVR1-Co39* was demonstrated to induce a hypersensitive response (HR) in rice genotypes carrying the R gene Pi-CO39(t) (Peyyala and Farman, 2006).

1.1.3.2 Virulence effectors of the Rice Blast Fungus

In addition to the avirulence effectors, where corresponding R-genes have been identified on side of the host, there exist effectors, where no corresponding R-gene has been found, so far. These so called virulence effectors are therefore not well characterised. One of the best known examples is the effectors Slp1 that is secreted by *M. oryzae* to biotrophic interfacial complex (BIC) to compete with the chitin-elicitor binding protein (CEBiP) for the binding of chitin. Such competitive binding for chitin can inhibit chitin-induced PTI, including the generation of reactive oxygen species and the expression of PR genes (Mentlak *et al.*, 2012). Four putative biotrophy-associated secreted (BAS) proteins, BAS1-4, have been identified from the interaction transcriptome collected during early stages of infection of rice by *M. oryzae* and are thought to be effectors (Mosquera *et al.*, 2009). Likewise, the effector MC69 is secreted during the early stage of infection. Since the deletion mutant *mc69* displays significant reduction in pathogenicity on both rice and barley, MC69 is interpreted as virulence effector of *M. oryzae* (Saitoh *et al.*, 2012). While most reported effectors are proteins, recently, cytokinin, produced by *M. oryzae* was shown virulence factor by repartitioning the transport of sugars and amino acids towards the infected site (Chanclud *et al.*, 2016).

No matter, whether a given effector is a protein or not, or whether it qualifies as avirulence or as virulence effector (a classification that depends on the presence of genetic loci from the host, anyway), the effector molecule has to reach its target site in the host, or, at a later stage, it has to move from cell to cell. This places the mechanisms for effector delivery to the host and transport within the host into the focus.

1.1.4 How Rice Blast Fungus effectors are delivered to the host cell and move within the host

1.1.4.1 Mechanisms of effector secretion

There exist two distinct mechanisms to deliver effectors to the cytoplasm of the host cell, space or to apoplastic space, respectively (Zhang and Xu, 2014). The delivery of effectors to the cytoplasm of the host is achieved via the biotrophic interfacial complex (BIC), a specific membrane-rich structure in the interface between host and fungus, (Khang *et al.*, 2010; Giraldo *et al.*, 2013). The delivery of effectors via the BIC is associated with a recently discovered type of secretion system including the fungal exocyst complex and t-SNAREs. Targeted deletion of the exocyst complex components *SEC5* and *EXO70* or mutation in t-SNARE component *SSO1* in *M. oryzae* caused impaired secretion of cytoplasmic effectors and culminated in a breakdown of pathogenicity (Giraldo *et al.*, 2013).

However, the apoplastic effectors are secreted via a separate pathway. They leave the fungal cell through the conventional ER-Golgi secretory system, i.e. independently of the BIC-mediated cytoplasmic effector secretion system. For instance, disruption of Golgi-dependent secretion by the exocytosis inhibitor Brefeldin A in *M. oryzae* inhibited the delivery of apoplastic effectors such as slp1 and Bas4, but this did not affect the secretion of cytoplasmic effectors to BIC such as Bas1, Bas107 and Pwl2 (Giraldo *et al.*, 2013).

1.1.4.2 Effector movement from cell to cell via plasmodesmata

Once effectors are secreted, they can move from cell to cell possibly via plasmodesmata (Zhang and Xu, 2014). The involvement of plasmodesmata is inferred from the strong

dependency of intercellular effector movement on molecular weight. For example, a fusion of Pwl2 with the dimeric fluorescent protein tdTomato with 68.3 kDa (i.e. larger than the size exclusion limit of plasmodesmata) was deficient in translocation to neighboring uninfected cell, while a fusion of Pwl with the monomeric mCherry with 39.3 KD (i.e. smaller than the size exclusion limit of plasmodesmata) was transported normally (Khang *et al.*, 2010). The observation that effectors spread ahead of invasive hyphae indicates that effectors serve as pioneering weapons to suppress plant immunity, thereby creating a permissive environment for hyphae proliferation (Khang *et al.*, 2010).

Effectors are, therefore, the central factor on side of the pathogen that help to overwhelm the basal immunity (PTI) of the host. Among these effectors, there are both proteinaceous and non-proteinaceous molecules which are typically fungal secondary metabolites produced in the biotrophic stage and are critical in virulence in the infection process. There are also secondary metabolites termed as toxins if produced in the necrotrophic stage in the infection. No matter whether these fungal secondary metabolites function as effectors or toxins, they are often the key players in the regulation of fungal virulence. Thus, an introduction of the fungal secondary metabolites in the next section will be given.

1.2 Fungal secondary metabolites

1.2.1 Diversity of fungal secondary metabolites

During the infection process, many pathogenic fungi produce secondary metabolites of low molecular weight. Some of these secondary metabolites are widely used in our daily life, such as the pharmaceutically important penicillin, statins, or cephalosporins (Aharonowitz *et al.*, 1992; Manzoni *et al.*, 1999). Other fungi produce notorious toxins, such as carcinogenic aflatoxin and trichothecenes (Klich, 2007; Desjardins *et al.*, 1993). These secondary metabolites are not required for the normal growth of the fungi, but

they are typically critical mediators of virulence in pathogenicity (Mobius and Hertweck, 2009). In spite of the complexity of chemical structures and functions of these secondary metabolites, they are generally biosynthesised from a limited number of precursors which derive from the primary metabolism. In general, they are classified into polyketides, produced by polyketide synthases (PKS), non-ribosomal peptides, generated by non-ribsosomal peptide synthetases (NRPS), and alkaloids, which are synthesised via dimethylallyl diphosphate tryptophan synthases (DMATS) (Pusztahelyi *et al.*, 2015). Additionally, there exist hybrid enzymes of a fungal type 1 PKS connected to NRPS module that can produce a combinatorial product (Collemare *et al.*, 2008). Fungal secondary metabolite enzymes are quite diverse. For example, in *M. oryzae*, 23 PKS, 8 NPRS, 10 PKS-NPRS and 3 DMATS have been identified; *Aspergillus niger* harbours 15 PKS, 12 NPRS and 5 PKS-NPRS; and in *Botrytis cinerea*, there are 17 PKS, 8 NPRS, 5 PKS-NPRS and 1 DMATS (Collemare *et al.*, 2008).

This huge diversity of fungal secondary metabolites is mirrored by a diversity of function. Some compounds act as repellents against other competitors, while others are virulence factors in pathogenicity (Macheleidt *et al.*, 2016). In the next section, the function of fungal secondary metabolites in virulence will be in the focus.

1.2.2 Fungal secondary metabolites in virulence

Pythopathogenic fungi include necrotrophic, biotrophic and hemibiotrophic fungi that use different strategies for infection and feeding on the plant tissue. Depending on the respective strategies secondary metabolites must play different function: While a necrotrophic pathogen will try to weaken and kill the plant cell, a biotrophic pathogen will rather reprogram plant signaling for their own sake. Even though necrotrophic pathogens use less complex infection strategies compared to biotrophic pathogens, their tools deployed for infection are basically the same, such as host selective toxins (HST) or effector proteins (Pusztahelyi *et al.*, 2015).

Numerous reports show the pivotal role of fungal secondary metabolites for virulence on the host. For example, the necrotrophic fungal pathogen *Cochliobolus heterostrophus* needs the polyketide T-toxin for successful colonisation of maize, since a loss-of-function strain for it's the synthesis gene *PKS1* eliminates both the accumulation of polyketide T-toxin and infection success (Yang *et al.*, 1996). For *M. oryzae*, the avirulence gene *ACE1* encoding a PKS-NPRS hybrid is expressed specifically during the penetration stage, and this gene product is recognised by the R protein *Pi33* in rice followed by induction of plant defence (Fudal *et al.*, 2007). Of course, the fungus does not produce this PKS-NPRS hybrid to activate plant defence, but is suggested to act as a pathogenicity effector, for which the host plant has evolved an appropriate receptor (Collemare *et al.*, 2008).

Fungal secondary metabolites are less known in biotrophic fungi; especially in obligate biotrophs such as the oomycetes causing Downy Mildews. One reason for this lack of information might be that the genes encoding secondary metabolic enzymes and transporters for toxin secretion, which are quite common in necrotrophic pathogens, have been partially or completely lost in obligate biotrophs (Pusztahelyi *et al.*, 2015). For hemi-biotrophic pathogens and necrotrophic pathogens, fungal secondary metabolites are frequently key virulence factors. The mechanisms of fungal secondary metabolites in virulence are quite diverse, as will be exemplarily discussed in the following section.

1.2.3 Mechanisms of fungal secondary metabolites in virulence

1.2.3.1 Fungal secondary metabolites that mimick phytohormones

One of the most efficient strategies for a pathogen would be to manipulate existing signal pathways of the host to facilitate infection. A classic example is the phytotoxin

coronatine in the hemibiotrophic bacterium *Pseudomonas syringae* that mimicks jasomonic acid to repress salicylic-acid signaling and, thus, the defence responses of the host (Zheng *et al.*, 2012). For the Rice Blast Fungus *M. oryzae* could cytokinin-like substances produced at the infection sites repartition sugar and amino acids from surrounding tissues, in order to obtain maximal nutrients from the rice host and to cause susceptibility (Chanclud *et al.*, 2016).

1.2.3.2 Fungal secondary metabolites that induce ROS in a light-sensitive manner

Necrotrophic fungi kill the host cell to feed on the dead tissue. Therefore, one of the strategies used by necrotrophic pathogens is to produce toxins which serve as photosensitizers to induce ROS generation, thereby causing necrosis and decomposition of the cell membrane. For example, *Cercospora nicotianae* produces a polyketide called cercosporin that can cause damage to plant cells in a light-induced manner and result in nutrient leakage to the apoplastic space where nutrients are available to fungal hyphae (Mobius and Hertweck, 2009). Fungi can avoid self-damage by rapid export and by use of quenchers (Daub *et al.*, 2005). The cercosporin biosynthesis gene *CTB1*, belonging to the PKS class, is strongly regulated by light and disruption of this gene leads to reduced necrotic lesion formation and reduced virulence on plants (Choquer *et al.*, 2007).

1.2.3.3 Fungal secondary metabolites that induce PCD resulting in susceptibility

For biotrophic fungi, ETI can be induced, when the effector is recognised by a R protein of the host, leading to HR and arrested colonisation. However, this mechanism can be hijacked by necrotrophic pathogens to establish effector-triggered susceptibility (ETS) by secretion of fungal secondary metabolites. For example, the host-specific toxin

victorin, produced by the necrotrophic pathogen *Cochliobolus victoriae* on oats can produce susceptibility by inducing outplaced PCD, as seen from DNA laddering and cell shrinkage (Curtis and Wolpert, 2004). Victorin is recognised by the protein encoded by R gene *Pc2* in oat, which actually confers to resistance against the biotrophic fungus *Puccinia coronate* in oat (Wang *et al.*, 2014). Further investigation of victorin-induced susceptibility led to the identification of the susceptibility locus, *LOV1*, encoding a homologue of a R gene identified in *Arabidopsis*, This shows a scenario, hwere a resistance gene product which is useful to ward off biotrophic pathogens by inducting HR, is abused by a necrotrophic fungal pathogen to kill the host (Lorang *et al.*, 2007).

1.2.3.4 Fungal secondary metabolites that disrupt membrane integrity

Certain secondary metabolites of the pathogen can inhibit enzymes which are involved in membrane lipid synthesis. A good example of this is the polyketide toxin fumonisin produced by *Fusarium*. This toxin functions as a sphingosine analogue to block the activity of ceramide synthase and sphinganine–N-acetyltransferase, thereby inhibiting biosynthesis of lipids and leading to membrane permeability (Williams *et al.*, 2007).

1.2.3.5 Fungal secondary metabolites that function as competitors for nutrients

Phytopathogens acquire nutrients from their hosts after infection, and some of these nutrients can be used by the pathogen to outcompete the host plant. For instance, iron as central player for cellular redox homeostasis and important cofactor for essential enzymes can be depleted by pathogen-produced NPRS siderophore toxins, such that the host is depleted from iron. In fact, deletion of siderophore biosynthesis genes in severeal pathogens reduced virulence and increased the sensitivity to ROS senstivity

(Haas *et al.*, 2008), indicating that siderophores are critical virulence factors.

1.2.4 Pyriculol

One of the secondary metabolites identified in the Rice Blast Fungus is pyriculol. Pyriculol is a salicylaldehyde polyketide that is produced by fungus *M. oryzae* even in culture medium, i.e. in the absence of the host. Pyriculol was originally identified in the culture broth of *Pyricularia oryzae cavara* using silica gel column chromatography. Application of this fraction on a rice leaf produced necrotic lesions, and growth of rice shoots and roots was inhibited from 50 ppm pyriculol (Iwasaki *et al.*, 1969). In this fraction, pyriculol, epipyriculol and tenuazonic acid were shown to cause similar brownish lesion on rice leaves, but also inhibited spore germination of *P. oryzae* (Kono *et al.*, 1991). The induction of necrotic lesions by pyriculol was suggested to be light-dependent. Under conditions of a day/night cycle, necrotic lesions could be induced by pyriculol (Iwasaki *et al.*, 1969; Kono *et al.*, 1991; Kim *et al.*, 1998), while under total darkness, “green islands” were seen (Lokeshwari and Suryanarayanan, 1992). The biosynthesis pathway of pyriculol in *M. oryzae* was later resolved (Fig. 3), finding that two enzymes *MoPKS19* and *MoC19OX* were required for the biosynthesis of the pyriculol and pyriculariol, both of which were tested to be able to induce necrotic lesions on rice leaves (Jacob *et al.*, 2017). In addition, there are also two transcription factors *MoC19TRF1* and *MoC19TRF2* which could negatively regulate *MoPKS19*, with *MoC19TRF1* having stronger effects (Jacob *et al.*, 2017). Even though pyriculol and pyriculariol were confirmed to be sufficient for the necrotic lesions on rice leaves using crude extract produced by different transgenic strains of *M. oryzae*, the infection assay showed that pyriculol was not required for the pathogenicity on rice cultivar Co39 (Jacob *et al.*, 2017). Thus, the function of pyriculol for the success of rice blast infection has remained a mystery.

The structure of pyriculol shows a striking similarity with the phytohormone salicylic

acid, leading to the question, whether modulation of hormonal signaling plays a role for the infection success of *M. oryzae*.

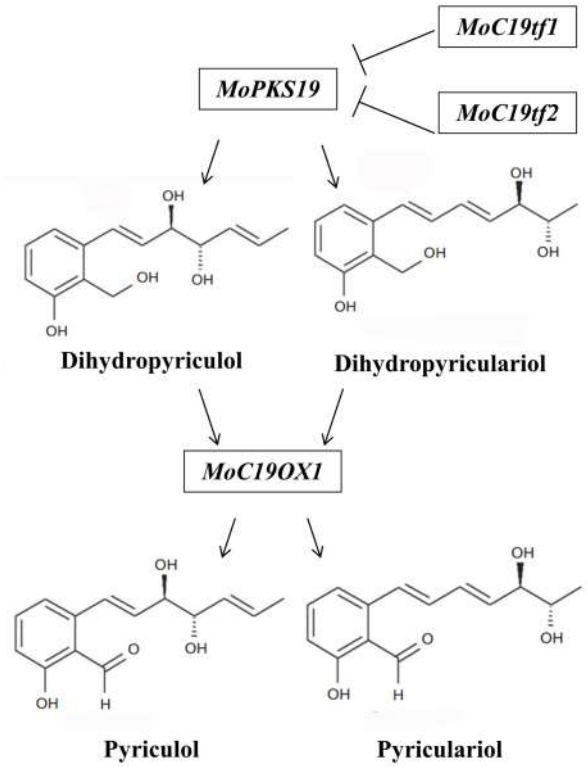


Fig. 3 Biosynthetic pathway of pyriculol and pyriculariol in rice blast fungus, *M. oryzae* (adapted from (Jacob *et al.*, 2017)). The gene *MoPKS19* encoded a polyketide synthase responsible for the production of the polyketides dihydropyriculol, dihydropyriculolariol, pyriculol and pyriculariol. The transcript level of *MoPKS19* was negatively regulated by two transcription factors *MoC19tf1* and *MoC19tf2*. Dihydropyriculol and dihydropyriculariol could be oxidised by *MoC19OX1* to be pyriculol and pyriculolariol.

1.3 Phytohormones involved in rice blast fungus defence

Plant hormones are not only important for the regulation of plant development and reproduction, but also participate in signaling during the immune responses. The signaling network deployed by plants in response to pathogen attack is complex and the interactions between phytohormones can be both, synergistic or antagonistic (Robert-Seilaniantz *et al.*, 2011). The crosstalk among phytohormones enables plants

to tailor their defence to specific pathogens or herbivores (Pieterse *et al.*, 2012). Typically, salicylic acid (SA) is recruited to fight against the biotrophic pathogens which feed on living tissues, while jasmonic acid (JA) and ethylene (ET) are effective against necrotrophic pathogens that kill plants first and the live on dead tissues (Bari and Jones, 2009). Although there exist exceptions, the two pathways are mutually antagonistic in most cases. Other phytohormones such as auxin, cytokinin, brassinosteroid, abscisic acid and gibberellic acid have also been reported to be involved in plant defence signaling by blending into the central SA, JA and ET pathway (Robert-Seilaniantz *et al.*, 2011) that are briefly described in the following paragraphs.

1.3.1 Salicylic acid (SA) in plant defence

SA is a natural phenolic compound of plants which is biosynthesized via the shikimate-phenylpropanoid pathway following two alternative branches in *Arabidopsis*. One branch runs through the phenylalanine ammonium lyase (PAL) triggered production of cinnamic acid, while the other branch uses isochorismate (ICS) (Vlot *et al.*, 2009). In rice, SA biosynthesis seems to rely mainly on the PAL pathway, since in the *OsPAL06* deletion mutant SA contents in the root are reduced by two thirds compared to wild type (Duan *et al.*, 2014). After biosynthesis, SA can be conjugated, for instance into SA β-glucoside (SAG), via the key enzyme *OsSGT1*. Conjugation is thought to play important roles in chemically induced plant resistance and the regulation of SA signaling (Umemura *et al.*, 2009). Downstream of SA biosynthesis, both, rice and *Arabidopsis,* share one master regulator, NPR1 (nonexpressor of pathogenesis-related genes 1). This protein is normally tethered in an oligomeric state in the cytosol, but in response to redox change can be translocated to nucleus, where it interacts with TGA transcription factors culminating in the activation of SA signaling genes (Caarls *et al.*, 2015). Recently, NPR1, together with its two paralogues NPR3 and NPR4, has been reported to be a receptors for SA,

although their role in regulating immune responses is antagonistic (Ding *et al.*, 2018). A second key regulator in SA signaling, complementing the activity of NPR1, is the transcription factor *OsWRKY45* which in rice can also act as master regulator independently of *OsNPR1* (Shimono *et al.*, 2007b; Sugano *et al.*, 2010). Following the activation of these regulators, pathogenesis-related (PR) genes and phytoalexins are induced to contribute to plant defence (Shimono *et al.*, 2007b; Daw *et al.*, 2008).

However, the role of SA in the pathogen response of rice is still not fully understood: For instance, there is no significant change of SA abundance upon pathogen attack, and the resting levels of this hormones are relatively high (up to >30 µg/g fresh weight), which means that the signaling seems to be fairly insensitive (Silverman *et al.*, 1995). In stark contrast, SA level in tobacco and *Arabidopsis* are lower than 100 ng/g fresh weight and can be induced by up to two magnitudes after pathogen infection (Malamy and Klessig, 1992). Unlike the more specific patterns seen in *Arabidopis*, treatment of SA functional analogs such as PBZ (probenazole), BTH (benzothiadiazole), and TDL (tiadinil) produce a broad-band spectrum of pathogen resistance, regardless of lifestyles, including hemibiotrophic fungal pathogen *M. oryzae*, the bacterial pathogen *Xanthomonas oryzae pv. oryzae*, the necrotrophic root pathogen *Pythium graminicola*, and the root-rot nematode *Hirschmanniella oryzae* (Shimono *et al.*, 2007b; Xu *et al.*, 2013; De Vleesschauwer *et al.*, 2012; Nahar *et al.*, 2012). This indicates that the role of SA in rice seems to extend beyond resistance against biotrophic pathogens.

It is also clear that SA plays a pivotal role in defence against the Rice Blast Fungus. Firstly, exogenous application of SA can enhance the accumulation of diterpenoid phytoalexins followed by resistance to *M. oryzae* (Daw *et al.*, 2008). Likewise, activation of SA signaling by the SA analogue BTH can augment rice diterpenoid phytoalexin accumulation via the *OsWRKY45* pathway (Akagi *et al.*, 2014). As to be

expected, the SA-biosynthesis deletion mutant *OsPAL06* accumulated lower levels of SA, and was less resistant to the Rice Blast Fungus correlated with reduced accumulation of phytoalexins (Duan *et al.*, 2014). Functional analogues of SA, such as PBZ and BTH are accepted by *OsSGT1* which converts SA to SAG regulating the SA signaling, and these analogues can boost defence against *M. oryzae* (Umemura *et al.*, 2009). Since the resting levels of SA are very high in rice, SA was proposed to act as preformed antioxidant interfering with fungus-induced oxidative burst. In fact, the SA-deficiency mutant NaG showed spontaneous necrotic lesions linked with reduced resistance to biotic and abiotic stresses (Yang *et al.*, 2004). Altogether, SA seems to promote resistance to the Rice Blast Fungus by induction of diterpenoid phytoalexin accumulation, PRs expression and regulation of oxidative balance.

1.3.2 Jasmonic acid (JA)

The biosynthesis of jasmonic acid starts from liberation of linolenic (18:2) and α-linolenic (18:3) acid from the chloroplast membrane, releasing substrates for the 9-lipoxygenases (9-LOX) and 13-lipoxygenases (13-LOX) which catalyze the formation of 9S-HPODE and 13S-HPODE (Feussner and Wasternack, 2002). In rice, the HPODEs are then catalysed by allene oxide synthase (*OsAOS1* and *OsAOS2*), and allene oxide cylase (*OsAOC*) into 12-oxo phytodienoic acid (12-OPDA) (Park *et al.*, 2002; Mei *et al.*, 2006; Riemann *et al.*, 2013). Subsequently, OPDA is transferred to peroxisomes where it is reduced by the enzyme of 12-oxo phytodienoic acid reductase (*OsOPR7*), followed by three steps of β-oxidation, resulting in the formation of JA (Tani *et al.*, 2008). JA requires further modification by the GH3 amido synthetase *OsJAR1* which conjugates JA to the amino acid isoleucine to yield JA-Ile, which is the active signal (Wakuta *et al.*, 2011; Shimizu *et al.*, 2013), recognised by the receptor complex SCF^{COI1} which acts as E3 ligase to target JAZ proteins for degradation via the 26S-proteasome pathway. The degradation of JAZ proteins releases downstream transcription factors such as *OsMyc2* from repression, thereby

activating JA-dependent gene expression (Ogawa *et al.*, 2017; Uji *et al.*, 2016). In rice, there are 15 JAZ-encoding genes, nine of which are wounding-responsive. Almost all of the JAZs are responsive to at least one type of abiotic stresses (Ye *et al.*, 2009).

JA and its derivatives are collectively termed as jasmonates. JA is reported to be involved in wounding, regulation of secondary metabolism and the responses to abiotic and biotic stress. In dicots, JA is generally responsible for defence against necrotrophic pathogens, while SA is more important for resistance against biotrophic pathogens. There exists a pronounced antagonistic relationship between the JA-dependent and SA-dependent pathways that seems to be evolutionarily conserved and has been reported for 17 plant species so far (Thaler *et al.*, 2012). In rice, JA is involved in defence against pathogens with various lifestyles, including hemibiotrophic pathogens *M. oryzae* and *Xanthomonas oryzae*, the necrotrophic pathogens *Rhizoctonia solani*, and the biotrophic root knot nematode *Meloidogyne graminicola* (Tani *et al.*, 2008; Riemann *et al.*, 2013; Yamada *et al.*, 2012; Taheri and Tarighi, 2010; Nahar *et al.*, 2012).

The function of JA in orchestrating the response to Rice Blast Fungus has been intensively studied: JA can not only induce the accumulation of reactive oxygen species, but also modulates diterpenoid and flavonoid phytoalexin production after elicitation with in $CuCl_2$ and chitin (Li *et al.*, 2014; Nojiri *et al.*, 1996; Rakwal *et al.*, 2014). Overexpression of *OsAOS2* enhanced the accumulation of JA and transcripts for PR gene expression during infection with the Rice Blast Fungus infection (Mei *et al.*, 2006). Conversely, the JA biosynthesis deletion mutants *osjar1* (*OsJAR1* insertion mutant), *cpm2*, and *hebiba* (*OsAOC* deletion mutants) displayed a higher susceptibility to Rice Blast Fungus accompanied by reduced accumulation of flavonoid phytoalexins, such as sakuranetin, while the diterpenoid phytoalexin production was not affected (Shimizu *et al.*, 2013; Riemann *et al.*, 2013). Taken

together, JA can enhance rice defence against Rice Blast Fungus linked with enhanced oxidative burst, induction of PR genes, and accumulation of the flavonoid phytoalexin sakuranetin.

1.3.3 Ethylene (ET)

Ethylene is a gaseous hormone and synthetized from the amino acid methionine, catalysed by SAM synthetase to form S-AdoMet, followed by catalysis through ACC synthase and ACC oxidase to produce ET (Wang *et al.*, 2002). For ET signaling, five receptors have been reported in *Arabidopsis*, ETR1, ETR2, EIN4, ERS1, and ERS2, which act as repressors of ET signaling in the absence of ET. In the presence of ET, these receptors are inactivated, leading to inactivation of another negative regulatory kinase CTR1, resulting in activation of EIN2, the positive regulator of ET downstream signaling (Wang *et al.*, 2002).

ET generally functions synergistically with JA in the defence against necrotrophic pathogens, but negatively interacts with SA during resistance to biotrophic pathogens (Derksen *et al.*, 2013). ET biosynthesis could be rapidly induced by PAMPs, in a pattern that was similar to that observed for JA and SA (Boller and Felix, 2009; Schwessinger and Ronald, 2012). However, ET and its co-product cyanide are also reported to be positive regulators of ETI against *M. oryzae* in rice (Iwai *et al.*, 2006). For instance, overexpression of *OsACS2* in rice leads to increased resistance to the hemibiotrophic fungus *M. oryzae*, but also to the necrotrophic fungus *R. solani*, while rice plants deficient in the ET signal transducer *OsEIN2* showed reduced resistance to *M. oryzae* (Helliwell *et al.*, 2013; Yang *et al.*, 2017). Taken together, these data support an important role of ET in the defence against a variety of pathogens in rice.

Even all of the three phytohormones mentioned above are by themselves modulating the response to the Rice Blast Fungus, their output in the plant is not independent, but

integrated into complex crosstalk that can be either antagonistic or synergistic. This crosstalk allows to tune plant adaptation to different stresses or to stress combination. In the next section, therefore, the crosstalk between SA and JA will be in the focus.

1.4 Crosstalk between SA and JA

The antagonism between SA and JA is evolutionarily conserved both in dicots and monocots, with SA acting in the defence against biotrophic pathogens, while JA acts in the defence against necrotrophic pathogens and herbivores (Thaler *et al.*, 2012). This antagonistic crosstalk has been reported for 6 wild and 11 crop species, and phylogenetic analysis indicates that this antagonism reaches back to the beginning of angiosperms (Thaler *et al.*, 2012). Functional counterparts of this antagonistic interaction between SA and JA seem to be present even in animals: The lipid-derived prostaglandins (structural analogues of JA) in animals can be effectively inhibited by aspirin (acetylsalicylic acid) (Flower, 2003). In most cases SA predominates JA signaling, and there exist several mechanisms, how this suppression of JA signaling is achieved (Caarls *et al.*, 2015).

1.4.1 How SA can suppress JA

1.4.1.1 SA can suppress JA biosynthesis

The repression of jasmonic acid biosynthesis genes by exogenous application of salicylic acid was reported in several plant species, such as tomato, flax and Arabidopsis. For example, the salicylic acid analogue aspirin was found to repress wound-induced jasmonic acid accumulation in tomato by targeting expression of allene oxide synthase (AOS) in detached leaves of tomato (Pena-Cortés *et al.*, 1993). This was further confirmed by a later report in which wound-induced AOS mRNA accumulation was inhibited by salicylic acid or aspirin in flax leaves, while enzyme activity of AOS was not affected by neither salicylic acid nor aspirin treatment (Harms *et al.*, 1998).

Even though the repression of jasmonic acid biosynthesis genes such as *LOX2*, *AOS*, *AOC2* and *OPR3* by salicylic acid was also described in *Arabidopsis*, it was later found that the real target of inhibition by salicylic acid was downstream of the jasmonic acid biosynthesis pathway, because the AOS deletion mutant *aos/dde2* was still showing salicylic-acid mediated suppression of the jasmonic acid-induced gene *PDF1.2* as compared to wild type Col-0 plants (Leon-Reyes *et al.*, 2010). So far, there is no evidence for stabilisation or induction of JAZ genes by SA, which would be an alternative mechanism to repress JA signaling. Degradation of JAZ proteins induced by JA is not affected by addition of SA in *Arabidopsis* (Van der Does *et al.*, 2013). Likewise, SA-mediated repression of JA induced response remains functional in the JA reception mutant *coi1-1* (coronatine insensitive 1), indicating that SA' suppression of JA is downstream of SCF^{COI1}-JAZ complexes (Van der Does *et al.*, 2013).

1.4.1.2 SA can control regulators to inhibit JA-dependent gene expression

The positive regulator in the SA signaling, NPR1, can, mediated by thioredoxins and glutaredoxins, regulate transcription factors such as TGA to convey the SA/JA crosstalk (Caarls *et al.*, 2015). For example, in the *NPR1-1* mutant of *Arabidopsis* which, as to be expected, is deficient in SA signaling, JA levels are elevated as well as the expression of JA responsive genes in response to the pathogen *Pseudomonas* DC3000 (Spoel *et al.*, 2003). NPR1 protein exists in the cytosol as oligomer, but it can be reduced and translocated to the nucleus as a monomer in response to redox change (Caarls *et al.*, 2015). However, it is the cytosolic localisation of NPR1 protein that is essential for regulation of the crosstalk between SA and JA. For instance, in plants that overexpress the fusion protein NPR1-HBD to block its nuclear import, SA-mediated suppression of JA induced gene expression was observed (Spoel *et al.*, 2003). This notion is further verified in rice (*Oryzae sativa*). For instance, overexpression of *OsNPR1* shows enhanced susceptibility to herbivore infestation,

concomitant with suppression of JA-related defence gene expression, but overexpression of a mutated form of *OsNPR1* renders it constitutively nucleus-localized and herbivore susceptibility is abolished and no inhibition of JA responsive genes is found (Yuan *et al.*, 2007). Taken together, cytosol-localised NPR1 can be deployed by SA to crosstalk with JA.

1.4.1.3 SA can induce degradation of JA responsive transcription factors

SA can also induce degradation of the transcription factors which are needed for the activation of JA responsive genes. Specifically, SA treatment was proposed to result in degradation of one ethylene-responsive factor ORA59 that plays a positive regulatory role in JA signaling (Van der Does *et al.*, 2013). *In-silico* promoter analysis of SA/JA crosstalk transcriptome finds that the presence of a GCC-box motif as potential target of SA to suppress JA responses. Further analysis using GCC-box fused to the b-glucuronidase reporter gene and overexpression of GCC-box binding transcription factor ORA59 reveals reduced GCC-GUS activity and declined accumulation of the ORA59 protein in response to SA treatment (Van der Does *et al.*, 2013). Collectively, SA can induce degradation of JA-responsive transcription factors by targeting GCC-motif.

1.4.1.4 SA can suppress JA-dependent gene expression through SA-inducible transcription factors

A further level of crosstalk might come from SA-induce expression of transcription factors that can suppress JA responses. For example, the expression of *WRKY70* in *Arabidopsis thaliana* is induced by SA, but repressed by JA, and overexpression of *WRKY70* impairs JA-dependent *PDF1.2* expression (Li *et al.*, 2004; Li *et al.*, 2006). Similarily, *OsWRKY13* overexpressing plants show enhanced resistance to bacterial

blight and fungal blast disease, with increased activation of SA inducible genes and concomitantly repressed expression of JA responsive genes (Qiu *et al.*, 2007b), indicating *OsWRKY13* as regulatory nod of SA/JA crosstalk.

1.4.2 How JA can suppress SA

Although SA often dominates over JA in the SA/JA antagonism, there are also reports of JA-mediated suppression of SA in plants. For example, in the *Arabidopsis coi1* mutant that is deficient in recognition of JA a higher resistance to the bacterial pathogen *P. syringae* is correlated with enhanced activation of the SA-dependent pathway (Kloek *et al.*, 2001). Conversely, coronatine, the structural mimic of JA, produced by the pathogen *P. syringae*, can activate JA-inducible NAC transcription factors inhibiting the SA biosynthesis key gene *ICS1* and promoting the SA catabolism factor *BSMT1* (Zheng *et al.*, 2012). Also in rice, there is evidence for suppression of SA by JA. In leaf blades that are directly sprayed with JA or wounded (inducing high JA levels) SA levels were reduced (Tamaoki *et al.*, 2013; Lee *et al.*, 2004).

1.4.3 SA and JA synergism

Even though SA/JA antagonism seems to be the predominant mode of crosstalk, cases of SA/JA synergism have also been described in both, monocots and dicots. For example, the *Arabidopsis* mutant *hrl1* (*hypersensitive response-like lesion 1*) shows spontaneous necrotic lesion formation and is more resistant to the oomycete *Peronospora parasitica* and the bacterial pathogen *P. syringae*, linked with synergistic interaction between SA, JA and ET during the regulation of cell death and defence responses (Devadas *et al.*, 2002). Likewise, the SA receptors NPR3 and NPR4 can interact with JAZ proteins, thereby inducing JA responsive genes during RPS2-mediated effector trigger immunity (Liu *et al.*, 2016). Synergistic interaction

has also reported in *Ginkgo biloba* cells in which SA and JA have complementary role in regulating the production of flavonol glycosides (Xu *et al.*, 2009). In the monocot rice, positive interaction between SA and JA seems to be more common. Rice plants with a deletion of the hydroperoxide lyase *OsHPL3* accumulate higher levels of SA, JA and also transcripts for both, JA- and SA-responsive defence genes(Tong *et al.*, 2012; Liu *et al.*, 2012). Likewise, rice plants overexpressing a JA and ET responsive factor, JERF1, show enhanced resistance to sheath blight caused by the pathogen *R. solani* linked with increased activities of two enzymes which are induced by both, the JA and SA pathway (Pan *et al.*, 2014). Further validated support comes from a microarray analysis in rice, where more than half of the defence-related genes were activated by both treatments (Garg *et al.*, 2012; Tamaoki *et al.*, 2013). While this synergy seems to contradict the general pattern of antagonistic crosstalk also found in rice, it seems that the mode of interaction is concentration dependent: Synergism between SA and JA occurs at low concentration, while antagonism takes place at high concentration (Mur *et al.*, 2006).

1.5 Scope of study

The polyketide pyriculol could be isolated from liquid culture of the Rice Blast Fungus and was identified as one of the most abundant fungal toxins. In the detached leaf assay, crude fungal extracts were able to induce necrotic lesions. Since polyketides are often key mediators for fungal virulence in both, nectrophic and biotrophic, fungi, pyriculol, as necrotic lesion-inducing polyketide was interesting, moreover, since it is structurally related to the phytohormone SA. Based on these preliminary data and considerations we wondered, whether pyriculol might affect other phytohormone such as JA (important in the defence against Rice Blast Fungus) in rice. This would be a case, where hijacking of one phytohormonal pathway would inhibit defence-related signaling of a second pathway, similar to the situation in *P. syringae*, where the JA mimic coronatine can down-regulate SA signaling to facilitate infection (Zheng *et al.*,

2012). To dissect the effect of pyriculol on JA such a scenario, pyriculol was applied in combination with a wounding treatment (which strongly induces JA biosynthesis and signaling).

These considerations led to the following questions were asked:

1. How is the necrotic lesion induced by pyriculol regulated?
2. What is effect of pyriculol application on biosynthesis and signaling of the defence phytohormone JA?
3. How does SA crosstalk JA?
4. Is there any role of pyriculol for the infection process of Rice Blast Fungus?

To address these questions, the following approaches were pursued:

In infection assays using pyriculol-related transgenic strains of *M. oryzae*, a potential role of pyriculol as virulence factor was tested. Since the mode of action of effectors (virulent or avirulent) is strongly dependent on the rice subspecies (*japonica* versus *indica*), and even the variety, a screening from a core-collection of rice varieties was required to establish the appropriate pair of host and pathogen for the infection test.

To date, pyriculol had been only detected in the liquid culture medium of rice blast fungus, while no report is published so far about its existence in the infected plants. It is therefore a realistic possibility that pyriculol is not induced at all during infection. To test, whether host defence is activated or inactivated by this polyketide, exogenous, purified pyriculol to rice was applied to rice in order to detect potential functions *in planta*.

2 Material and Methods

2.1 Chemicals

Pyriculol and pyriculariol (80% in purity) were obtained from the Institut für Biotechnologie und Wirkstoff-Forschung gGmbH (IBWF) and the extraction and purification process was described (Jacob *et al.*, 2017). Methanol (Roth, Karlsruhe, Germany) was used as the solvent for pyriculol and pyriculariol and therefore also used as the solvent control in the presence of pyriculol treatment; 50 μM diphenylene-iodonium chloride (DPI, Sigma-Aldrich, Deisenhofen, Germany) was deployed as an inhibitor for respiratory burst oxidase homolog (Rboh), aplasma-localized NADPH oxidase; 1mM ascorbic acid (Roth, Karlsruhe, Germany) was used as a common antioxidant; 1mM methyl jasmonate (Sigma-Aldrich, Deisenhofen, Germany) and salicylic acid (Sigma-Aldrich, Deisenhofen, Germany) were applied as phytohormones; 0.35 g/l of MURASHIGE & SKOOG medium (MS medium) (Duchefa Biochemie, Haarlem, Netherlands) was used as nutrients in the hydroponic culture; 5% sodium hypochlorite (Roth, Karlsruhe, Germany) and 70% (v/v) ethanol (Roth, Karlsruhe, Germany) were used for sterilization of rice seeds; 5% (w/v) gelatin (Roth, Karlsruhe, Germany) served as the surfactant for the fungal spore inoculation; 0.04% (w/v) phyto agar (Duchefa Biochemie, Haarlem, Netherlands) acted as the solid medium for holding the seeds.

2.2 Seed sterilization, sowing and hydroponic culture of rice seedlings

Selected rice seeds with good quality were surface sterilized firstly with 70% ethanol once, then rinsed with sterilized water twice. Subsequently, the seeds were further soaked in 5% sodium hypochlorite and incubated on a shaker (IKA, Staufen, Germany)

at 250 rpm for 20 min. The sterilized seeds were sown with a flame-sterilized tweezer into Magenta boxes filled with 100 ml autoclaved 0.4% phyto agar on a UV-irradiated clean bench (Thermo Fisher Scientific, Massachusetts, USA). Following this, Magenta boxes were incubated in a plant chamber (CLF Plant Climatics, Wertingen , Germany) (12 h in light at 28 °C and 12 h in darkness at 28 °C) for 7 days, then transplanted into a hydroponic culture system consisting of a glass bottle with liquid medium (0.35 g/L MS medium) and a floating plate (Roth, Karlsruhe, Germany) on which rice seedlings were fixed.

2.3 Pyriculol assay on detached leaf segments

After 7 days of growth in the Magenta boxes, Nihonmasari seedlings of comparable size were transplanted to the hydroponic culture system for another 2 weeks at the same culture condition. During this period, the liquid MS medium was changed every 5 days in case of algae growth. Then, the fifth leaf from 3 weeks old rice plants was cut into around 5 cm long segments and on each leaf segment, a glass rod was used to make a wounding site without piercing the leaf. These wounding sites served as the positions where chemicals were applied. For each wounding position, 5 µl of the following solutions (including double-distilled water, 1% methanol, 1 mM ascorbic acid, 1 mM JA, 1 mM SA, 50 µM DPI, 0.64 mM pyriculol, mixture of 0.64 mM pyriculol and 1 mM ascorbic acid, mixture of 0.64 mM pyriculol and 1 mM JA, mixture of 0.64 mM pyriculol and 1 mM SA, mixture of 0.64 mM pyriculol and 50 µM DPI) was applied. After application of the chemicals，the leaf segments were incubated in a petri dish filled with 20 ml 0.35 g/l MS solution for 24 h in a plant chamber at the same condition (in a light and darkness cycle). 24 h of incubation later, leaf segments were pasted onto a label paper and covered with a transparent plastic foil and then subjected to scanning. Obtained pictures were analyzed using ImageJ (free at https://imagej.nih.gov/ij/).

For the similar treatment in darkness, after application of the solutions (including

double-distilled water, 5% methanol, 0.64 mM pyriculol and 3.2 mM pyriculol) on leaf segments, the petri dishes containing treated leaf segments and 20 ml of 0.35 g/l MS solution were wrapped in black plastic foil and then put in a paper carton. It was subsequently incubated in the chamber under the same culture condition. 3 days after the chemical application, leaves were pasted onto a label paper and covered with a transparent plastic foil and scanned to pictures. The pictures were then analyzed with ImageJ (https://imagej.nih.gov/ij/).

2.4 Exogenous application of pyriculol and SA in combination with wounding treatment

Rice seeds of Nihonmasari were sterilized, sowed and hydroponically cultured as mentioned above. When the rice seedlings were cultured in the hydroponic system for 7 days, they were subjected to homogeneous treatment of the following solutions (mock solution (1% methanol + 0.5% gelatin), 0.64 mM SA dissolved in mock solution and 0.64 mM pyriculol dissolved in mock solution) by using micro-sprayers until the liquid ran off from the leaves. 0.5 h after the solution treatment, the third leaf blades were wounded by deploying a scissor to cut six times from one direction towards the midvein. 0.5 h, 1 h and 24 h after wounding treatment, samples were taken and stored immediately in liquid nitrogen.

2.5 Culture of *M. oryzae* strains and preparation of spore suspension

The *M. oryzae* strains were cultured on rice flour agar medium composed of 2% (w /v) rice flour, 0.25% (w/v) yeast extract and 1.5% agar (w/v) for 10 days under the fluorescent light (12 h light per day, 26 °C) before harvest of spores. Spore suspension was prepared using 3 ml sterilized double-distilled water to flush the petri dish plate on which rice blast fungus sporulated. Then a plastic scraper was used to remove spores

from the culture medium and spore suspension was filtered through 2 layers of gauze into a glass tube mounted on ice (low temperature prevents spore germination). The concentration of obtained spore suspension was microscopically determined using a counting chamber (Neubauer chamber, Marienfeld, Germany).

2.6 The effect of pyriculol and pyriculariol on spore germination of rice blast fungus

For spore germination tests, the first step was to adjust the concentration of Gy11 spore suspension to be 5×10^4 spores/ml (also the concentration for inoculation), since high concentration of spore suspension is self-inhibiting in germination. Then the spore suspension was used to mix with pyriculol to make 50 µl of spore suspension with different concentrations of pyriculol (0, 0.02 mM, 0.04 mM, 0.08 mM, 0.16 mM, 0.32 mM and 0.64 mM) and also different concentrations of solvent control (0.03% methanol, 0.06% methanol, 0.125% methanol, 0.25% methanol, 0.5% methanol, 1% methanol). 50 µl of the mixture was pipetted onto a glass slide and subsequently covered with a cover slide. The slides were placed in darkness under the room temperature for overnight. Germination rate was quantified by averaging the rate of germinated spores in at least five microscopic fields.

2.7 Rice growth in the green house and inoculation with rice blast fungus

Rice plants were grown in the green house (Montpellier, France) in a day/night cycle with temperature being 20 °C at night and 30 °C at the daytime. Seeds were sown in pots filled with compost containing 7/8 Neuhaus compost no. 9 and 1/8 pozzolana. Soil was maintained humid with water every day and was given fertilizer solution (1.5 g/L NPK (17-7-22), 0.25 g/L QUELARTAL Fe (6% w/v) and 0.25 g/L Hortrilon) every week.

Inoculation was conducted using calibrated concentration of spore suspension (5×10^4 spores/ml) mixed with 0.5% gelatin. The 3-week old rice plants were placed on a turnplate and spore suspension was then gun-sprayed onto rice leaf blades. In order to be homogeneous in infection, the turnplate should rotate at a constant speed. After spraying of spores, inoculated rice plants were moved into a dew chamber (100% humidity, 25 °C in darkness) for 16 h. After this, rice plants were transferred to a growth chamber (12 h in darkness at 25 °C and 12 h in light at 30 °C) for 6 days. Then leaves (at the same developmental stage) with symptoms were sampled, pasted onto a label paper, covered with transparent plastic foil and then scanned to pictures for symptom analysis.

2.8 Exogenous application of pyriculol in combination with Gy11 spores on rice plants

Three weeks old Nihonmasari rice plants were gun-sprayed with the following suspensions: mock solution (0.5% gelatin and 0.25% methanol), Gy11 spore suspension (at concentration of 5×10^4 spores/ml) in mock solution, 40 μM pyriculol in mock solution, mixture of 40 μM pyriculol and Gy11 spore suspension, 160 μM pyriculol in mock solution, mixture of 160 μM pyriculol and Gy11 spore suspension, 40 μM pyricurariol in mock solution, mixture of 40 μM pyricurariol and Gy11 spore suspension, 160 μM pyricurariol in mock solution and mixture of 160 μM pyricurariol and Gy11 spore suspension. Then the inoculated plants were treated as mentioned above. At 2 days after inoculation, the infected leaves at the same developmental stage (normally the topmost fully expanded leaves) were sampled in liquid nitrogen for RNA extraction. At 7 days after inoculation, the leaves were sampled for symptom analysis as mentioned above.

2.9 Symptom classification and quantification

The obtained pictures of symptoms were firstly artificially classified into different lesion types, according to the general disease severity (Fig. 13). Based on the classification criterion, ImageJ was deployed to calculate the number and the area of each type of lesions and the results were then normalized to the leaf area to obtain lesion number/ leaf area and lesion area/ leaf area for each lesion type.

2.10 Rice varieties and *M. oryzae* strains used for the initial infection screen

Eight rice varieties were used in the infection experiment (Table 1). All seeds were from CIRAD-Center for Biological Resource (Montpellier, France). Plants were cultured as mentioned above. Three weeks old rice plants were inoculated with the 7 *M. oryzae* strains (Table 2**)**. Quantification of the symptoms was done using ImageJ to count the number of lesions for each lesion type and percentage of each lesion type was calculated to obtain the dominant lesion type (with the biggest percentage in all lesion types).

Table 1 Rice varieties used for the initial screen analysis.

rice varieties (Japonica)	rice varieties (Indica)
Azucena	Co39
Nipponbare	IR64
Sariceltik	Bala
Maratelli	Kasalath

Table 2 *M. oryzae* strains used for the infection assay.

Strains	Description	Pyriculol and pyriculariol production in vitro
MoWT 70-15	*M. oryzae* 70-15 wild type strain	normal pyriculol and pyriculariol production
ΔMoC19tf1	*M. oryzae* 70-15 transcription factor 1 (tf1) repressing polyketide synthesase 19 (PKS19)	more pyriculol and pyriculariol production putatively than MoWT 70-15
ΔMoC19tf2	*M. oryzae* 70-15 transcription factor 2 (tf2) repressing PKS19	more pyriculol and pyriculariol production putatively than MoWT 70-15
ΔMoPKS19	*M. oryzae* 70-15 PKS19 deletion strain	no pyriculol and pyriculariol production
ΔMoC19OX1	*M. oryzae* 70-15 oxidase deletion strain	no pyriculol and pyriculariol
ΔMoEF1::C19OX1	*M. oryzae* 70-15 oxidase overexpression strain	more pyriculol and pyriculariol than MoWT 70-15
ΔMoC19OX1/OX1	*M. oryzae* 70-15 oxidase deletion/ complementation strains	Comparable amount of pyriculol and pyriculariol production to MoWT 70-15

2.12 RNA extraction, cDNA synthesis and Real-Time PCR

RNA extraction was performed using the innuprep Plant RNA extraction kit of AnalyticJena. cDNA was synthesized according the following steps: 1 µg total RNA was used to synthesize the first strand of cDNA, then 2 µl of 40 µM oligo-dT and 1 µl of 10 mM dNTP were added in sterile microtubes. Final volume was adjusted to 16 µl with nuclease-free water, followed by a step at 70 °C for 5 min and incubated on ice immediately after a short spin. After this, each tube was supplemented with 2 µl of

10×RT buffer (500 mM Tris-HCl (pH 8.3), 750 mM KCl, 30 mM MgCl2 and 100 mM dTT), 1 µl RNAase inhibitor (10 U/µl) and 1µl M-Mulv reverse transcriptase (200 U/µl). The final volume for each tube was 20 µl. After all the reagents were mixed well, tubes were incubated at 42 °C for 1 hour, then 90 °C for 10 min to inactivate the enzyme. Synthesized cDNA was kept at -20 °C in a freezer. Real-Time PCR was conducted on real-time PCR detection system CFX-96 (Biorad, California, United States) using the following reagents in 20 µl volume: 1 µl cDNA (1/10 dilution), 4 µl GoTaq buffer, 11.75 µl nuclease-free H_2O, 0.4 µl dNTP (10 mM), 0.4 µl forward primer, 0.4 µl reverse primer, 1 µl $MgCl_2$ (50 mM), 0.1 µl GoTaq Pol and 0.95 µl SybrGreen. The expression of the genes was normalized to the expression of two house-keeping genes GAPDH and Ubiquitin 10. The information of primers used in the experiment are shown below (Table 3)

Table 3 Primers used in this experiment for real-time PCR

Gene name	Locus ID	Forward primer (5'-3')	Reverse Primer (5'-3')	Reference
OsJAZ8	Os09g0439200	GAAGGCTCAACAGCTGACCAT	TTGGTGGACGGGAAGTTCTC	(Cai *et al.*, 2014)
OsJAZ9	Os03g0180800	GGCCGGTCGAGTTGGAA	GGTCAGGCTCGGCGAAAT	(Cai *et al.*, 2014)
OsJAZ10	Os03g0181100	TCTTCCCACCCCGTCAAAT	CCTCGCTGGTGCTTTGCT	(Cai *et al.*, 2014)
OsJAZ11	Os03g0180900	CAGCCTTGCCTACCAGACATG	GACGATCCTGTTCTTCCTCTTCTC	
OsJAZ13	Os10g0391400	ACACGTCAGCTTTAATCCCATAATT	GAATAATCGTGCACTGTACAAATGC	(Cai *et al.*, 2014)

OsAOS1	Os03g0766900	CACCGTCACC TCGCTCAAGA AG	ACTCCGTATC CGTACAAGCT GATTG	
OsAOS2	Os03g0225900	GGAGGAAGCT GCTGCAATAC	GGAGGTTGAA GCTTTGGTGA	
OsAOC	Os03g0438100	TGCCTCAACA ACTTCACCAA CTA	CACATGCCGC AATTAACACT AAA	
OsJAR1	Os05g0586200	AGGAGGCATC AAAGTTCCTG G	CTCAGCTCCC AGAAGATCAC G	
OsOPR7	Os08g0459600	CTCAACCACC GGTTTCCTCA	TCCATGCATC AGTCTGCTCT	
Ubiquitin 10	Os02g0161900	TGGTCAGTAA TCAGCCAGTT TGG	GCACCACAAA TACTTGACGA ACAG	(Jain *et al.*, 2006)
GAPDH	Os04g0486600	AAGCCAGCAT CCTATGATCA GATT	CGTAACCCAG AATACCCTTG AGTTT	(Jain *et al.*, 2006)
OsPR1a	Os07g0129200	GTATGCTATGC TACGTGTTTAT GC	GCAAATACGG CTGACAGTAC AG	(Mitsuhara *et al.*, 2008)
OsPR1b	Os01g0382000	ACGCCTTCAC GGTCCATAC	AAACAGAAA GAAACAGAG GGAGTAC	(Mitsuhara *et al.*, 2008)
OsPBZ1	Os12g0555000	ATGAAGCTTA ACCCTGCCGC	GTCTCCGTCG AGTGTGACTT G	

OsSGT1	Os09g0518200	GCCAGAAATG CCATGTGTGA AGGT	CGGTCCACTC CAATCTTAGC GATGA	
OsWRKY45	Os05g0322900	CGGGTAAAAC GATCGAAAGA	TTTCGAAAGC GGAAGAACA G	(Shimono *et al.*, 2007)
OsCPS2	Os02g0570900	CGAGGAGCTT ACTGTACGC	TGAGCAGATC TCGATTGTG	(Toyomasu *et al.*, 2008)
OsCPS4	Os04g0178300	GTGTTGTAGC GTTGAAGTCA	CAATCTCAAA TCCAACTAGC A	(Toyomasu *et al.*, 2008)

3 Results

3.1 Pyriculol induced necrotic lesions

3.1.1 Pyriculol induced necrotic lesions under light condition in a dose-dependent manner

Pyriculol was recorded to be the causal agent to induce necrotic lesions when rice was grown under light (Kim *et al.*, 1998; Jacob *et al.*, 2017). The necrotic lesions were quite similar with the symptoms caused by rice blast fungus infection. This might indicate that pyriculol was one of the key virulence factors for the rice blast fungus. In order to be clear about how sensitive pyriculol was in induction of the necrotic symptoms under light condition, purified pyriculol (80% pure) was applied exogenously on rice leaf segments to investigate the resulting symptoms. Pyriculol was applied at seven concentrations (mock control- 5% methanol, 0.16 mM, 0.32 mM, 0.64 mM, 0.8 mM, 1.6 mM and 6.4 mM), and examined after 24 h of light exposure. Anecrotic ring was observed when the concentration of pyriculol was above 0.64 mM, while the methanol control did not induce any necrotic lesions (Fig. 4A). The size of the lesions increased with the concentration of pyriculol applied (Fig. 4B). The percentage of necrotic ring in necrotic lesions peaked around 50% when the concentration of pyriculol was between 0.64 mM and 1.6 mM (Fig. 4C). To sum up, pyriculol could indeed induced necrotic lesions under light condition, when applied at the appropriate concentration.

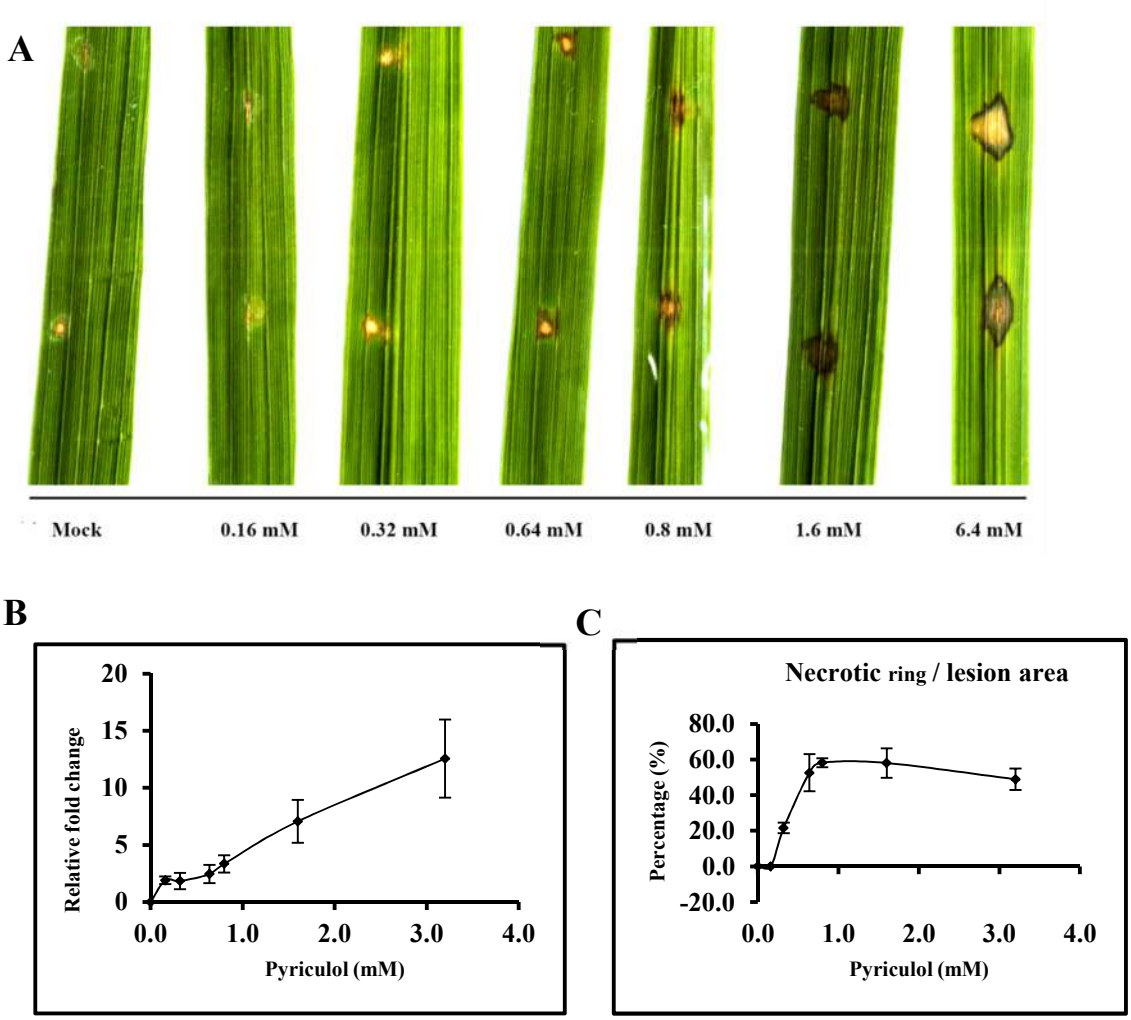


Fig. 4 Pyriculol induced necrotic lesions in a dose-dependent manner under light condition on fifth detached leaf segments of 3 weeks old Nihonmasari seedlings. A Representative symptoms at 1 day post application (1 dpa) under light condition. A series of concentrations of pyriculol at 0.16 mM, 0.32 mM, 0.64 mM, 0.8 mM, 1.6 mM and 6.4 mM was applied on wounded sites of leaf segments. In mock control 5% methanol was used, which was the solvent concentration of 6.4 mM pyriculol. **B** The lesion size increased with increasing concentration of pyriculol. Lesion size was quantified using ImageJ and relative lesion area was plotted using mock as control. Error bars indicated standard error of three replicates. **C** The percentage of necrotic lesion area occupied in each lesion depending on the concentration of pyriculol is depicted. Error bars indicated standard error of three replicates.

3.1.2 Pyriculol-induced necrotic ring could be attenuated by SA and DPI

Necrotic lesions caused by external stimuli is typically due to the excessive accumulation of reactive oxygen species (ROS) which are generated from different subcellular locations (plasma membrane, chloroplast, and mitochondria etc.). Plasma membrane-localised NADPH oxidases (respiratory burst oxidase homologues (RBOHs)) play an important role for ROS generation, which could be effectively inhibited by diphenyleneiodonium (DPI). DPI was also reported to potently inhibit mitochondrial-derived ROS production (Kim *et al.*, 1998). Except the specific ROS inhibitor, there are also some general antioxidants, such as ascorbic acid. In addition, SA had both pro-oxidant and anti-oxidant role in response to stresses in a temporally controlled manner, while JA could also inhibit ROS generation under ozone stress (Herrera-Vasquez *et al.*, 2015; Rao *et al.*, 2000b).

In order to investigate the effect of the above-mentioned different compounds (DPI, ascorbic acid, SA and JA) on formation of necrotic ring induced by pyriculol, combined treatment using pyriculol together with other compounds was done on detached leaf segment. Symptoms were observed 24 h after the application. Firstly, in the control group without pyriculol, 1 mM jasmonic acid and 1 mM salicylic acid could slightly increase the necrotic area to around 5%, while 1 mM ascorbic acid and 50 μM DPI induced approximately 30% of necrotic area (Fig. 5B). Water control and mock treatment (1% methanol as the solvent of pyriculol) did not result in necrosis. Secondly, in the group with pyriculol treatment, pyriuclol alone could result in 90% necrotic area in the lesions and addition of ascorbic acid or jasmonic acid did not affect necrotic symptoms caused by pyriculol (Fig. 5B). However, 1mM salicylic acid and 50 μM DPI could strongly reduce the necrotic area caused by 0.64 mM pyriculol to 40% and 30%, respectively (Fig. 5B).

To summarise, SA and DPI could reduce necrotic area induced by pyriculol under the light condition.

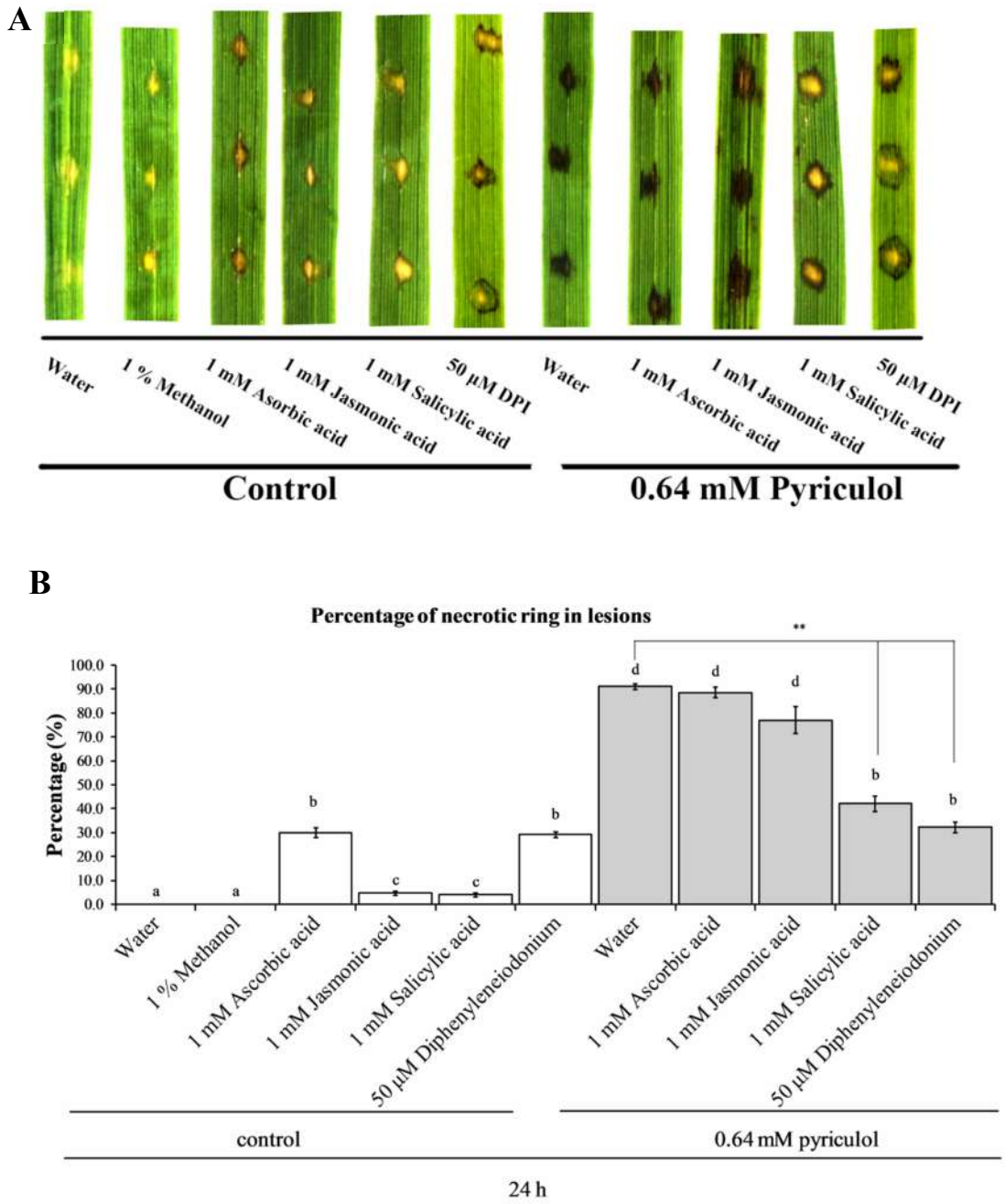


Fig. 5 Pyriculol induced necrotic ring was reduced by Saliylic acid and DPI under light condition on fifth detached leaf segments of 3 weeks old Nihonmasari seedlings. A Representative symptoms induced by different compounds in combination with 0.64 mM pyriculol after 24 h under light condition. **B** Quantification of the percentage of necrotic ring in each lesion

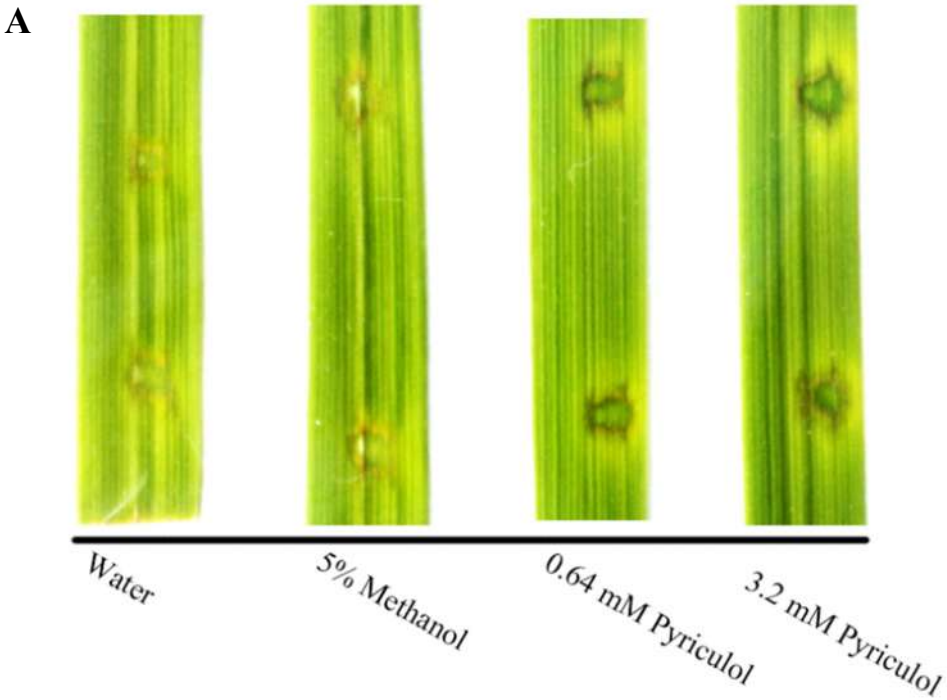
induced by the compounds using ImageJ. Error bars indicate the standard error of three replicates. Different letters above the bars indicate significant differences ($p < 0.05$, Fisher's LSD test) and "**" indicates very significant difference between treatments ($p < 0.01$, Fisher's LSD test).

3.1.3 Pyriculol induced "green island" symptoms in darkness

Even though pyriculol could induce necrotic lesions, it was suggested that the necrosis induced by pyriculol was light-dependent. Specifically, under light condition necrosis, while under darkness "green island" symptoms were induced by pyriculol (Lokeshwari and Suryanarayanan, 1992). "Green island" symptom is used as a term to describe the formation of an area of green tissue at the site (where pyriculol was applied) surrounded by neighboring yellow tissue (Walters *et al.*, 2008). This discrepancy in the symptoms between light and dark condition might indicate that chlorophyll-derived ROS accumulation was involved in generation of necrosis under light condition.

To confirm whether pyriculol-induced necrosis is light dependent, treatment of 3-week old leaf segments with pyriculol was conducted in complete darkness. Firstly, treatment of leaf segments with different concentration (0.64 mM and 3.2 mM) of pyriculol produced slight necrotic ring making up approximately 3% of lesion area at 3 days after application (Fig. 6B). Also, the symptom was similar with the described "green island" symptom with green tissue at the center and senescent tissue at the peripheral area.

Altogether, pyriculol could induce "green island" symptoms and also slight necrotic ring under dark condition in the detached leaf assay.



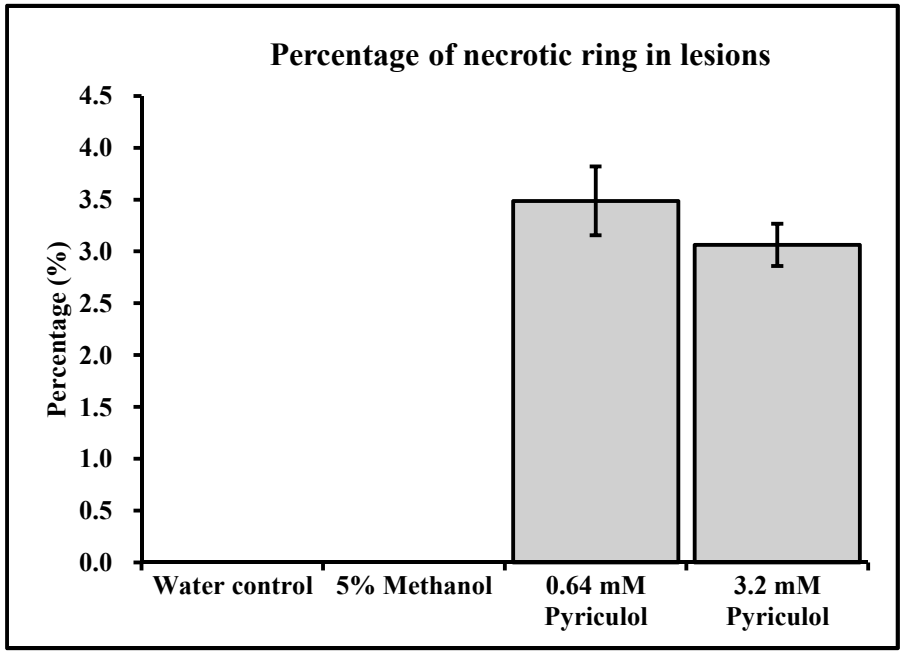


Fig. 6 Pyriculol induced necrotic ring on leaf segment under complete dark condition at 3 days after application on fifth detached leaf segments from 3 weeks old Nihonmasari seedlings. **A** Representative symptoms induced by pyriculol treatment in leaf segments of Nihonmasari. **B** The percentage of necrotic ring was induced by pyriculol. Error bars indicate standard error of three replicates

3.2 Impact of pyriculol and salicylic acid on JA-related gene expression

3.2.1 Pyriculol and salicylic acid inhibited the expression of JA biosynthesis genes

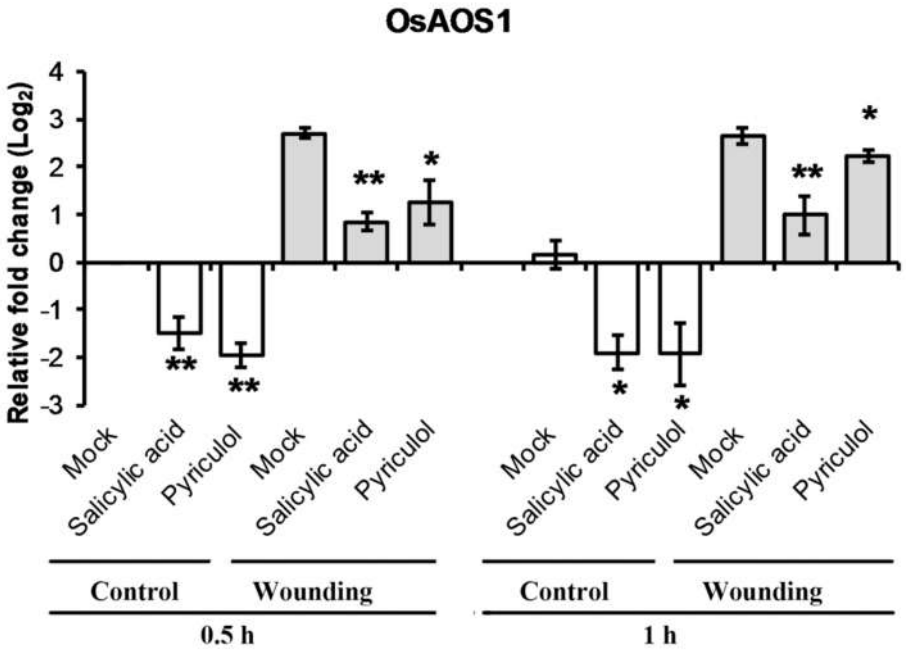
It is generally known that SA could repress JA's biosynthesis and jasmonic acid-dependent genes. Additionally, based on the structural similarity between pyriculol and SA and also a preliminary experiment that showed that pyriculol could inhibit JA biosynthesis and signaling genes (data not shown), a hypothesis that pyriculol and SA may share some similarity in the crosstalk with JA pathway was postulated. In order to test this hypothesis, a comparative analysis using the same concentration of pyriculol and SA (0.64 mM) in combination with wounding treatment was conducted. Wounding treatment was used as an effective way to induce JA biosynthesis and signaling gene expression.

In the results, the JA biosynthesis gene *OsAOS1* was repressed by both pyriculol and SA in the control and wounding treatment at both time points (0.5 h and 1 h). Specifically, it was suppressed by approximately 70% in the control by both pyriculol and SA, while it was down-regulated by 25% - 50% in the wounding treatment (Fig. 7A). As for *OsAOS2*, it was only repressed at 0.5 h by both pyriculol and SA, but not at 1 h. The amplitude of suppression by pyriculol and SA was roughly 65% in the control, whereas its expression was reduced around by 20% in the wounding treatment (Fig. 7B). *OsAOC* was also suppressed by around 50% in the control by pyriculol and SA at 0.5 h and 1 h, while its expression was suppressed by approximately 60% in the case of SA and by about 40% in the case of pyriculol under the wounding treatment at 0.5 h (Fig. 7C). The expression of *OsOPR7* expression was suppressed in both control and wounding treatment by pyriculol and SA at both time points (0.5 h and 1 h). In detail,

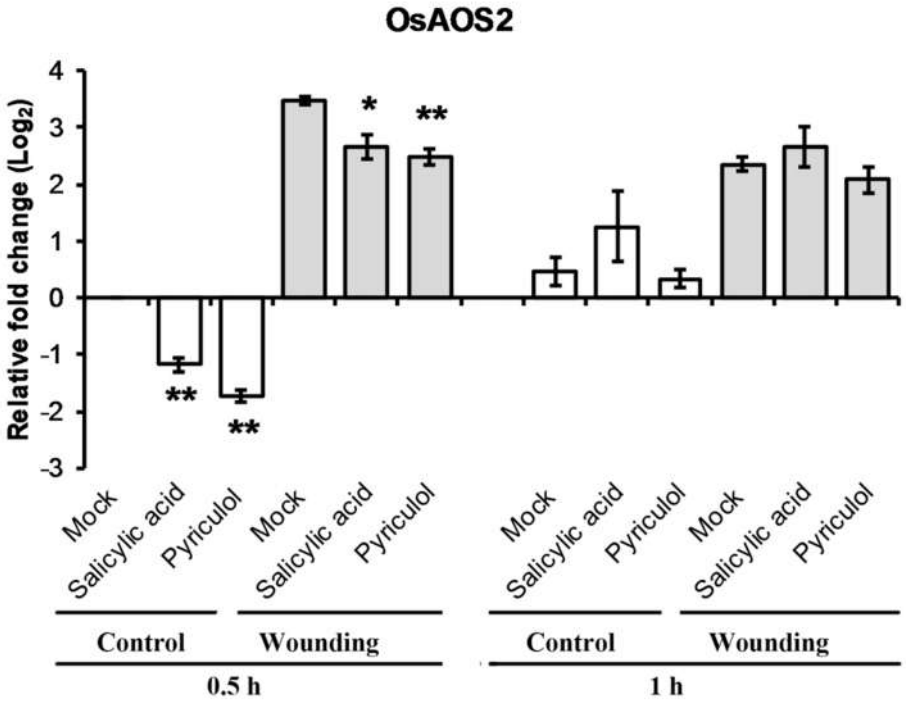
OsOPR7 was repressed by 50% - 75% by SA and pyriculol in the control at 0.5 h and 1 h, while its gene expression was reduced by about 70% at 0.5 h and by 60% at 1 h by SA and pyriculol (Fig. 7D). *OsJAR1* expression was not affected by SA at both time points, but suppressed by approximately 50% by pyriculol at 0.5 h in the control rather than in the wounding treatment (Fig. 7E).

Taken together, results showed that both pyriculol and salicylic acid could suppress jasmonic acid biosynthesis genes in the control and in the wounding treatment, such as *OsAOS1, OsAOS2, OsAOC* and *OsOPR7*. As for *OsJAR1*, its expression could only be inhibited by pyriculol at 0.5 h in the control but not by SA.

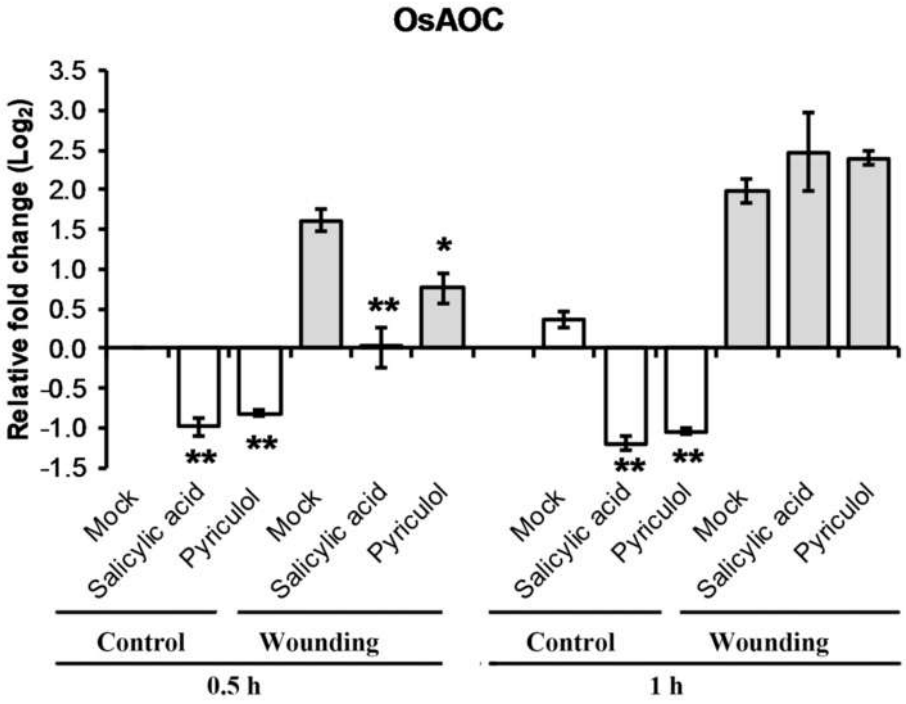
A



B


OsAOS2
Relative fold change (Log2)
4
3
2
1
0
-1
-2
-3
*
**
**
**
Mock
Salicylic acid
Pyriculol
Mock
Salicylic acid
Pyriculol
Mock
Salicylic acid
Pyriculol
Mock
Salicylic acid
Pyriculol
Control
Wounding
Control
Wounding
0.5 h
1 h

C


OsAOC
Relative fold change (Log2)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
**
**
**
*
**
**
Mock
Salicylic acid
Pyriculol
Mock
Salicylic acid
Pyriculol
Mock
Salicylic acid
Pyriculol
Mock
Salicylic acid
Pyriculol
Control
Wounding
Control
Wounding
0.5 h
1 h

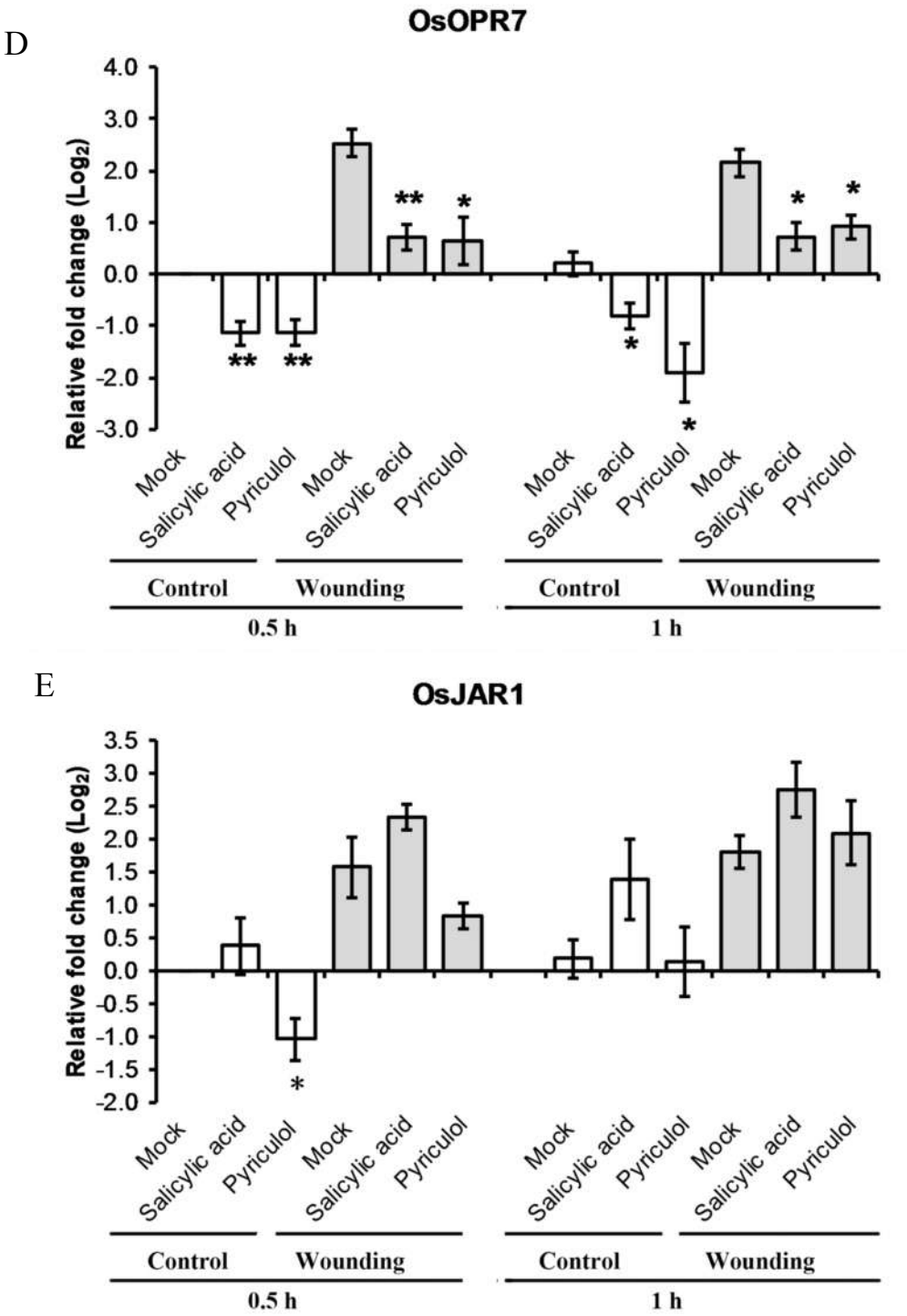


Fig. 7 Wounding induced jasmonic acid biosynthesis genes were repressed by pyriculol and salicylic acid. The concentration of pyriculol and salicylic acid used here was 0.64 mM. Error bars indicated the standard error of three replicates. "*" and "**" denoted significant differences ($p < 0.05$, student's t-test, two tailed) and very significant differences ($p < 0.01$, student's t-test, two tailed) between the chemical treatments and the mock treatment.

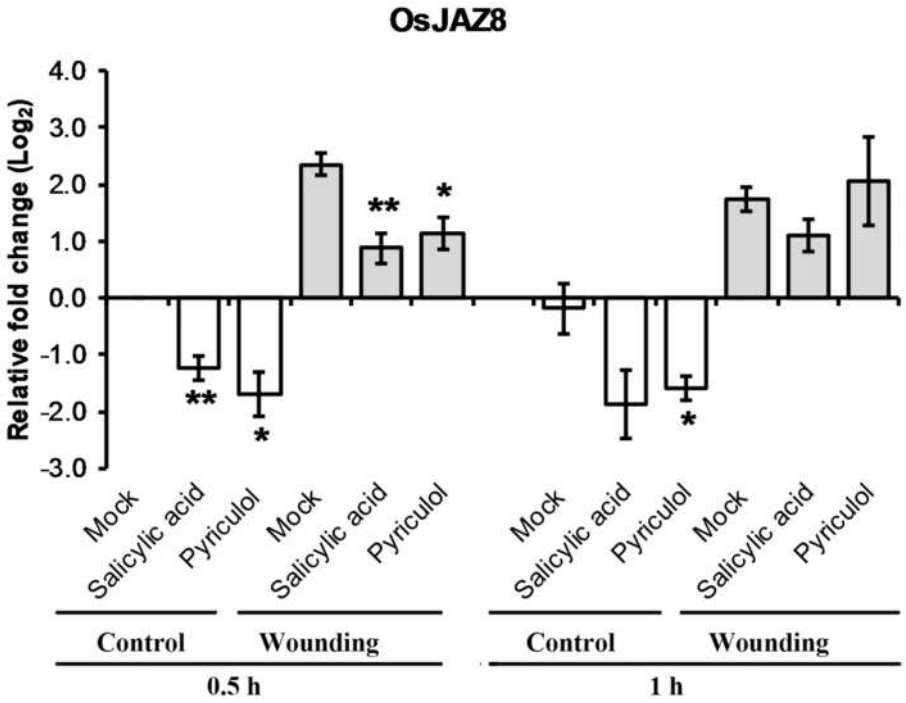
3.2.2 Pyriculol inhibited the expreesion of wounding-induced JA signaling genes

Similarly, signaling genes in the jasmonic acid pathway were also examined. JAZ proteins are repressors of jasmonic acid signaling, but they could be directed for degradation in the presence of jasmonic acid isoleucine. The level of JAZ gene transcripts could be an indicator of the extent to which JAZ proteins are degraded. In the presence of JA, the JAZ proteins are degraded and JAZ transcripts are newly biosynthesized to replenish. Thus, the more JA is present, the more JAZ transcripts are expressed. In the study, four JAZ genes (*OsJAZ8, OsJAZ9, OsJAZ11* and *OsJAZ13*) were selected since they were strongly responsive to wounding treatment in the preliminary test (data not shown).

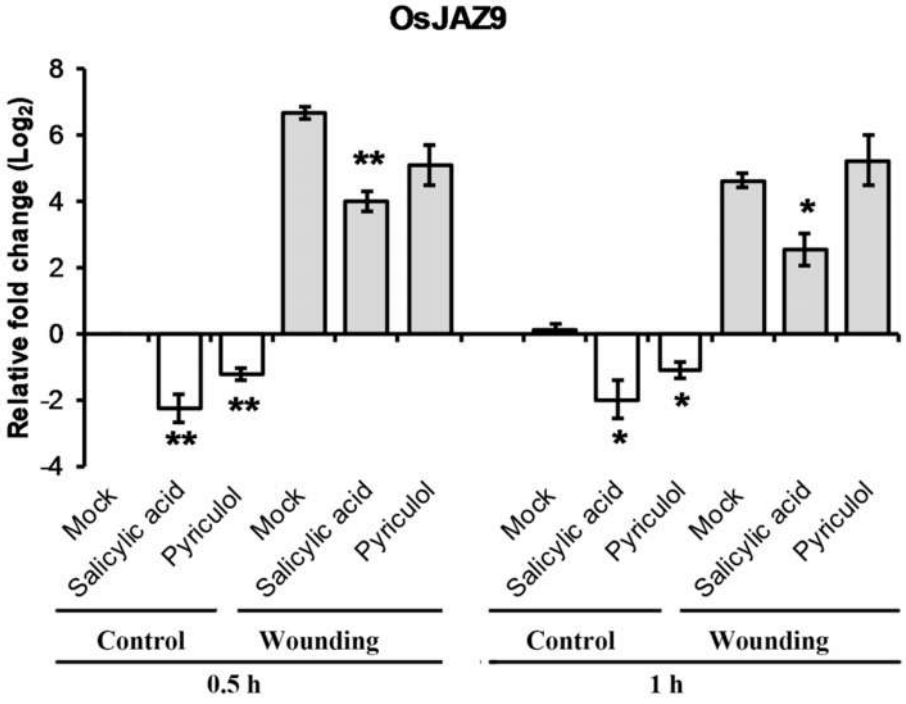
Firstly, at 0.5 h, the expression of *OsJAZ8* was repressed by 55% - 65% by SA and pyriculol in the control, while its expression was reduced by around 60% in the wounding treatment. Furthermore, at 1 h, *OsJAZ8* expression was suppressed by approximately 65% by pyriculol in the control only, but not by SA (Fig. 8A). As for *OsJAZ9* in the control, its expression was repressed by around 80% by SA, while pyriculol could reduce its expression by around 50% at both 0.5 h and 1 h. In the presence of wounding treatment, the gene expression of *OsJAZ9* was inhibited by SA by around 80% at 0.5 h and around 60% at 1 h (Fig. 8B). For *OsJAZ11*, its expression was inhibited by SA and pyriculol in the control (at 0.5 h) by around 30% and 60%, respectively. In the presence of wounding treatment, the expression *OsJAZ11* in mock treatment was repressed by SA and pyriculol at 0.5 h by approximately 60% (Fig. 8C). As for *OsJAZ13*, its expression was repressed by SA and pyriculol by about 50% and 30% in the control, respectively. In the wounding treatment, an inhibition of around 50% by SA was observed, but not by pyriculol (Fig. 8D).

To summarise, the transcript level of some JAZ genes was down-regulated by both pyriculol and SA in the control and wounding treatment, such as *OsJAZ8, OsJAZ9, OsJAZ11* and *OsJAZ13*.

A



B



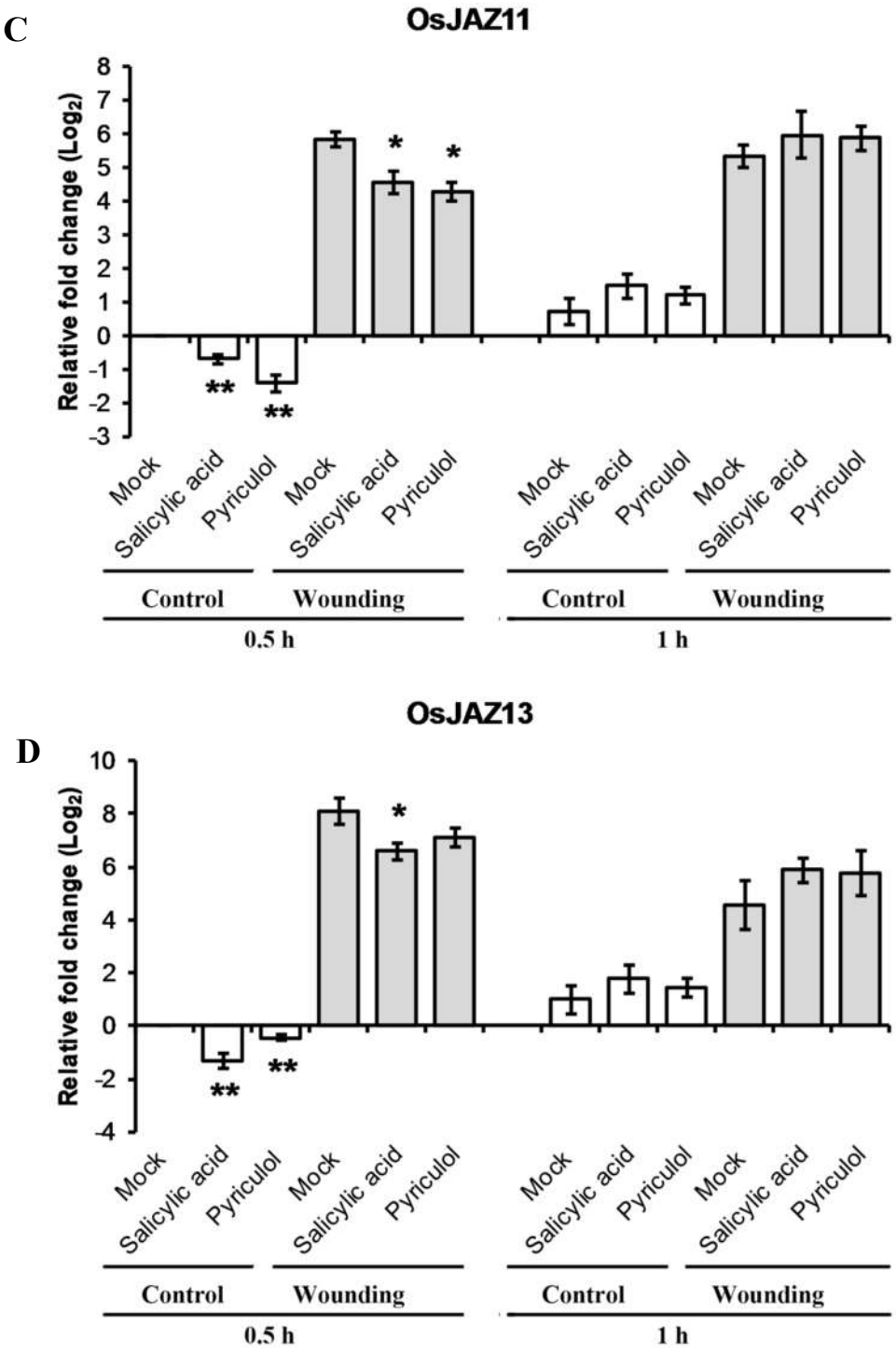


Fig. 8 Wounding induced jasmonic acid biosynthesis genes were repressed by pyriculol and salicylic acid on 2 weeks old rice seedlings (Nihonmasari). The concentration of pyriculol and salicylic acid used here was 0.64 mM**.** Error bars indicated the standard error of three replicates. "*" and "**" denoted significant differences ($p < 0.05$, student's t-test, two tailed) and very significant differences ($p < 0.01$, student's t-test, two tailed) between the chemical treatment and the mock treatment.

3.3 Pyriculol induced the expression of SA responsive genes

Pyriculol and SA shared some similarity in the molecular structure and both of them were demonstrated to have similar inhibitory effect on JA biosynthesis and signaling genes. It would be possible that pyriculol could function in the SA pathway. Thus, in order to check if pyriculol could induce or enhance salicylic acid regulating genes, two genes (*OsWRKY45* and *OsSGT1*) that were responsive to SA treatment were examined here. *OsWRKY45* encodes one of transcription factors with WRKY domain and is activated in the SA pathway in rice, while *OsSGT1* encodes a SA glucosyltransferase which catalyzes SA into SA-O-beta-glucoside (SAG).

Results showed that SA could strongly induce *OsWRKY45* expression by around 45 times at both time points (0.5 h and 1 h) in the control and in the wounding treatment, compared to pyriculol's relatively mild activation of 4 – 8 times (Fig. 9A). This suggested that the induction of *OsWRKY45* expression by pyriculol and SA was independent of wounding treatment, even though wounding treatment only could also induce *OsWRKY45* expression by 8 times at 0.5 h and by 4 times at 1 h, respectively (Fig. 9A). As for the gene *OsSGT1*, its expression was induced by pyriculol, SA and wounding. Specifically, *OsSGT1* could be strongly induced by SA by approximately 45 times (at 0.5 h) and 64 times (at 1 h), while it was also induced by pyriculol by around 16 times (at 0.5 h) and 32 times (at 1 h) (Fig. 9B). In the wounding treatment, SA could induce its expression by approximately 64 times (at 0.5 h) and 128 times (at 1 h), while pyriculol induced the expression by roughly 16 times (at 0.5 h) and 64 times (at 1 h) (Fig. 9B). This indicated that *OsSGT1* expression induced by both SA and pyriculol could be enhanced during wounding.

To sum up, the expression of *OsWRKY45* and *OsSGT1* were induced by salicylic acid and pyriculol and wounding treatment alone. In the case of *OsWRKY45*, its induction

by SA and pyriculol was independent of wounding treatment, while *OsSGT1* expression induced by SA and pyriculol could be enhanced in the wounding treatment.

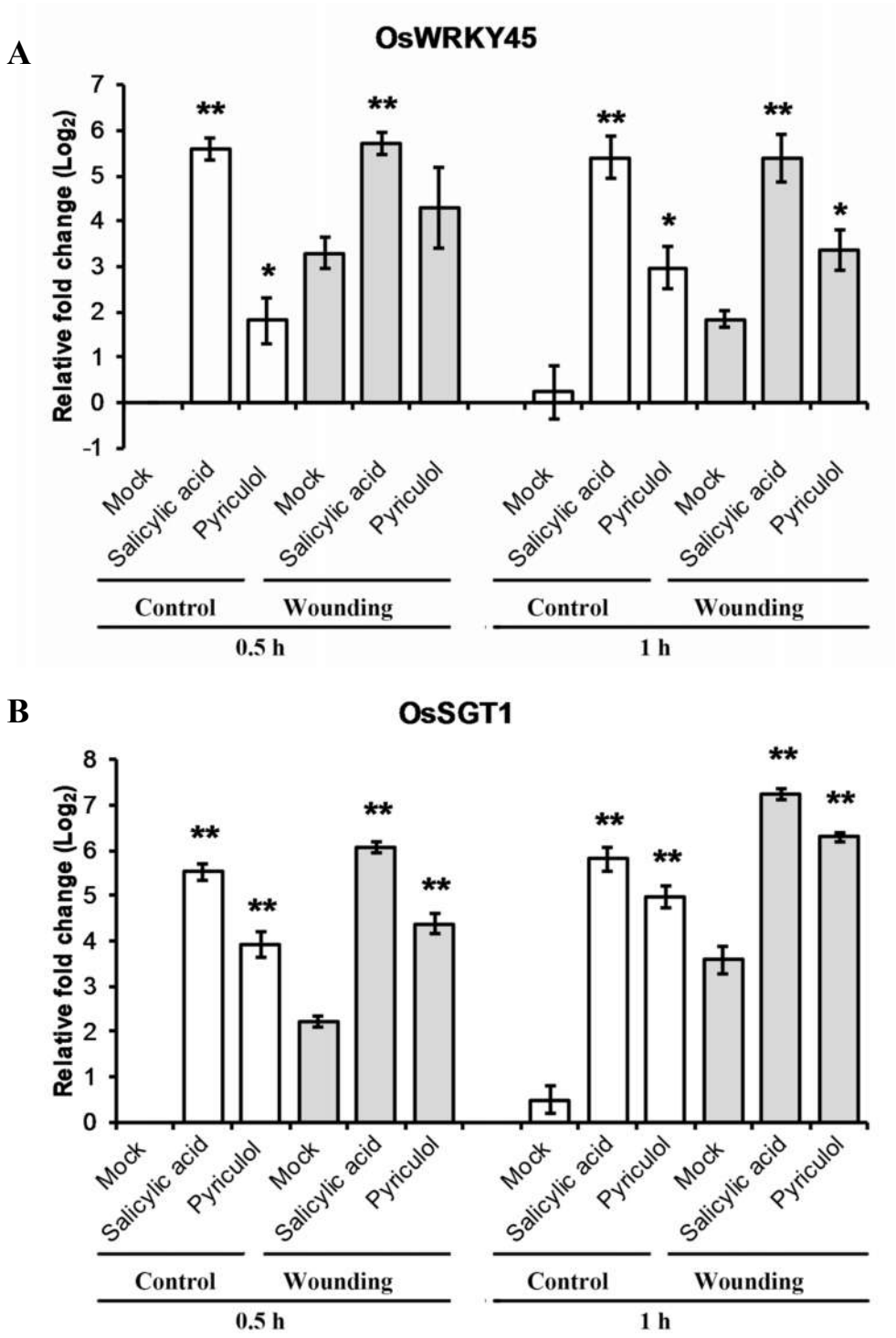


Fig. 9 Pyriculol and salicylic acid could induce or enhance salicylic acid responsive gene expression. The concentration of pyriculol and salicylic acid used here was 0.64 mM**.** Error bars indicated the standard error of three replicates. "*" and "**" denoted significant difference ($p < 0.05$, student's t-test, two tailed) and very significant difference ($p < 0.01$, student's t-test, two tailed)

between the chemical treatment and the mock treatment.

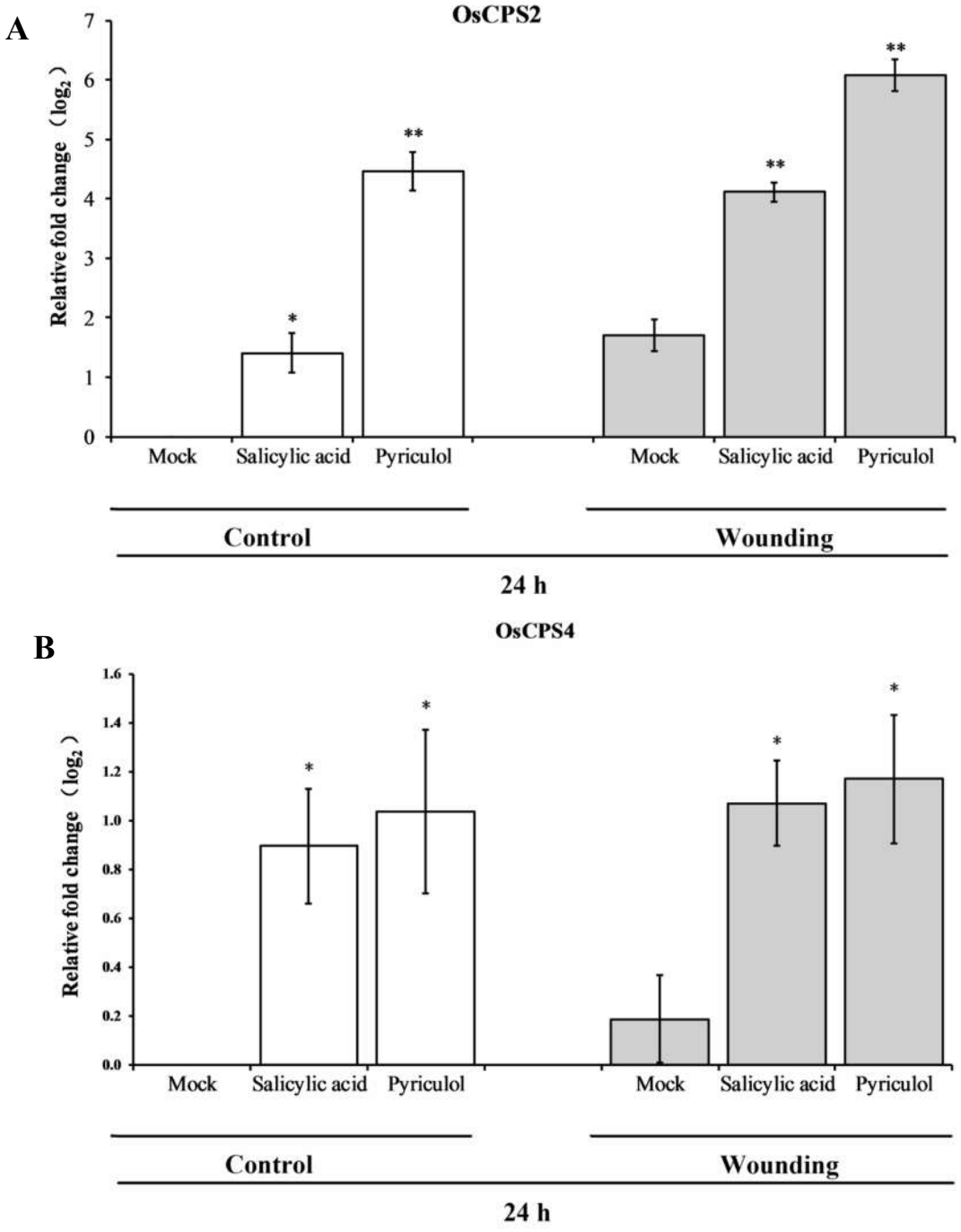
3.4 Pyriculol induced expression of plant defence genes

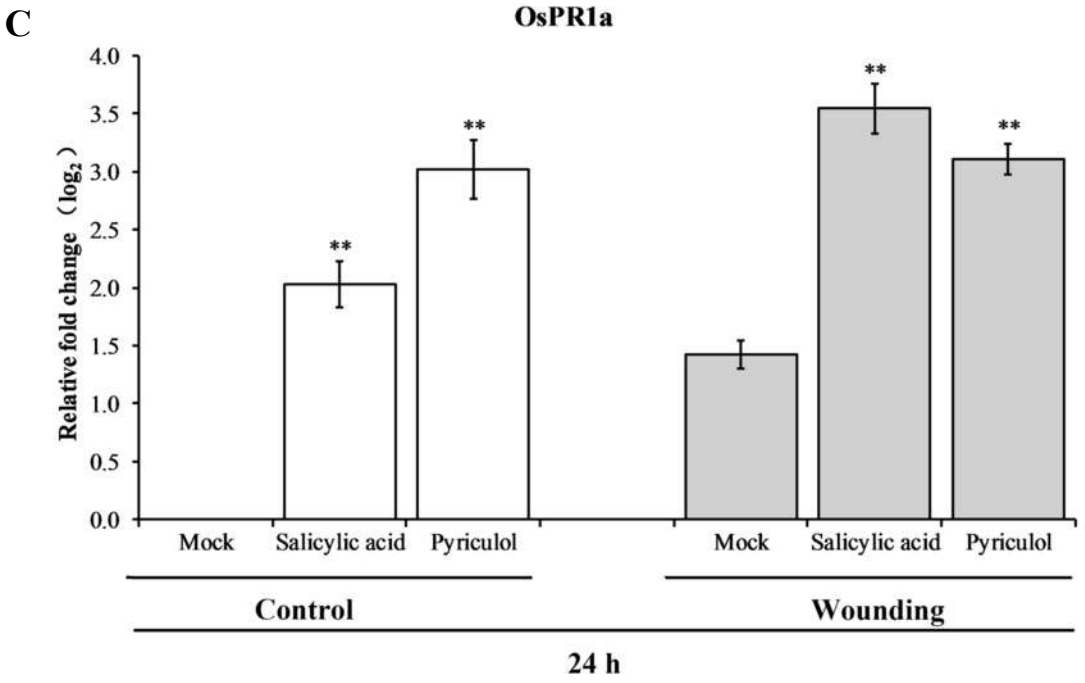
Pyriculol could activate SA signaling genes but repress JA biosynthesis and signaling genes in the control and in the wounding treatment at early time points (0.5 h and 1 h). However, it was not clear how this would affect the output of plant defence genes which were downstream of SA and JA signaling. Hence, in this study, a number of defence genes (i.e. *OsCPS2, OsCPS4, OsPR1a, OsPR1b* and *OsPBZ1*) in rice were selected for the test.

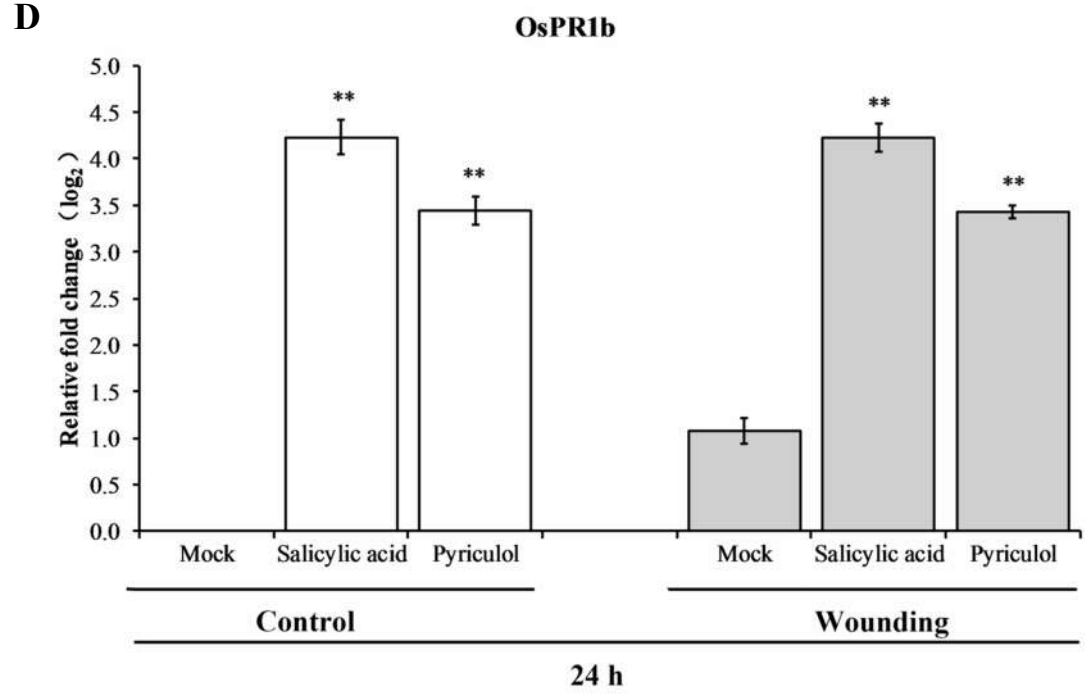
In the results, *OsCPS2* could be induced by approximately 2 times and 22 times by SA and pyriculol, respectively. This could be further enhanced in the presence of wounding to about 16 times and 64 times, respectively (Fig. 10A). As for *OsCPS4*, its expression was slightly stimulated by SA and pyriculol by around 1.8 times and 2 times at 24 h, but it was not affected by wounding treatment (Fig. 10B). *OsPR1a* was induced by about 4 times and 8 times by SA and pyriculol, separately. This induction by SA and pyriculol was enhanced in the wounding treatment to roughly 11 times and 8 times (Fig. 10C). For *OsPR1b*, its expression reached 19 times and 9 times compared to mock treatment by SA and pyriculol, individually. *OsPR1b* expression was not affected in the presence of wounding treatment, even though wounding alone induced about 2 times accumulation (Fig. 10D). *OsPBZ1* expression accumulated by 3 times and 2 times under the treatment of SA and pyriculol, respectively. This accumulation by SA and pyriculol was slightly augmented to 3.5 times and 4 times in the wounding treatment (Fig. 10E).

All in all, both SA and pyriculol could induce expression of genes which are important in plant defence, such as *OsCPS2, OsCPS4, OsPR1a, OsPR1b* and *OsPBZ1*. In some cases, this induction by SA and pyriculol could further be enhanced in the presence of

wounding, such as *OsCPS2* and *OsPBZ1*.




C
OsPR1a
Relative fold change (log2)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
**
Mock
Salicylic acid
Pyriculol
Mock
Salicylic acid
Pyriculol
Control
Wounding
24 h


D
OsPR1b
Relative fold change (log2)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
**
**
Mock
Salicylic acid
Pyriculol
Mock
Salicylic acid
Pyriculol
Control
Wounding
24 h

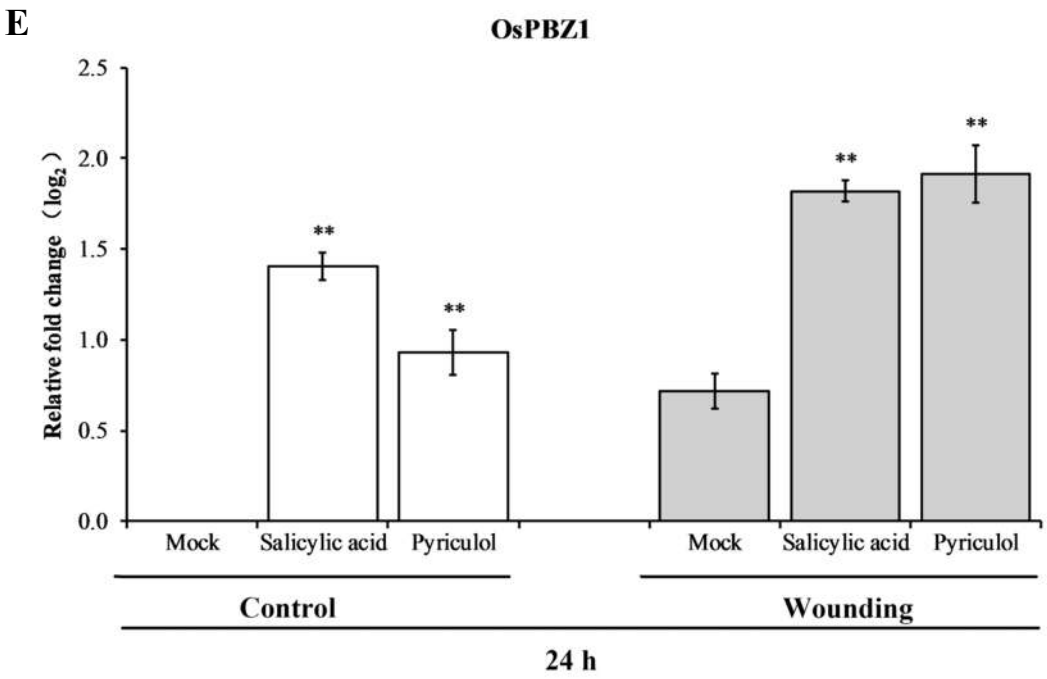


Fig. 10 Pyriculol and salicylic acid could induce and enhance wounding-induced PR gene expression on two weeks old rice seedlings (Nihonmasari). The concentration of pyriculol and salicylic acid used here was 0.64 mM**.** Error bars indicated the standard error of three replicates. "*" and "**" denoted significant difference ($p < 0.05$, student's t-test, two tailed) and very significant difference ($p < 0.01$, student's t-test, two tailed) between the chemical treatment and the mock treatment.

3.5 Exogenous application of pyriculol and pyriculariol in combination with rice blast fungus Gy11 spore inoculation

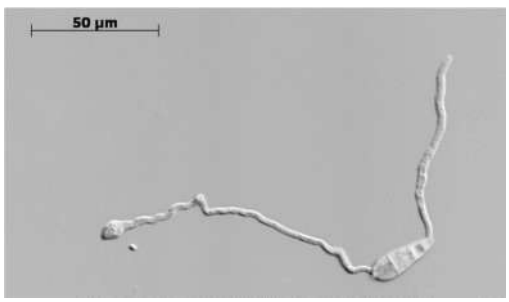
Pyriculol was shown to be able to repress the gene expression in JA biosynthesis and signaling (Fig. 7A-E and Fig. 8A-D), but induce SA responsive gene expression (Fig. 9A-B). Moreover, pyriculol could induce some defence gene expression which could also be activated by SA (Fig. 10A-E). The question was that how exogenous application of pyriculol or its structural isomer pyriulariol would affect the performance of rice in the presence of rice blast fungus infection. In this part, exogenous application of pyriculol and pyriculariol together with rice blast fungus strain Gy11 (virulent strain) spores was done to answer this question.

3.5.1 High concentration of pyriculariol inhibited spore germination

To start with, selection of an appropriate concentration at which pyriculol and pyriculariol did not affect the spore germination was required, since pyriculol and pyriculariol solution and fungal spore suspension were gun-sprayed on rice seedlings at the same time. Thus, a germination rate test was done by incubating different concentrations of pyriculariol or pyriculol with Gy11 spores. Results showed that when the concentration of pyriculariol was below 0.16 mM, spore germination rate was quite comparable with that in the methanol solvent control and that in water, indicating that no inhibition of spore germination was found (Fig. 11C). However, when the concentration increased to 0.32 mM, approximately 50% of spores was inhibited to germinate and when the concentration reached 0.64 mM, spore germination was almost completely inhibited (Fig. 11C).

The germination test was done using only pyriculariol; therefore an additional germination assay using both pyriuclol and pyriculariol was conducted. It showed that when the concentration of pyriculol and pyriculariol was below 0.16 mM, no inhibition of spore germination was found when compared to the germination in methanol (solvent control) (Fig. 11D).

A



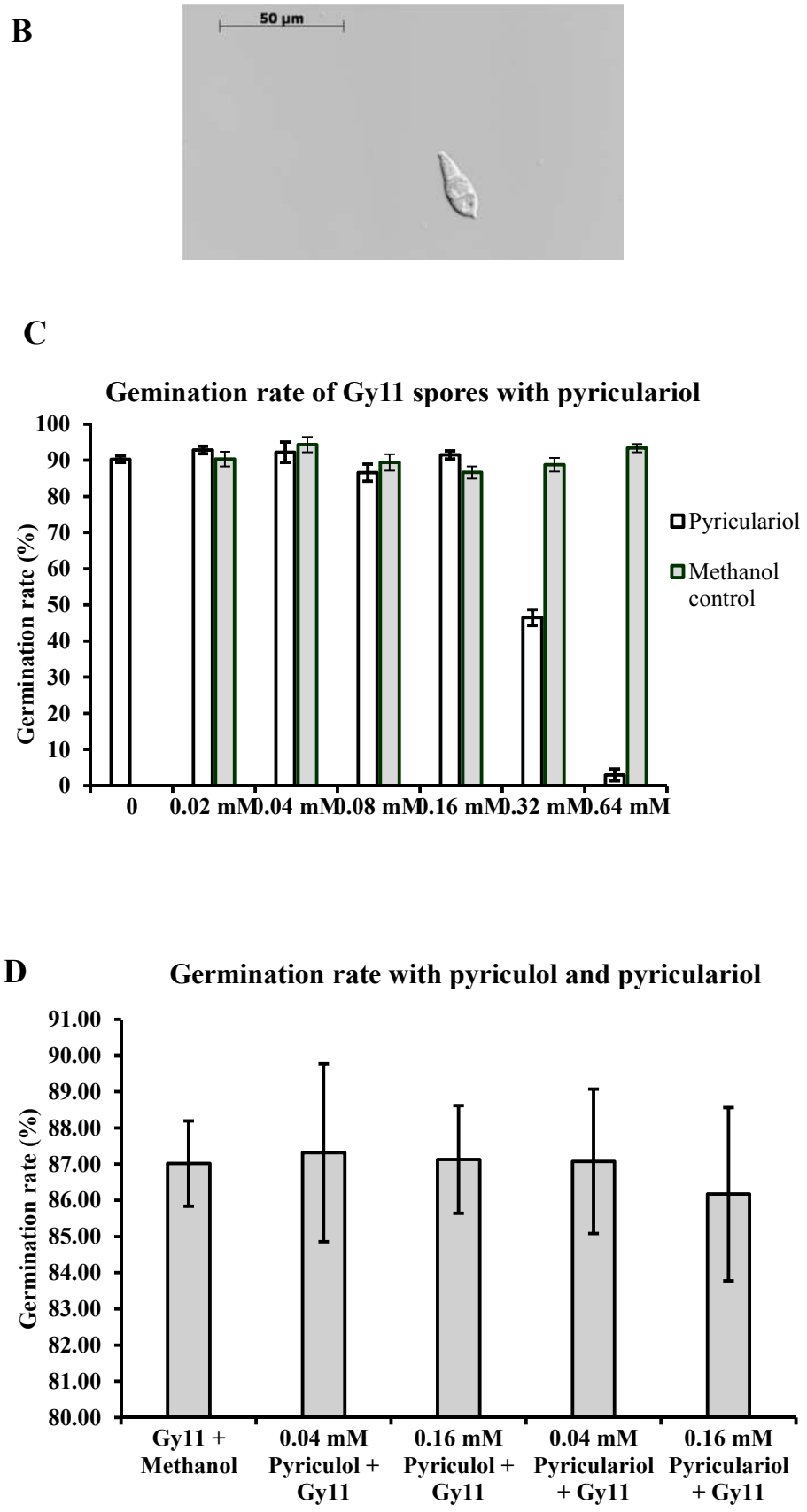


Fig. 11 Germination rate of spores of rice blast fungus strain Gy11 treated with pyriculol and pyriculariol. A Spores of *M. oryzae* before germination, **B** Germinated spores with appresorium. **C**

Germination rate under the treatment of a series of concentrations of pyriculariol. Error bars represented standard error of three replicates. **D** Germination rate under the treatment of relatively low concentration of pyriculol and pyriculariol. Error bars represented standard error of three replicates.

3.5.2 Classification of symptoms

Before quantification of the symptoms, a classification of lesion types was made. In general, necrotic lesions were regarded as resistant lesions, while whitish lesions were susceptible lesions. Specifically, necrotic lesions were due to programmed cell death (hypersensitive response from the host), while whitish lesions were indications for disease-induced cell death. Based on this, the symptoms were classified as follows. “Type 1”: no lesions formed as a result of rice blast fungus infection; “Type 2”: tiny necrotic lesions formed，indicating strong hypersensitive response from the host； “Type 3”: necrotic lesions with relatively bigger lesion size and small whitish area in the center of lesions; “Type 4”: the lesion size was relatively bigger than that in “Type 3”, but with smaller percentage of necrotic ring and bigger part of whitish center area; “Type 5”: the lesion size was relatively bigger than that in “Type 4”; “Type 6”: the lesion size was comparable to that in “Type 5”, but the lesions were completely whitish with no necrotic ring (Fig. 12). Hence, disease severity increases from “Type 1” to “Type 5” lesions.

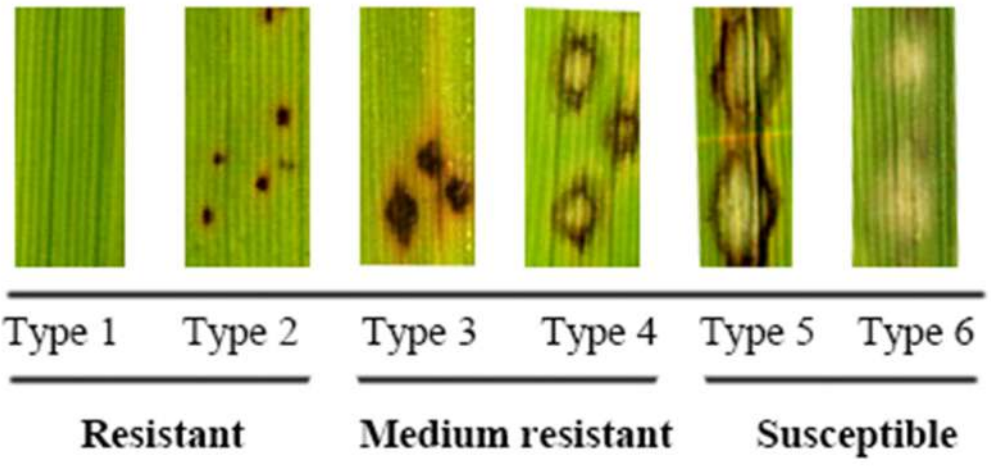


Fig. 12 Symptom classification based on disease severity. Symptoms were produced on three-week old rice plants inoculated with rice blast fungus strain 70-15 at 6 days post inoculation.

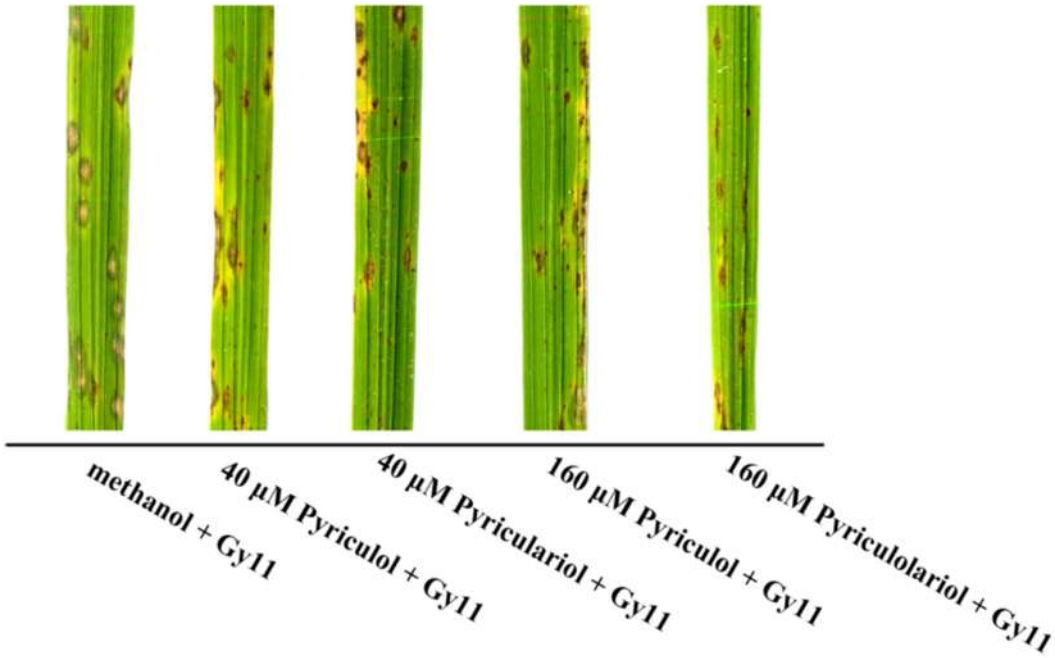
3.5.3 Exogenous application of pyriculol and pyriculariol enhanced rice resistance in the presence of rice blast fungus infection

To find out that exogenous application of pyriculol or pyriculariol enhanced plant defences or susceptibility in the presence of rice blast fungus inoculation, infection assay using Gy11 (virulent strain) spore suspension in combination with pyriculol and pyriculariol was conducted.

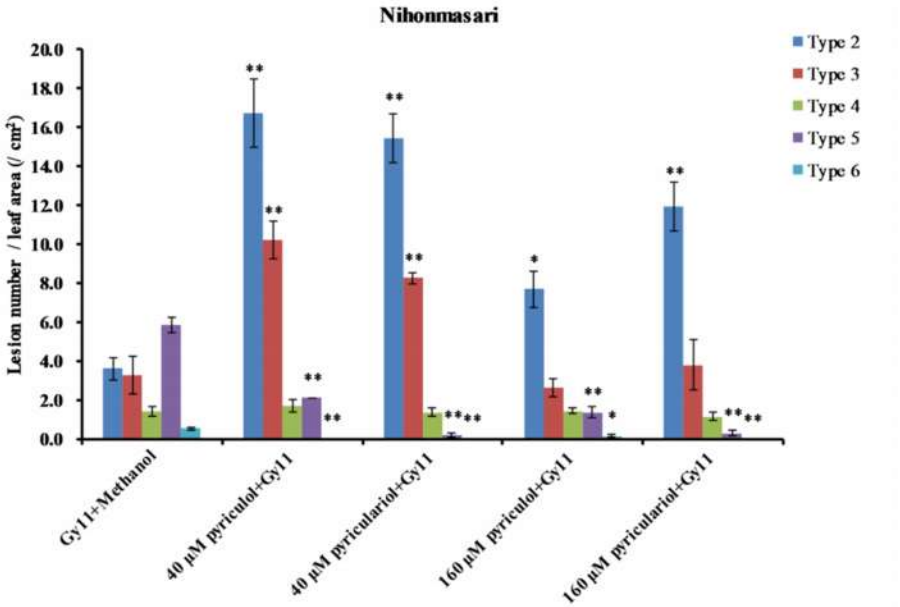
In terms of the lesion number per leaf area, pyriculol and pyriculariol (at concentrations of 40 μM and 160 μM) treated rice plants in combination with Gy11 infection showed fewer “Type 5” and “Type 6” lesions compared to that treated with solvent control in combination with Gy11 infection, but more “Type 2” lesions (Fig. 13B). Likewise, in terms of lesion area per leaf area, pyriculol and pyriculariol treatment at both concentrations (40 μM and 160 μM) could significantly reduce the “Type 5” and “Type 6” lesion area compared to methanol (solvent control) treated plants, but could increase the area of “Type 2” lesions (Fig. 13C).

Taken together, both pyriculol and pyriculariol treatment could significantly increase resistance to rice blast fungus infection.

A


methanol + Gy11
40 µM Pyriculol + Gy11
40 µM Pyriculariol + Gy11
160 µM Pyriculol + Gy11
160 µM Pyriculolariol + Gy11

B


Nihonmasari
Lesion number / leaf area (/ cm²)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Type 2
Type 3
Type 4
Type 5
Type 6
Gy11+Methanol
40 µM pyriculol+Gy11
40 µM pyriculariol+Gy11
160 µM pyriculol+Gy11
160 µM pyriculariol+Gy11

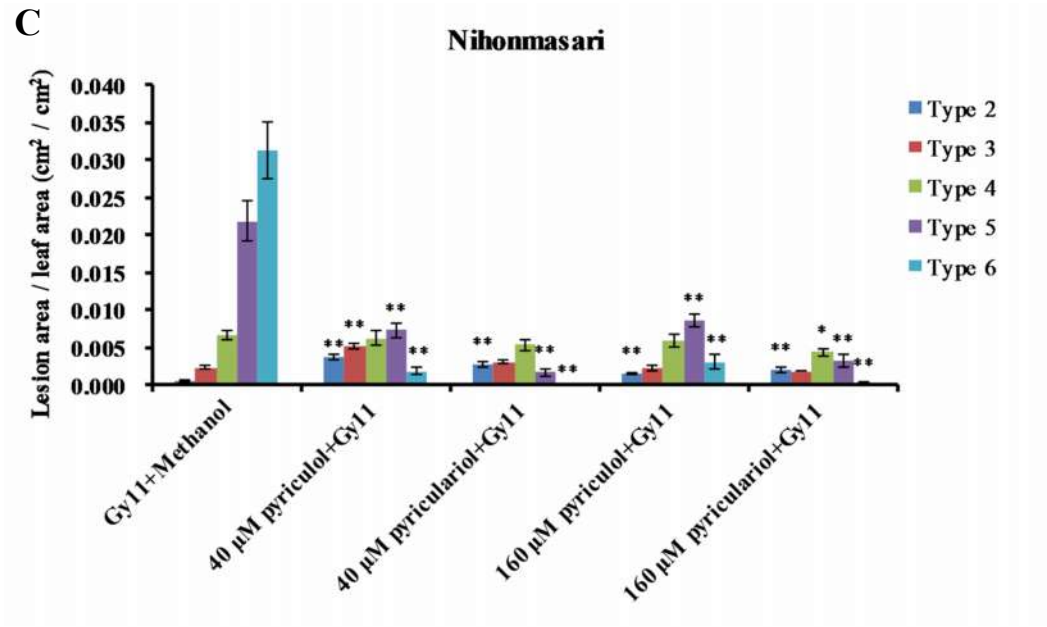


Fig. 13 Representative symptoms and quantification of symptoms of rice blast fungus strain Gy11 spore inoculation in combination of pyriculol and pyriculariol. In **A,** the symptoms were evaluated at 6 days after inoculation. In **B** and **C**, error bars represented standard error of three replicates. "*" and "**" denoted significant difference ($p < 0.05$, student's t-test, two tailed) and very significant difference ($p < 0.01$, student's t-test, two tailed) when compared to Gy11 inoculation.

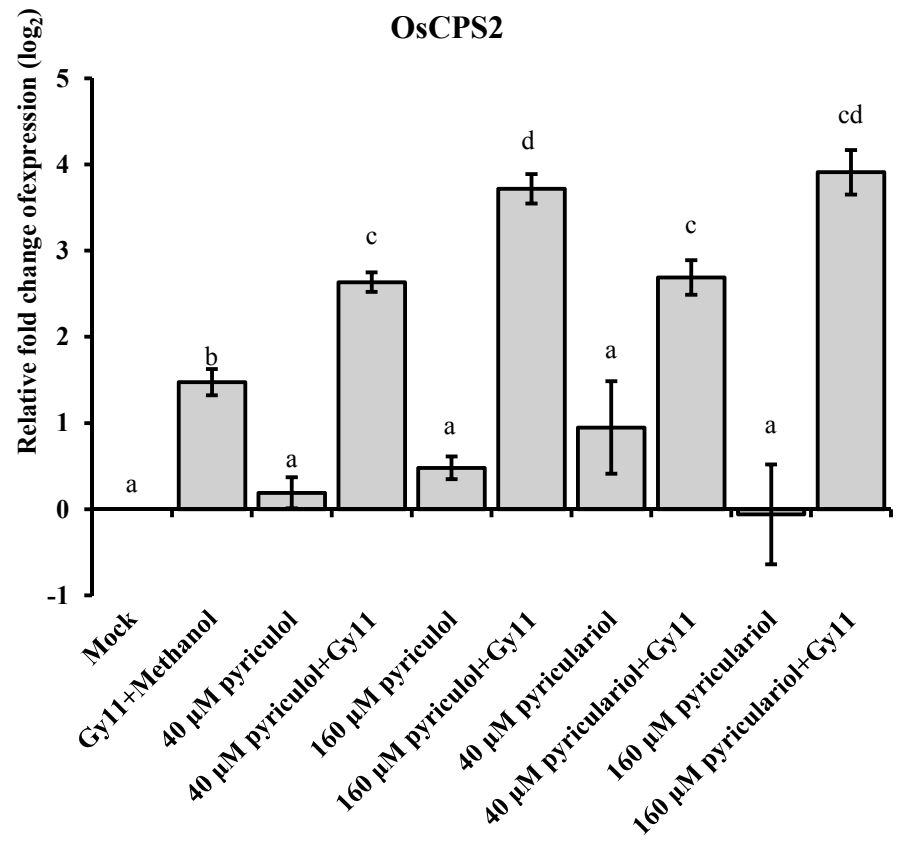
3.5.4 Pyriculol and pyriculariol application enhanced plant defence gene expression under infection of rice blast fungus

After the examination of the symptoms, the expression analysis for some defence-related genes was also conducted. Consistent with the symptom quantification, the expression of defence genes such as *OsCPS2, OsCPS4, OsPR1a, OsPR1b* and *OsPBZ1*, in the presence of rice blast fungus inoculation, was enhanced by pyriculol or pyriculariol treatment after 2 days of inoculation. Specifically, *OsCPS2* was induced by around 3 times by Gy11 infection at 2 days post inoculation (2 dpi). This induction was further enhanced to around 12 times in the presence of pyriculol and pyriculariol at both concentrations (40 μM and 160 μM) (Fig. 14A). As for *OsCPS4*, it was induced by pyriculol and pyriculariol alone at the higher concentration of 160 μM and also induced

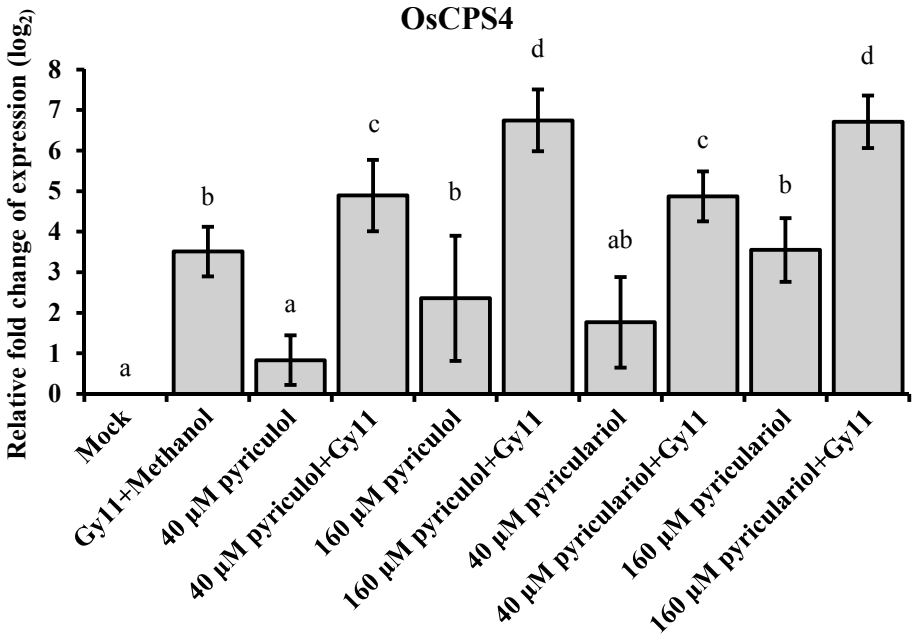
by Gy11 infection. This induction of *OsCPS4* by Gy11 was enhanced by pyriculol and pyriculariol to approximately 30 times (at concentration of 40 μM) and 100 times (at concentration of 160 μM) (Fig. 14B). *OsPR1a* was strongly induced by approximately 48 times by Gy11 infection and also induced by 160 μM pyriculol, 40 μM pyriculariol and 160 μM pyriculariol by about 3 times, 2 times and 8 times, respectively. *OsPR1a* accumulation induced by Gy11 infection was further elevated by 160 μM pyriculariol to roughly 128 times (Fig. 14C). *OsPR1b* was significantly activated by about 32 times by Gy11 infection and by 160 μM pyriculariol. This accumulation by Gy11 infection was enhanced by both concentrations of pyriculol and pyriculariol to around 64 – 128 times (Fig. 14D). Similarly, *OsPBZ1* expression was triggered by Gy11 infection by around 8 times and also induced by 160 μM pyriculol and 160 μM pyriculariol by about 3 times. This expression induced by Gy11 infection could be augmented by pyriculol and pyriculariol (40 μM and 160 μM) to about 16 – 32 times (Fig. 14E).

All in all, all defence genes (*OsCPS2, OsCPS4, OsPR1a, OsPR1b* and *OsPBZ1*) tested were activated by Gy11 infection and this could further be enhanced in the presence of pyriculol and pyriculariol treatment. In addition, some defence genes were induced by pyriculol or pyriculariol treatment alone, such as *OsCPS4, OsPR1a, OsPR1b* and *OsPBZ1*.

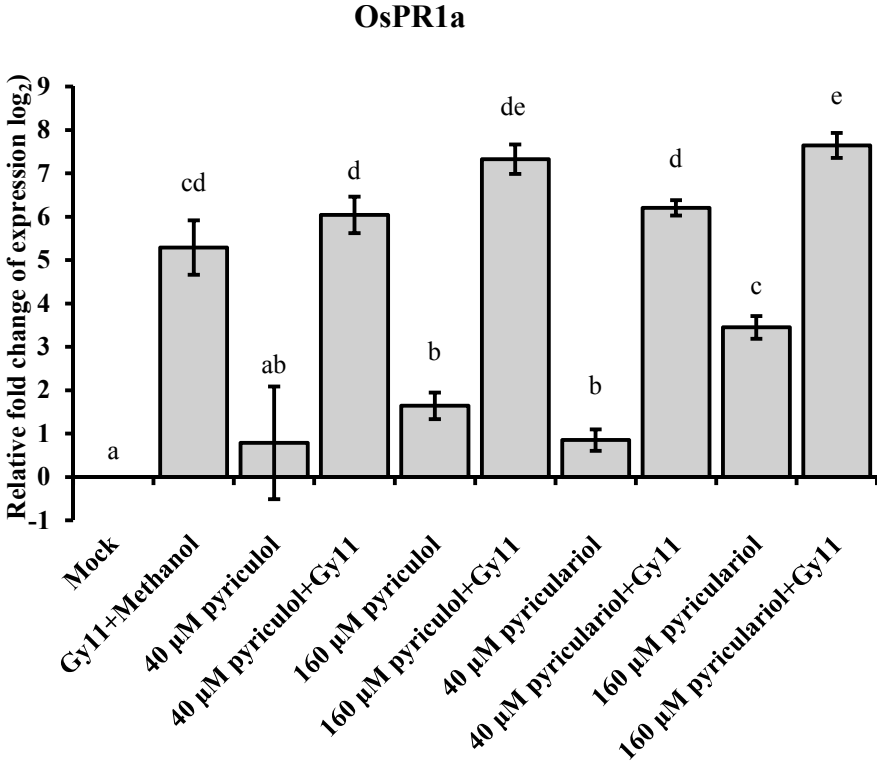
A


OsCPS2
Relative fold change ofexpression (log2)
5
4
3
2
1
0
-1
a
b
a
c
a
d
a
c
a
cd
Mock
Gy11+Methanol
40 µM pyriculol
40 µM pyriculol+Gy11
160 µM pyriculol
160 µM pyriculol+Gy11
40 µM pyriculariol
40 µM pyriculariol+Gy11
160 µM pyriculariol
160 µM pyriculariol+Gy11

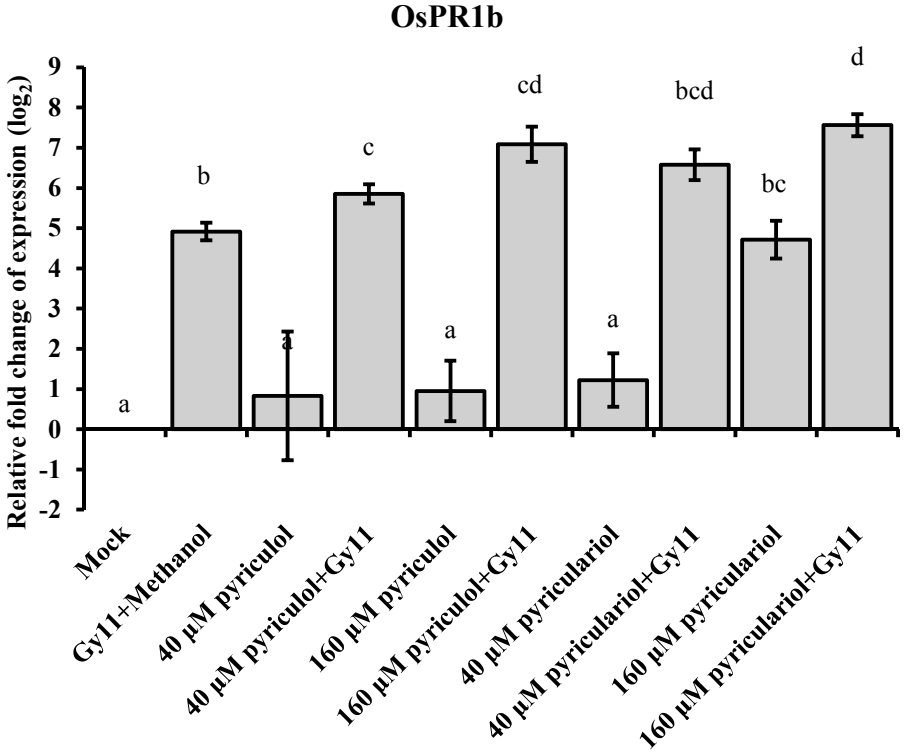
B


OsCPS4
Relative fold change of expression (log2)
8
7
6
5
4
3
2
1
0
a
b
a
c
b
d
ab
c
b
d
Mock
Gy11+Methanol
40 µM pyriculol
40 µM pyriculol+Gy11
160 µM pyriculol
160 µM pyriculol+Gy11
40 µM pyriculariol
40 µM pyriculariol+Gy11
160 µM pyriculariol
160 µM pyriculariol+Gy11

C


OsPR1a
Relative fold change of expression log2
9
8
7
6
5
4
3
2
1
0
-1
a
cd
ab
d
b
de
b
d
c
e
Mock
Gy11+Methanol
40 µM pyriculol
40 µM pyriculol+Gy11
160 µM pyriculol
160 µM pyriculol+Gy11
40 µM pyriculariol
40 µM pyriculariol+Gy11
160 µM pyriculariol
160 µM pyriculariol+Gy11

D


OsPR1b
Relative fold change of expression (log2)
9
8
7
6
5
4
3
2
1
0
-1
-2
a
b
a
c
a
cd
a
bcd
bc
d
Mock
Gy11+Methanol
40 µM pyriculol
40 µM pyriculol+Gy11
160 µM pyriculol
160 µM pyriculol+Gy11
40 µM pyriculariol
40 µM pyriculariol+Gy11
160 µM pyriculariol
160 µM pyriculariol+Gy11

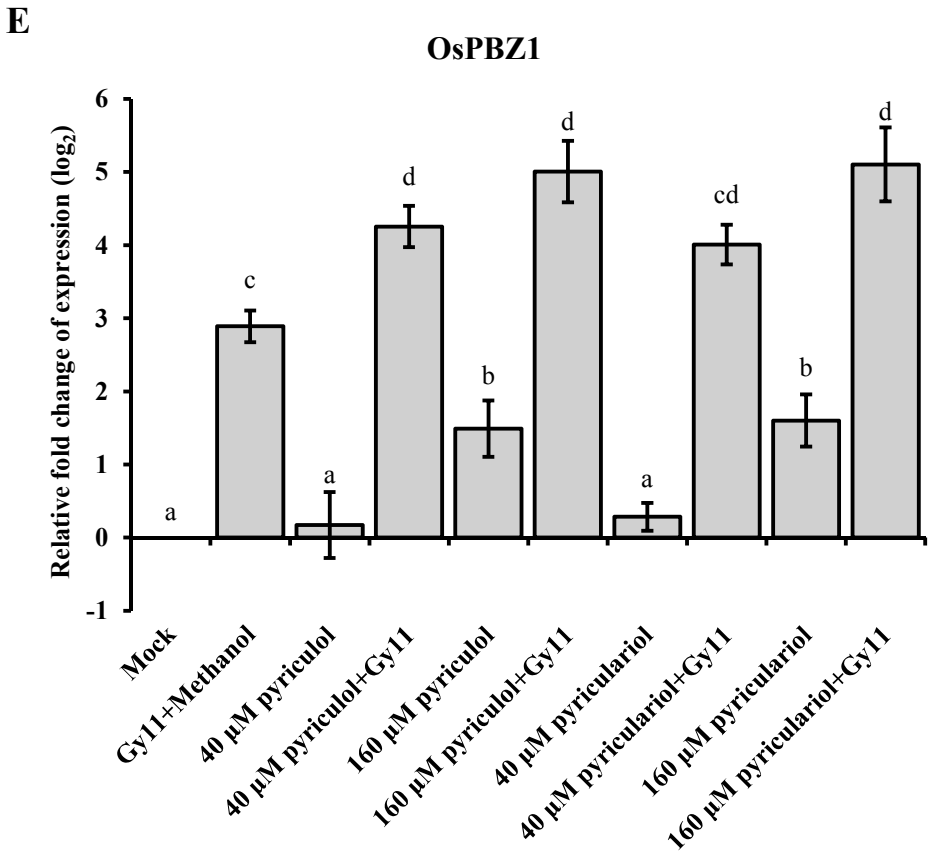


Fig. 14 Pyriculol and pyriculariol could enhance rice blast fungus induced defence gene expression. Error bars represented standard error of three replicates. Different letters in the graphs indicated significant difference between treatments ($p < 0.05$, Fisher's LSD test).

3.6 Pyriculol-related transgenic strain infection assay *in planta*

3.6.1 Initial screen using different rice varieties

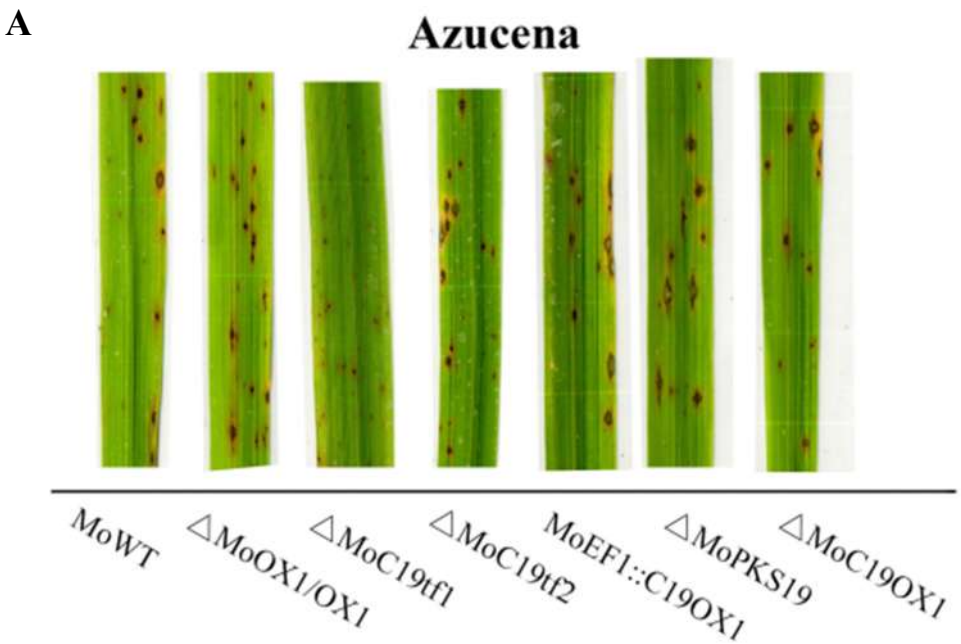
Pyriculol could repress JA biosynthesis and signaling genes, but could up-regulate SA responsive genes. In addition, JA has been reported to play important roles in rice blast fungus defence. The hypothesis that pyriculol was a secreted effector to manipulate SA signaling to repress the host's JA responses was formulated. To test this, 7 fungal strains which were genetically modified (in genes that were required for pyriculol biosynthesis and regulation) were utilized to infect 8 rice varieties. Among these eight rice varieties, four varieties belong to the subspecies Japonica (Acuzena, Nipponbare, Sariceltik and Maratelli) and the other four are in the subspecies Indica (Co39, IR64, Bala and

Kasalath). The idea of using 8 varieties was based on the following observation: Indica subspecies were reported to have 2 times higher level of SA constitutively compared to Japonica subspecies (Vergne *et al.*, 2010); therefore Indica subspecies would be less sensitive to pyriculol (might function as SA mimicry) manipulation in the infection assay. For the 7 transgenic strains used in this infection assay, they were described (in Table 2) in the material and method part.

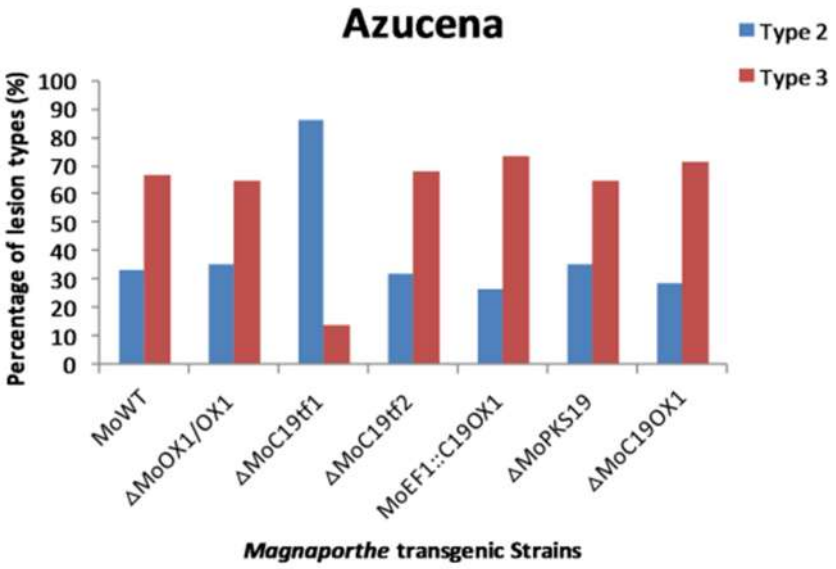
Generally speaking, there was no significant difference in terms of the dominant lesion types produced between the wild type strain MoWT, complementation strain MoC19OX1/OX1, deletion strains ΔMoPKS19 and ΔMoC19OX1, and over-expressor MoEF1::C19OX1 across the eight rice varieties. Specifically, Azucena had dominant lesion type of "Type 3" after infection by all strains except the strain ΔMoC19tf1 which resulted in the dominant lesion type of "Type 2" (Fig. 15B); In Nipponbare (Japonica), the dominant lesion type was "Type 4" lesion infected by all strains except the strain ΔMoC19tf1 that produced "Type 3" lesion as the dominant lesion (Fig. 15D), while in Sariceltik (Japonica) and Maratelli (Japonica), the dominant lesion type was "Type 6" after infection by all the strains except ΔMoC19tf1 (with "Type 4" as dominant lesion type) and ΔMoC19tf2 (with "Type 5" as dominant lesion type) (Fig. 15F and 15H).Comparatively, in Co39 (Indica), there were dominant lesion type of "Type 4" as a result of infection by all strains except the strain ΔMoC19tf1 which produced "Type 2" lesion as the dominant lesion (Fig. 15J). In IR64 (Indica) and Bala (Indica), there were no lesion formed as the result of infection by all strains (Fig. 15L and 15N), indicating the two varietes were the most resistant of all varieties tested. In Kasalath (Indica), the dominant lesion type was "Type 3" by infection of all strains except the strain ΔMoC19tf1 that resulted in "Type 2" as the dominant lesion type (Fig. 15P).

To sum up, pyriculol was not required in the infection process, since n the wild type strain MoWT, complementation strain MoC19OX1/OX1, deletion strains ΔMoPKS19

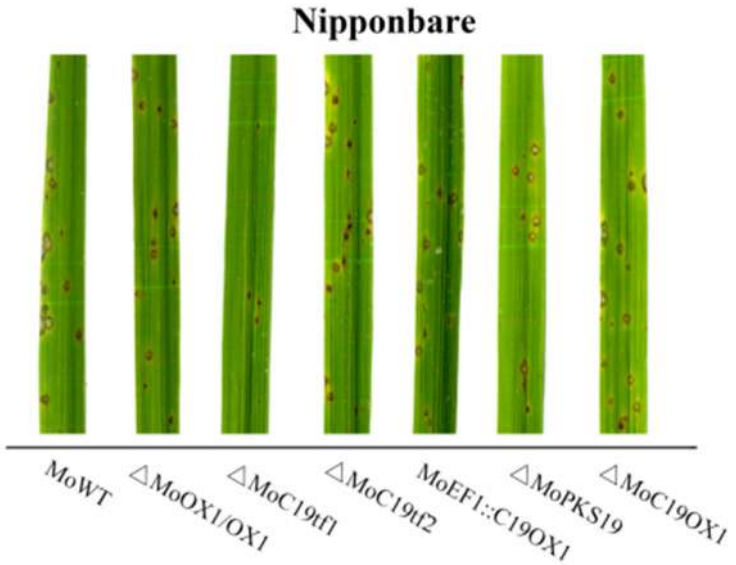
and ΔMoC19OX1, and over-expressor MoEF1::C19OX1 did not show significant difference in induction of lesions on all the eight rice varieties. However, two additional deletion strains ΔMoC19tf1 and ΔMoC19tf2 which were deleted in two transcription factors which are negative regulators of the key biosynthesis enzyme MoPKS19, displayed reduced susceptibility in some rice varieties when compared to the wild type control. Specifically, ΔMoC19tf1 induced lower score of lesion type in all rice varieties except IR64 and Bala on which no symptoms were found under the infection of all strains. Also, ΔMoC19tf2 induced lower score of lesion type only on two very susceptible rice varieties Maratelli and Sariceltik.



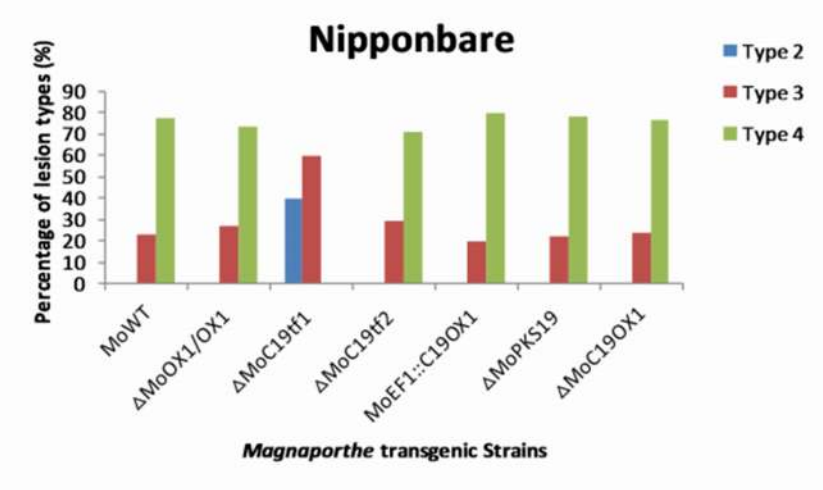
B


Azucena
Type 2
Type 3
Percentage of lesion types (%)
100
90
80
70
60
50
40
30
20
10
0
MoWT
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1
Magnaporthe transgenic Strains

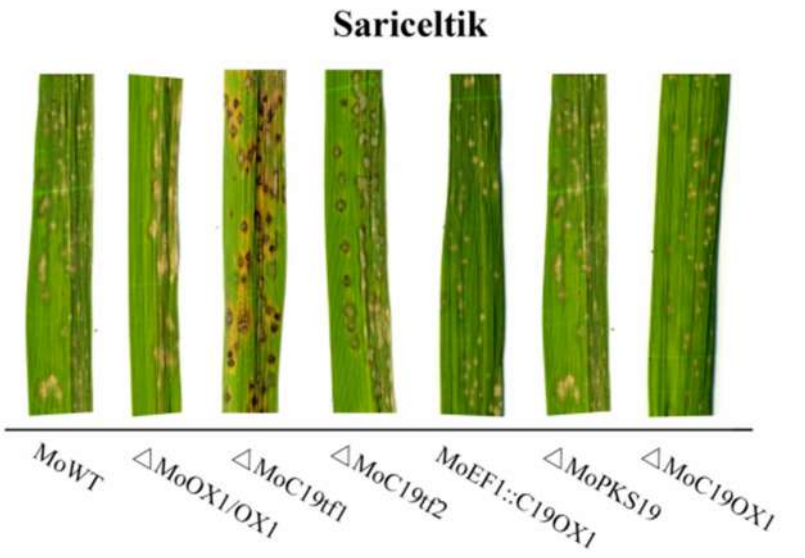
C


Nipponbare
MoWT
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1

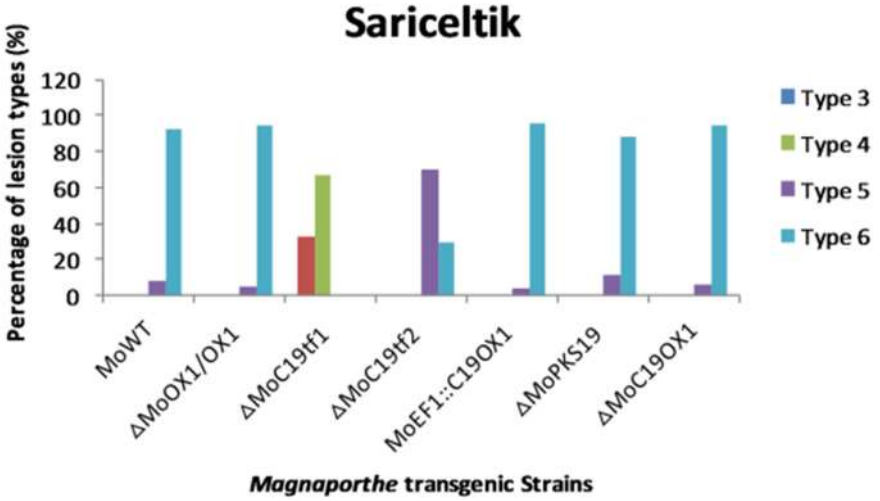
D


Nipponbare
Type 2
Type 3
Type 4
Percentage of lesion types (%)
90
80
70
60
50
40
30
20
10
0
MoWT
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1
Magnaporthe transgenic Strains

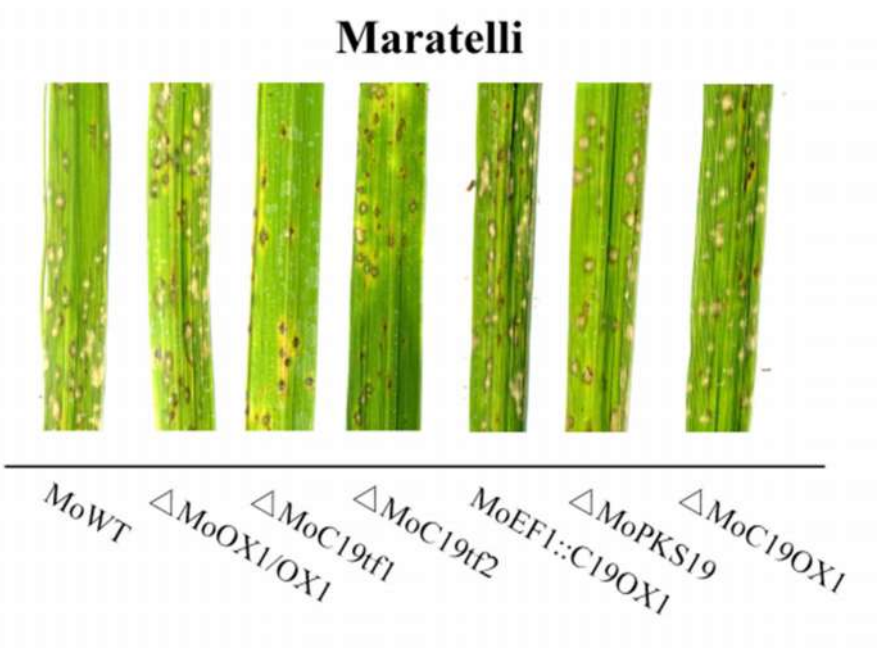
E


Sariceltik
MoWT
△MoOX1/OX1
△MoC19tf1
△MoC19tf2
MoEF1::C19OX1
△MoPKS19
△MoC19OX1

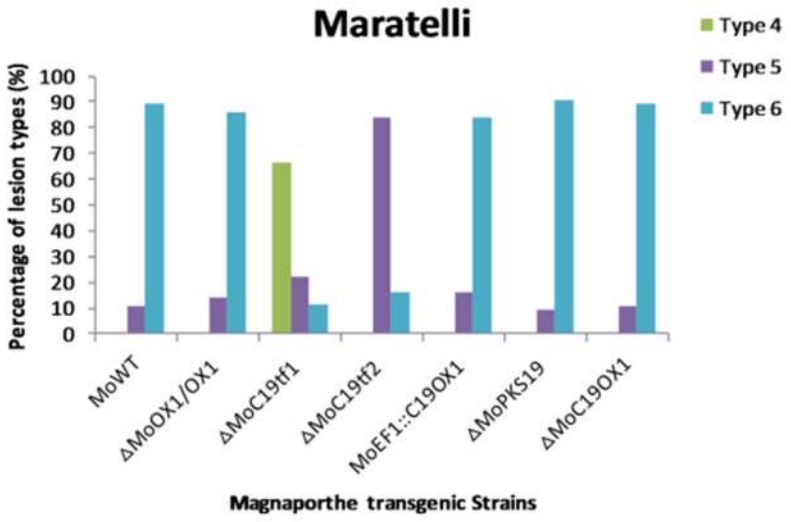
F


Sariceltik
Percentage of lesion types (%)
120
100
80
60
40
20
0
Type 3
Type 4
Type 5
Type 6
MoWT
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1
Magnaporthe transgenic Strains

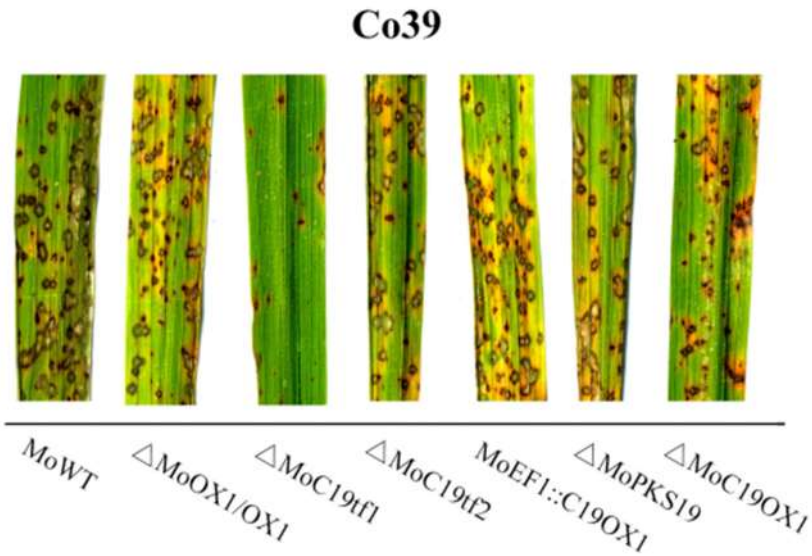
G


Maratelli
MoWT
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1

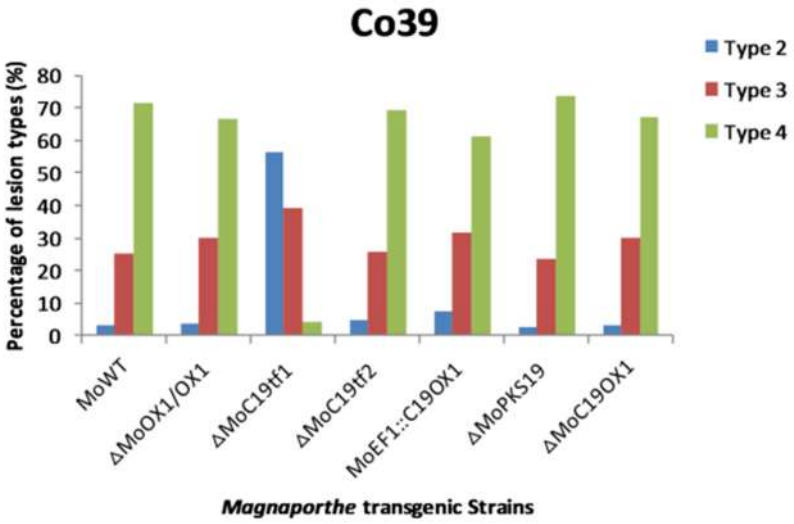
H


Maratelli
Type 4
Type 5
Type 6
Percentage of lesion types (%)
100
90
80
70
60
50
40
30
20
10
0
MoWT
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1
Magnaporthe transgenic Strains

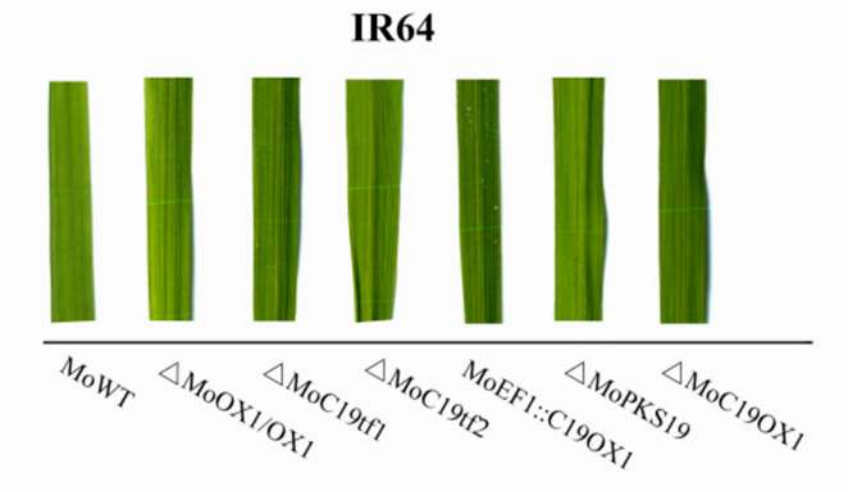
I


Co39
MoWT
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1

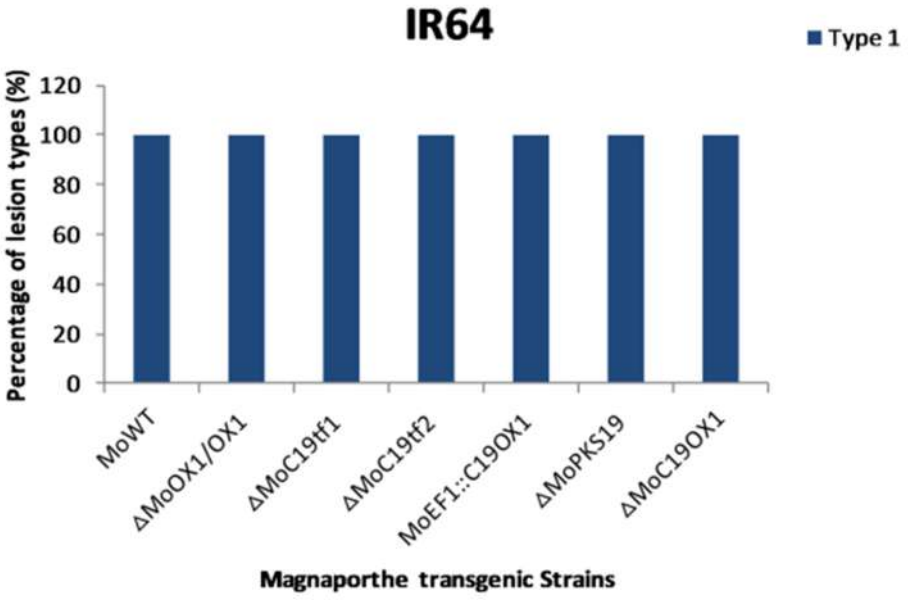
J


Co39
Type 2
Type 3
Type 4
Percentage of lesion types (%)
80
70
60
50
40
30
20
10
0
MoWT
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1
Magnaporthe transgenic Strains

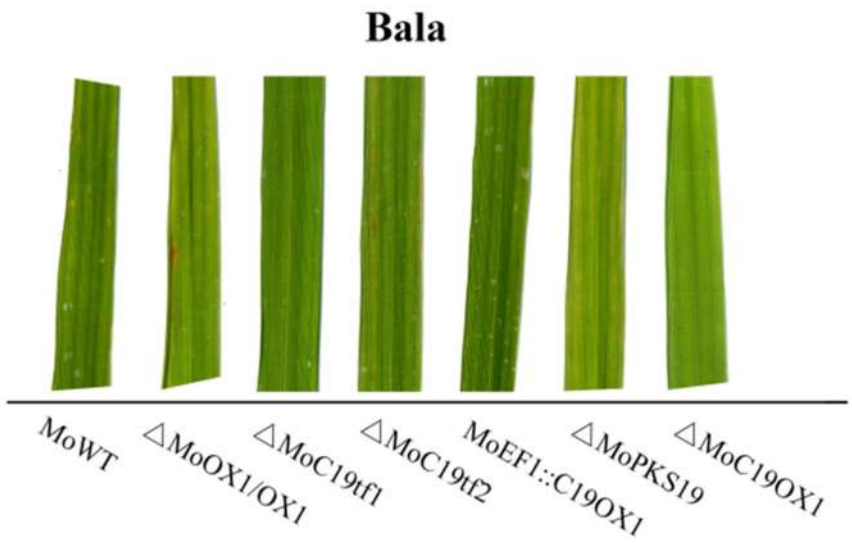
K


IR64
MoWT
△MoOX1/OX1
△MoC19tf1
△MoC19tf2
MoEF1::C19OX1
△MoPKS19
△MoC19OX1

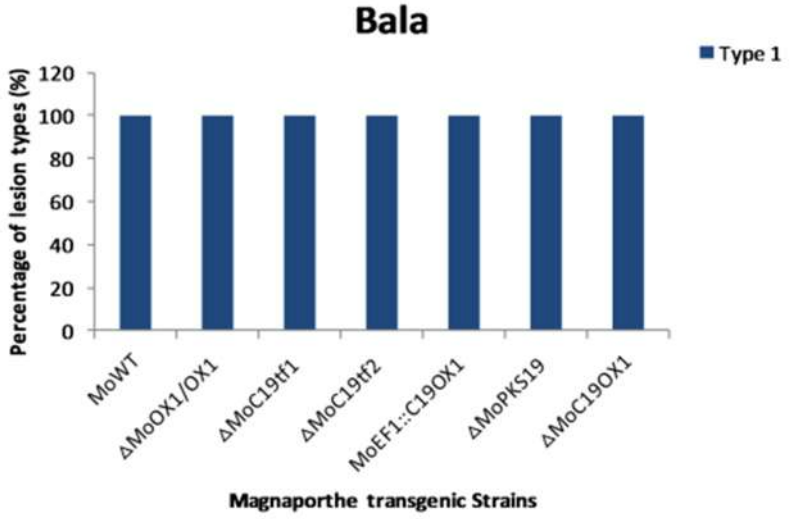
L


IR64
Type 1
Percentage of lesion types (%)
120
100
80
60
40
20
0
MoWT
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1
Magnaporthe transgenic Strains

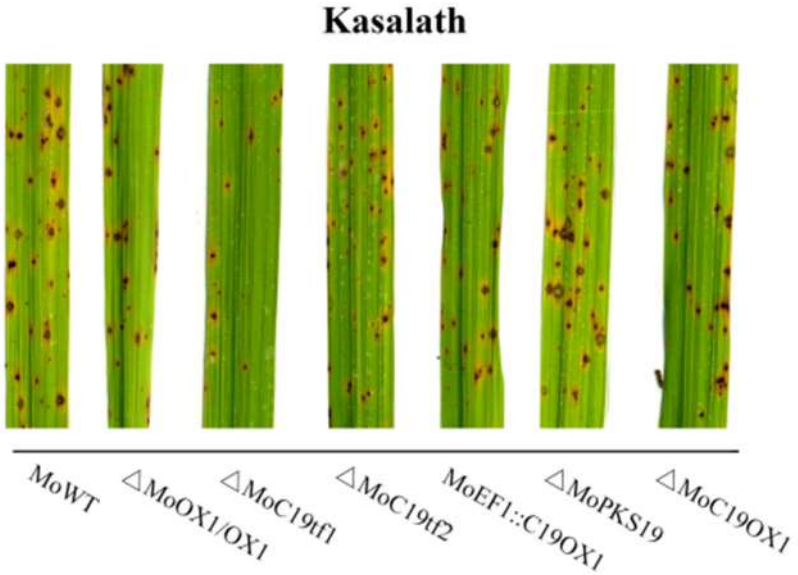
M


Bala
MoWT
△MoOX1/OX1
△MoC19tf1
△MoC19tf2
MoEF1::C19OX1
△MoPKS19
△MoC19OX1

N


Bala
Type 1
Percentage of lesion types (%)
120
100
80
60
40
20
0
MoWT
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1
Magnaporthe transgenic Strains

O


Kasalath
MoWT
△MoOX1/OX1
△MoC19tf1
△MoC19tf2
MoEF1::C19OX1
△MoPKS19
△MoC19OX1

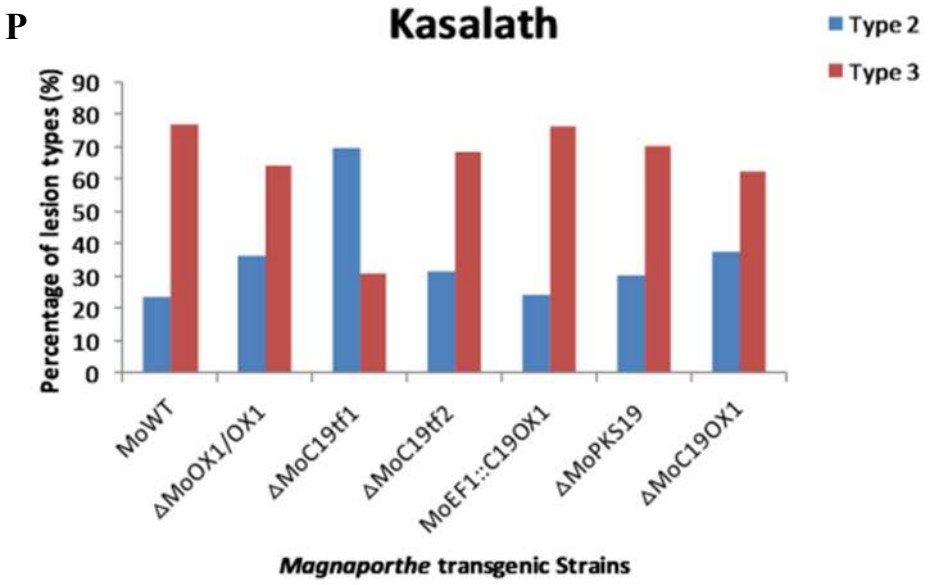


Fig. 16 Representative symptoms and quantification data produced by seven *Magnaporthe* transgenic strains on 8 rice varieties. A and **B:** Azucena; **C** and **D**: Nipponbare; **E** and **F**: Sariceltik; **G** and **H**: Maratelli; **I** and **J**: Co39; **K** and **L**: IR64; **M** and **N**: Bala; **O** and **P**: Kasalath.

3.6.1 Two selected rice varieties for symptom quantification and gene expression analysis

3.6.2.1 Symptom quantification on the rice varieties of Maratelli and Co39

Based on the results from the initial screen, two rice varieties (one from Japonica and one from Indica) were selected for detailed analysis to verify the observation. One variety from Japonica was Maratelli that showed difference in terms of the pathogenicity between some fungal genotypes, such as ΔMoC19tf1 and ΔMoC19tf2, as compared to MoWT. The other rice variety, belonging to Indica subspecies was Co39, and has been used in previous experiments (Jacob et al. 2016).

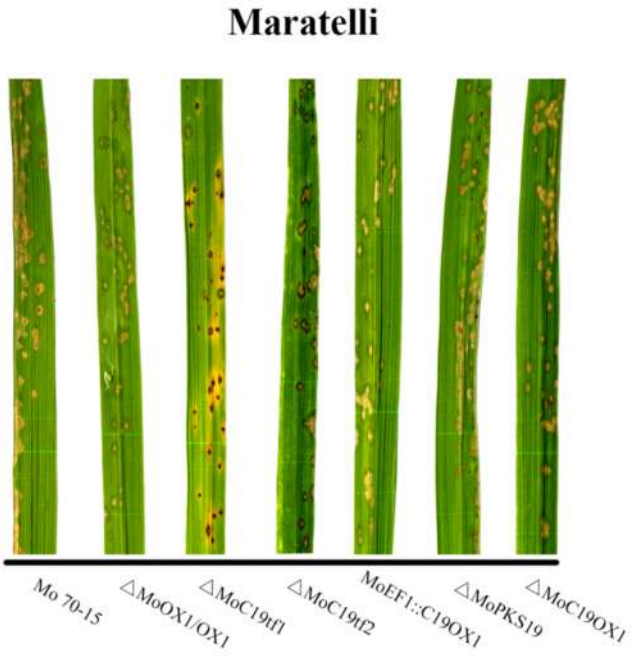
For Maratelli, two strains ΔMoC19tf1 and △MoC19tf2 showed reduced virulence

compared to the wild type strain MoWT and the complementation strain $MoC19OX_1/OX_1$. Specifically, strain ΔMoC19tf1 resulted in more "type 2" and "type 3" lesions, but less "type 6" lesions both in lesion number and lesion area per leaf area. Similarly, strain ΔMoC19tf2 produced more "type 5" lesions and less "type 6" lesions in terms of both lesion number and lesion area per leaf area. For strains other than ΔMoC19tf1 and ΔMoC19tf2, they produced no significant difference in formation of lesion number and lesion area per leaf area (Fig. 16B-C).

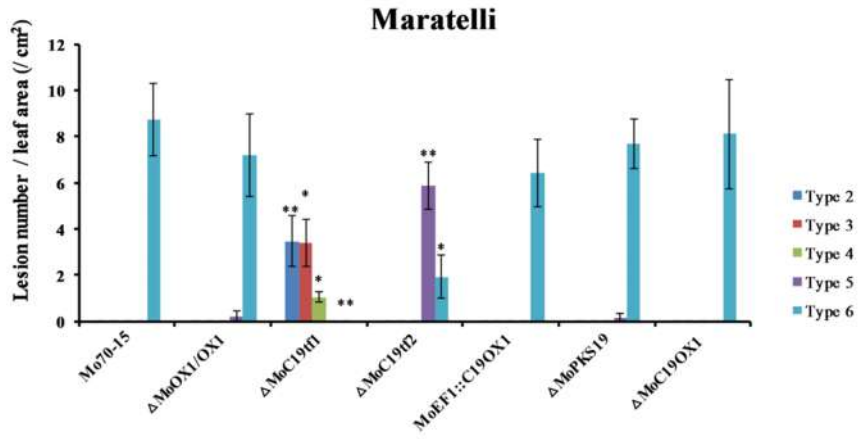
For Co39, only one strain ΔMoC19tf1 displayed reduced virulence compared to wild type strain MoWT, since more "type 2" but less "type 4" lesions were produced in both lesion number and lesion area per leaf area. For the other strains except ΔMoC19tf1, there was no significant difference in the pathogenicity as compared to the wild type strain MoWT (Fig. 16E-F).

To summarize, whether on Maratelli or on Co39, ΔMoC19tf1 was the most avirulent strain among all the fungal strains tested here. In addition, ΔMoC19tf2 was the second most avirulent strain following ΔMoC19tf1 when tested on the variety of Maratelli. Most importantly, except for ΔMoC19tf1 and ΔMoC19tf2, all other strains were demonstrated to have comparable virulence in induction of lesions.

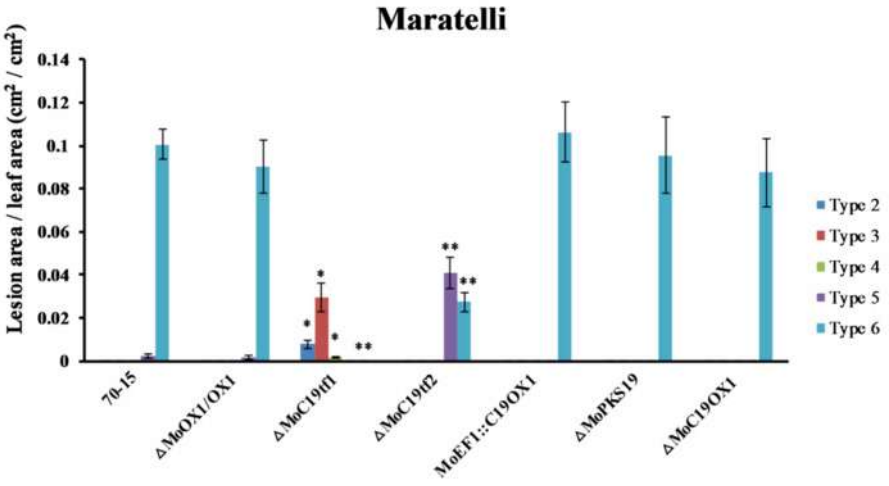
A



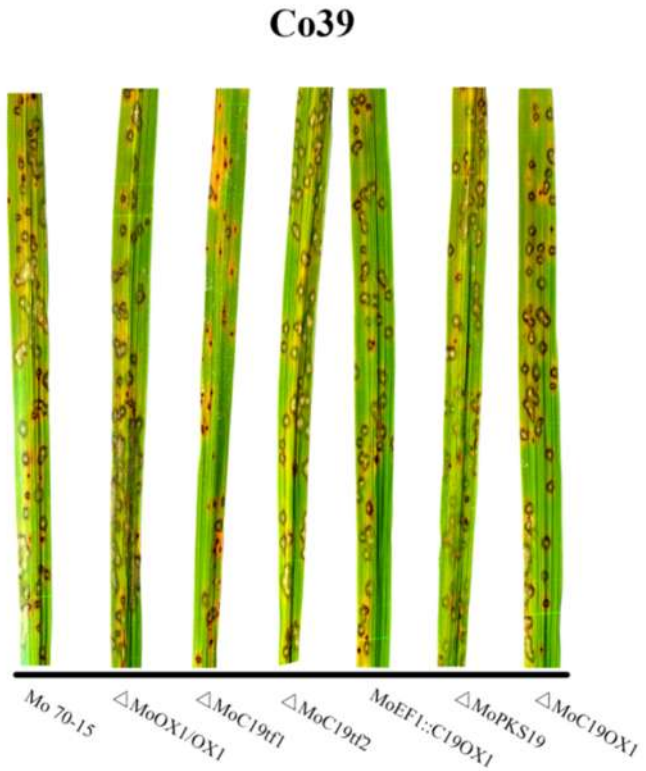
B


Maratelli
Lesion number / leaf area (/ cm²)
12
10
8
6
4
2
0
Type 2
Type 3
Type 4
Type 5
Type 6
Mo70-15
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1

C


Maratelli
Lesion area / leaf area (cm² / cm²)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Type 2
Type 3
Type 4
Type 5
Type 6
70-15
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1

D


Co39
Mo 70-15
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1

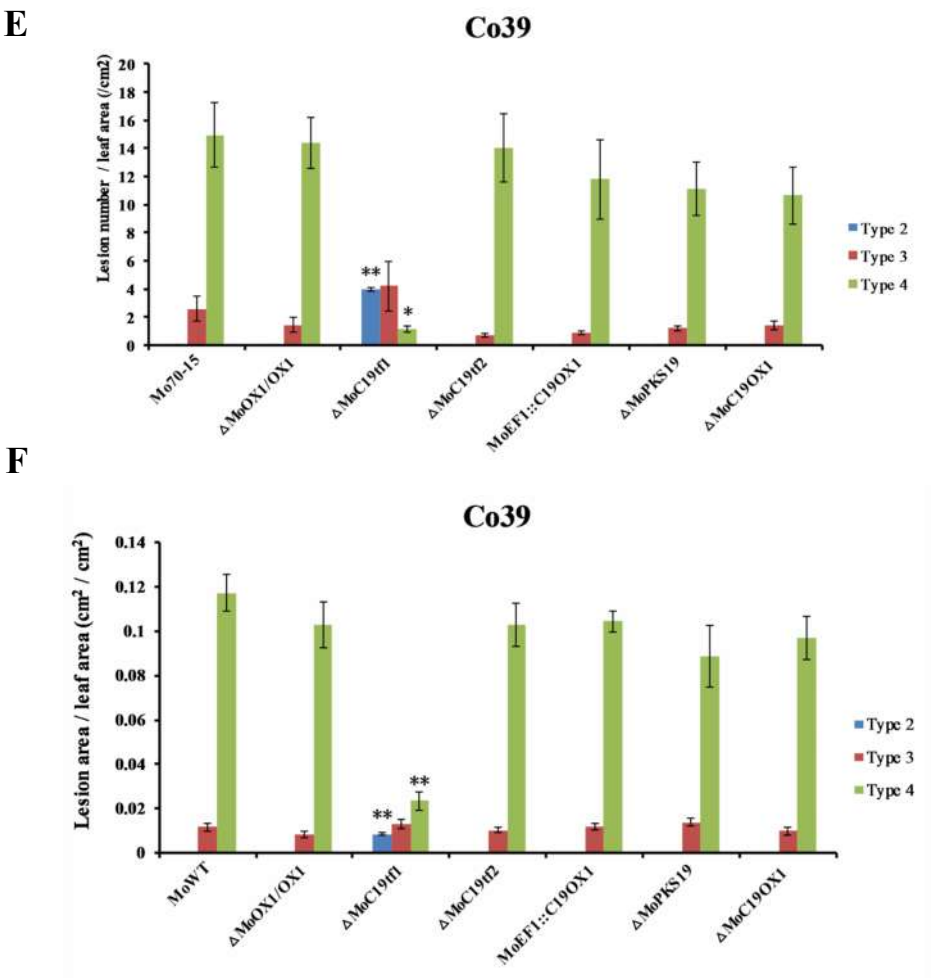


Fig. 16 Representative symptoms produced by seven *Magnaporthe* strains on Maratelli and Co39 Error bars represented standard error of three replicates. "*" and "**" denoted significant difference ($p < 0.05$, student's t-test, two tailed) and very significant difference ($p < 0.01$, student's t-test, two tailed) between transgenic strains and wild type strain MoWT.

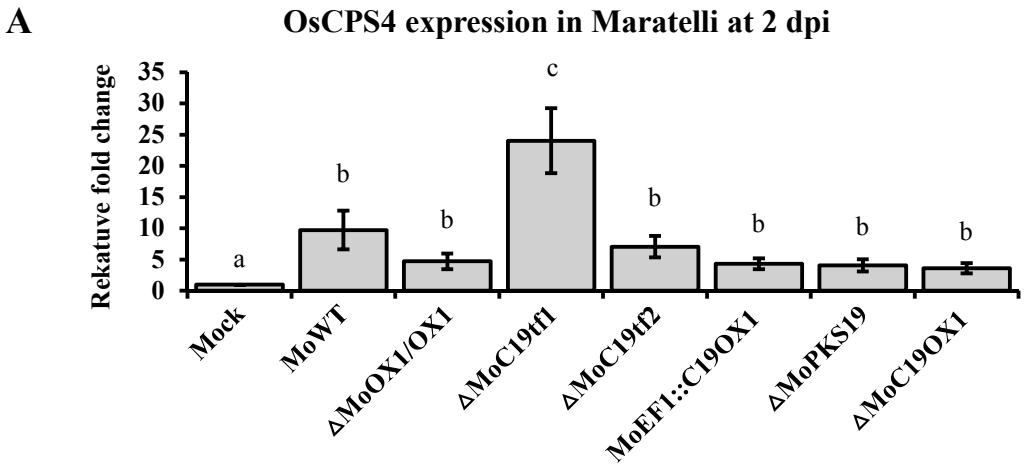
3.6.2.2 Defence gene expression analysis on the rice varieties of Maratelli and Co39

Now that the symptoms have been quantified, further examination of the defence gene expression would be needed to consolidate the results. The sample harvested from two different varieties (Maratelli and Co39) at 2 dpi was used for the gene expression analysis.

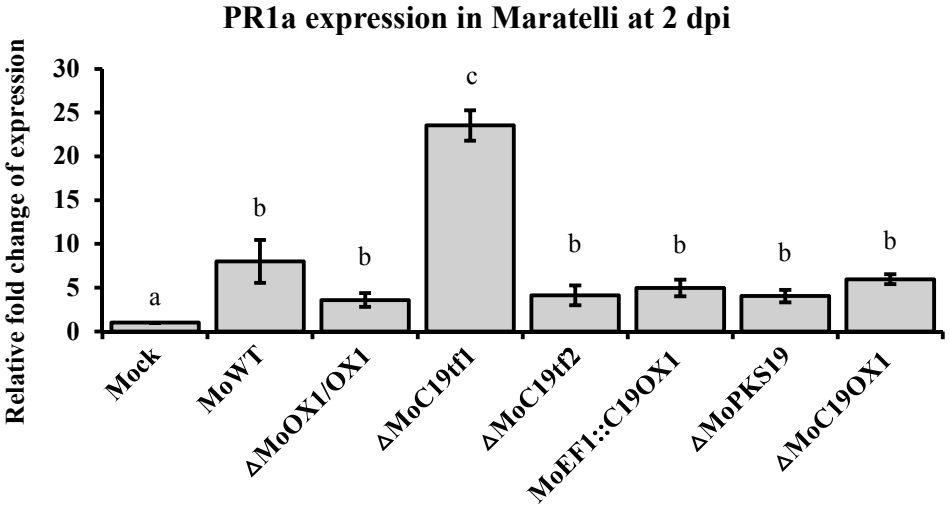
In the rice variety of Maratelli, the expression of *OsCPS4, OsPR1a* and *OsPBZ1* was induced by all genotypes of rice blast fungus tested here, but reached to the highest to be approximately 24 times, 23 times and 19 times under the infection of ΔMoC19tf1, respectively, as compared to that in mock treatment. There was no significant difference in the expression level of *OsCPS4, OsPR1a* and *OsPBZ1* infected by fungal genotypes other than ΔMoC19tf1 (Fig. 17A-C).

In the rice variety Co39, the gene expression of *OsCPS4, OsPR1a* and *OsPBZ1* accumulated to highest level (around 26, 5 and 7 times of that in mock treatment, respectively) under the infection of ΔMoC19tf1. This was followed by the second highest induction (approximately 10, 2.5 and 5 times relative to that in mock treatment, respectively) under the infection of the fungal genotype of ΔMoC19tf2. As for other fungal genotypes other than ΔMoC19tf1 and ΔMoC19tf2, they showed no significant difference in induction of *OsCPS4, OsPR1a* and *OsPBZ1* expression (Fig. 17D-F).

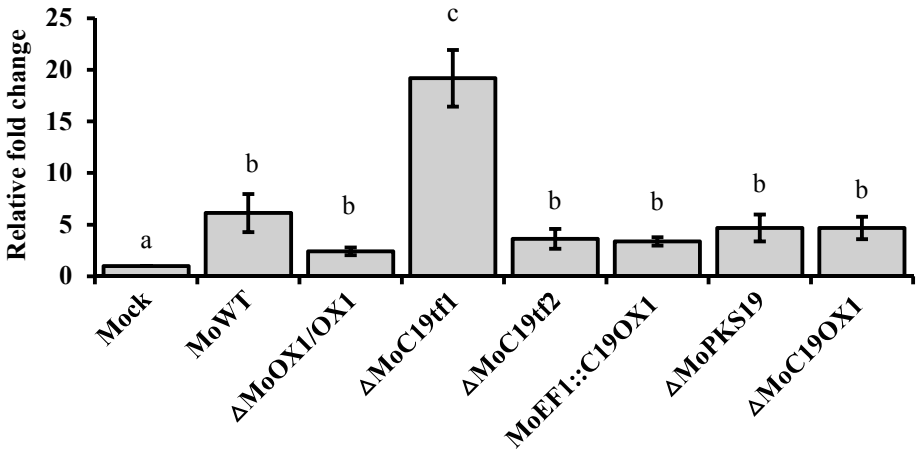
Taken together, no matter in rice variety of Maratelli or Co39, ΔMoC19tf1 was the most avirulent strain causing the highest induction of the defence genes such as *OsCPS4, OsPR1a* and *OsPBZ1*.



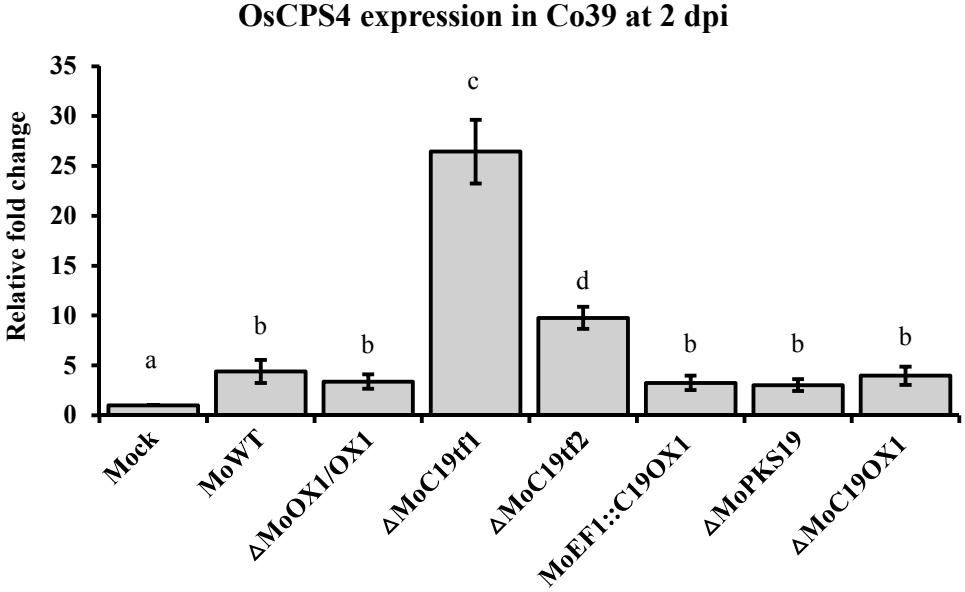
B


PR1a expression in Maratelli at 2 dpi
Relative fold change of expression
30
25
20
15
10
5
0
a
b
b
c
b
b
b
b
Mock
MoWT
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1

C


OsPBZ1 expression in Maratelli at 2 dpi
Relative fold change
25
20
15
10
5
0
a
b
b
c
b
b
b
b
Mock
MoWT
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1

D


OsCPS4 expression in Co39 at 2 dpi
Relative fold change
35
30
25
20
15
10
5
0
a
b
b
c
d
b
b
b
Mock
MoWT
ΔMoOX1/OX1
ΔMoC19tf1
ΔMoC19tf2
MoEF1::C19OX1
ΔMoPKS19
ΔMoC19OX1

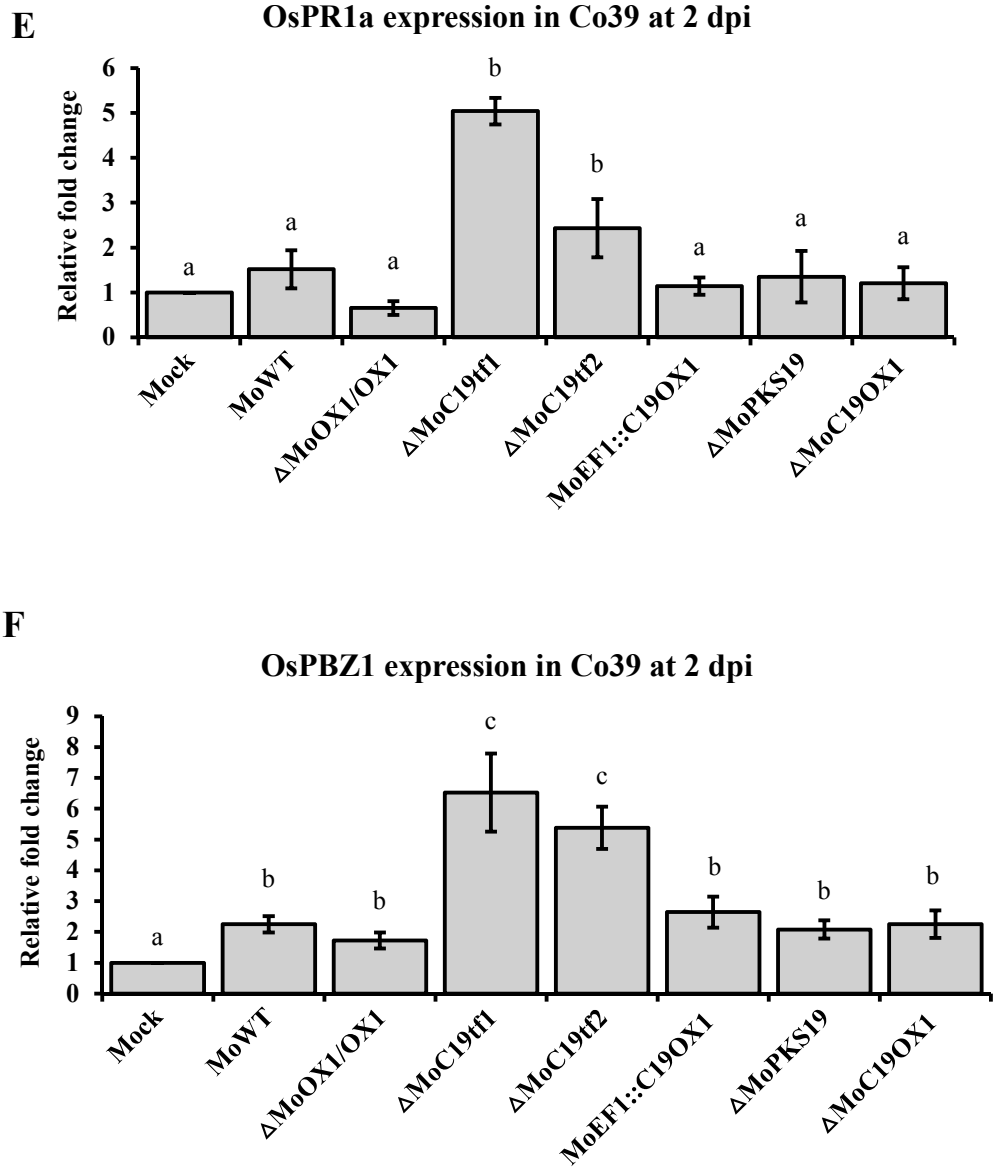


Fig. 17 Expression pattern of selected defence genes at 2 days post inoculation on Maratelli and Co39. Different letters indicated significant difference between treatments ($p < 0.05$, Fisher's LSD test). Error bars represented standard error of three replicates.

4 Discussion

In order to investigate the possible function of pyriculol in the infection process of rice blast fungus, isolated pyriculol compound (80% in purity) was used for *in vitro* assay by exogenous application and pyriculol-related transgenic fungal strains were also used in the infection *in planta*. In the first part, pyriculol induced necrotic ring in a light-dependent manner in the leaf segment assay and this necrosis could be mitigated by co-treatment with SA (a phytohormone with anti-oxidative role) and DPI (NADPH oxidases inhibitor to inhibit apoplastic ROS generation). It was postulated that light-dependent ROS generation might play a role in regulation of pyriculol-induced necrosis on the detached leaf. Yet, it is still not clear whether necrosis induced by pyriculol would lead to defence or susceptibility in the presense of pathogen infection. In the second part of this research, pyriculol was able to down-regulate the gene expression in JA biosynthesis and signaling pathway, but up-regulated SA dependent signaling gene expression. In addition, exogenous application of pyriculol could enhance rice host's resistance and defence genes expression in the presence of rice blast fungus infection. However, no significant difference in terms of the virulence in causing disease symptoms was found between pyriculol-overproducing, pyriculol-deletion and wild type fungal strains which were used to infect different rice varieties, indicating that pyriculol was not involved in the infection process. Taken all together, the possible function of pyriculol for rice blast fungus is discussed below.

4.1 Pyriculol-induced necrotic lesions are light-dependent

Pyriculol (0.64 mM) could induce necrotic lesions under light condition after 24 h of incubation on detached leaf, reaching around 90% in terms of the percentage of necrotic ring in the lesion (Fig. 5B), while under darkness this percentage was merely

about 4% after 3 days of incubation (Fig. 6B). The necrotic ring might indicate that hypersensitive response was triggered by pyriculol, since induction of necrotic lesions or localized cell death was one of the most important symptoms during hypersensitive response (HR) or programmed cell death (PCD). Yet, more evidence (such as DNA laddering etc.) would be needed to verify whether this induction of necrosis was truly because of PCD (Zurbriggen *et al.*, 2010). Necrosis is typically induced by excessive accumulation of reactive oxygen species (ROS) including singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (Baxter *et al.*, 2014). The formation of the ROS could take place in chloroplasts, mitochondria, apoplast and in peroxisomes (Shapiguzov *et al.*, 2012). The excessive accumulation of ROS could result in lipid peroxidation, protein oxidation, nucleic acid damage and eventually programmed cell death visualized as necrosis (Das and Roychoudhury, 2014). ROS generation in chloroplast is the result of photoreduction of oxygen to superoxide radical and this is associated with light-harvesting systems that are the main sources of ROS generation in chloroplast; therefore this process is dependent on the light reaction (Das and Roychoudhury, 2014). In this study, the difference of the percentage of necrotic ring in lesions induced by pyriculol between light condition and dark condition was probably due to the involvement of chloroplast-derived ROS production. It is very likely that other source of ROS (especially apoplastic ROS) plays a role in the necrosis formation by initiation of ROS signal, which is strongly amplified by the chloroplast under the light condition (Shapiguzov *et al.*, 2012). In comparison, a necrotic ring was also slightly induced in darkness, showing that other sources of ROS generation indepedent of light condition might also be involved in necrosis formation, such as ROS generation from mitochondria and apoplast. Even though mitochondria was identified as the dominant source of ROS generation in animal cells (Marchi *et al.*, 2012), the role of this organelle in ROS accumulation in plants was less important (Shapiguzov *et al.*, 2012). The ROS generation in mitochondria of plants occurs at the complex Ⅰ and complex Ⅲ in the electron

transfer chain, but at a relatively smaller scale (Noctor *et al.*, 2007). Other sources (e.g. apoplastic ROS and ROS in peroxisome) might also be involved in the necrosis induced pyriculol in the dark condition. The possible explanation for this is that without the light-activating chloroplast to amplify the ROS signal, there is only slight necrosis formation under dark condition. To sum up, necrotic lesion formation induced by light might be caused predominantly by chloroplast-derived ROS accumulation, possibly through amplification of the ROS signal in the apoplast. In contrast, the slight induction of necrotic ring in darkness might be caused by ROS from mitochondria, apoplast or peroxisome, but the necrosis produced in darkness is too subtle to further investigate.

Except ROS from chloroplasts and mitochondria in plants, ROS generated in the apoplast by plasma membrane-bound NADPH oxidase was one of the most studied ROS in plants, since NADPH oxidase-mediated ROS was one of the crucial signaling components for plants to cope with pathogen attack as well as abiotic stresses (Kwak *et al.*, 2003). This could be blocked by diphenyleneiodonium chloride (DPI), an inhibitor of NADPH oxidases, so it was possible to investigate if apoplastic ROS produced by NADPH oxidases was related to the necrosis induced by pyriculol (Li and Trush, 1998). In addition, SA was a stress-responsive phytohormone and was involved in maintaining the balance of oxidative burst caused by abiotic stress and pathogenic fungus attack (Yang *et al.*, 2004); therefore SA was thought to play a general anti-oxidative role in stress responses. In the next section, the effect of DPI and SA on necrosis formation induced by pyriculol under light condition will be focused.

4.1.1 Pyriculol induced necrotic lesions under light condition could be reduced by DPI and SA

Firstly, DPI could reduce pyriculol-induced necrotic ring percentage from around 90%

to 30% under the light condition (Fig. 5B). This suggested that ROS generation through plasma membrane-bound NADPH oxidases was also involved in induction of necrosis by pyriculol under light, since DPI was the inhibitor of NADPH oxidases that generated ROS via reduction of O_2 to O_2^- (Li and Trush, 1998). However, DPI alone also induced 30% necrotic ring (Fig. 5B), indicating DPI's role in promoting oxidative stress under this condition. This point has been reported in animal cells in which oxidative stresses were induced by DPI application (Riganti *et al.*, 2004).

Moreover, SA could strongly inhibit necrosis induced by pyriculol, suggesting that SA might play pivotal role in mediating oxidative stress caused by pyriculol. SA has been reported to be important in regulation oxidative stress in plants, both biotic and abiotic stress (Mostofa and Fujita, 2013; Yang *et al.*, 2004). SA could act both a pro-oxidant and an antioxidant and this biphasic redox dynamics is temporally controlled (Herrera-Vasquez *et al.*, 2015). On the one hand, SA can effectively inhibit catalase and cytosolic ascorbate peroxidase, two key enzymes in detoxification of H_2O_2 to H_2O and O_2 (Conrath *et al.*, 1995; Durner and Klessig, 1995). As the result of the inhibitory effect of SA on catalase and peroxidase, accumulation of H_2O_2 could result in oxidative stress to plants, which was also reflected in this study that SA alone could induce slight necrotic ring after 24 h of treatment. On the other hand, SA could also promote plants' ROS scavenging capability by enhancing GSH level and the ratio GSH/GSSG (the reducing power) (Mateo *et al.*, 2006). Many reports have published that the interaction of SA and GSH played important role in anti-oxidative responses, such as in cadmium stress (Guo *et al.*, 2007), salinity stress (Kim *et al.*, 2018), high light stress (Kusumi *et al.*, 2006), copper stress (Mostofa and Fujita, 2013) and rice blast fungus infection (Yang *et al.*, 2004). This was consistent with the results described in this study where SA could inhibit pyriculol-induced necrosis possibly due to SA's enhancement of plants' anti-oxidative ability.

Ascorbic acid (vitamin C) did not alleviate the necrosis percentage induced by pyriculol. On the contrary, ascorbic acid alone induced the necrotic area to roughly 30%. This might indicate that ascorbic acid did not reduce ROS generation induced by pyriculol, but slightly promoted oxidative stress in the absence of pyriculol treatment. Ascorbic acid was used as a common anti-oxidant both in mammals and plants. It can react with ROS to form mono-dehydroascorbate (MDHA) radical with loss of one electron and to form dehydroascorbate (DHA) with the loss of second electron (Smirnoff, 2018). These oxidized ascorbates are relatively unreactive and thus cause no damage to cells. However, due to electron donating property of ascorbate, it can also cause radical generation and therefore act as a pro-oxidant. This might explain that ascorbic acid only could induce necrosis under the light condition, possibly because ascorbic acid could promote hydroxyl production via the Fenton reaction in the presence of metal ions, such as Fe^{+3} and Cu^{2+} (Smirnoff, 2018). In addition, ascorbic acid (1 mM) did not reduce the necrotic area induced by pyriculol. This might indicate that the concentration (1 mM) used here was not enough to scavenge the ROS produced by pyriculol, since it was reported ascorbic acid should reach a high physiological concentration of 10 mM to effectively scavenge ROS *in vivo* (Jackson *et al.*, 1998) and 10 - 25 mM of ascorbic acid was measured in chloroplasts of spinach leaves (Foyer *et al.*, 1983).

JA (1 mM) alone did neither induce any necrotic lesions nor affected the necrosis induced by pyriculol, despite the fact that JA was positively related to the alleviation of oxidative stress induced by salt (Abouelsaad and Renault, 2018), ozone (Örvar *et al.*, 1997; Rao *et al.*, 2000) and drought (Wang, 1999; Bandurska *et al.*, 2003).

Altogether, DPI and SA could significantly alleviate the necrotic ring percentage triggered by pyriculol application under light condition. This highlighted the involvement of plasma membrane-localized NADPH oxidases in generation of ROS

in the apoplast and also the excessive accumulation of ROS in the chloroplast (light-dependent) to cause oxidative stresses. How did the apoplastic ROS signal relate to the ROS signal in chloroplast in the induction of necrosis? One possible explanation for this was that ROS in the apoplast via NADPH oxidases was the early signal induced by pyriculol treatment; subsequently the signal was relayed to the chloroplast via cytosolic signaling (e.g. Ca^{2+}); then the ROS signal was amplified in chloroplast and transmitted to nucleus to initiate cell death process and to affect gene expression (Shapiguzov *et al.*, 2012). In the following section, the expression of JA-related genes and was investigated.

4.2 Pyriculol and SA inhibited the gene expression of JA biosynthesis and signaling at early wounding stage

Pyriculol is a fungal polyketide isolated from rice blast fungus culture medium. It is structurally related to the phytohormone SA by sharing the salicylaldehyde group. In a preliminary assay, it was found to be able to inhibit JA pathway (data not shown). Also, SA was known to have the antagonistic relationship with JA pathway either by inhibiting JA biosynthesis or JA-dependent gene expression across a vast number of plant species (Thaler *et al.*, 2012). In this study, a comparative experiment was done using both SA and pyriculol in combination with wounding treatment, with the hypothesis that pyriculol might function as SA mimicry to inhibit the JA pathway. Wounding treatment can effectively activate JA-dependent pathways and JA has been reported to be required for the defence against rice blast fungus through the induction of PR genes or phytoalexins (Riemann *et al.*, 2013; Mei *et al.*, 2006). The strategy of mimicking one phytohormone to down-regulate the other important defence hormone was frequently utilised by pathogenic fungi to their best advantage (Chanclud *et al.*, 2016). The results showed that the transcript of JA biosynthesis genes, such as *OsAOS1, OsAOS2, OsAOC, OsOPR7* and *OsJAR1* were inhibited by both pyriculol and SA (Fig. 7A-E). Also, the genes, *OsJAZ8, OsJAZ9, OsJAZ11* and *OsJAZ13* that

play roles in JA signaling were repressed (Fig. 8A-D). Even though there are no reports about pyriculol's inhibitory effect on JA, the repression of JA biosynthesis genes by exogenous application of SA was frequently reported in other plant species, such as in tomato, flax and *Arabidopsis*. For example, SA analog aspirin was found to repress wound-induced jasmonic acid accumulation in tomato by targeting *AOS* gene expression in tomato detached leaves (Pena-Cortés *et al.*, 1993). This was further consolidated by a later report in which wound-induced *AOS* mRNA accumulation was inhibited by SA or aspirin in flax leaves, but surprisingly, the enzyme activity of *AOS* was not affected by either salicylic acid or aspirin (Harms *et al.*, 1998). Even though the repression of JA biosynthesis gene expression such as *LOX2, AOS, AOC2* and *OPR3* by SA was also described in *Arabidopsis*, it was revealed that the real target of inhibition by SA was downstream of JA biosynthesis pathway and this was confirmed by deploying a *AOS* deletion mutant *aos/dde2* in which SA-directed suppression of JA-induced *PDF1.2* was not affected as compared to wild type Col-0 plants (Leon-Reyes *et al.*, 2010). Downstream of JA biosynthesis, degradation of JAZ repressor proteins could be targeted by SA. If JAZ proteins were stabilised by SA, fewer JAZ proteins would be degraded and fewer transcripts of *JAZs* would be newly synthesised. Such mechanism of enhancing the stability of repressor proteins was found in the crosstalk between SA and auxin, where SA could increase the stability of AUX-IAA (the negative regulator of auxin signaling) to inhibit auxin dependent response (Wang *et al.*, 2007). However, the stability of JAZ proteins in the crosstalk of SA and JA was not affected in *Arabidopsis* and it was the GCC promoter motif of JA-dependent transcription factor ORA59 (downstream of SCF^{COI1}-JAZ complex) that was targeted by SA to suppress JA (Van der Does *et al.*, 2013). In other words, JA-dependent transcription factor accumulation such as ORA59 could be strongly inhibited by SA and thus inhibiting JA dependent responses. The explanation of SA's down-regulation of JA biosynthesis and JAZ genes in this study might be positive feedback loop in which repression of JA responsive transcription factors caused

repression of upstream biosynthesis and subsequent JAZ gene expression. The positive feedback loop in regulating JA biosynthesis in plants was frequently reviewed (Browse, 2009; Wasternack and Hause, 2013).

Taken together, both pyriculol and SA could suppress JA-related gene expression, but the mechanism of how this was regulated was still unraveled in rice. Pyriculol showed similar pattern as SA in terms of the repressive effect on JA's biosynthesis and signaling genes, suggesting that pyriculol might act as SA mimicry to suppress JA-dependent gene expression. This provoked us to explore how pyriculol could affect SA-responsive genes.

4.3 Pyriculol induced the expression of SA responsive genes

The gene *OsWRKY45* was found to be strongly activated by SA, wounding and by pyriculol in a relatively milder manner (Fig. 9A). It has been reported that *OsWRKY45* was the key regulator of benzothiadiazole (BTH) induced rice blast disease resistance which was dependent on SA-OsWRKY45 pathway, but independent of SA-NPR1 pathway (Shimono *et al.*, 2007b; Nakayama *et al.*, 2013). It was plausible to observe that SA could strongly induce *OsWRKY45* expression, while pyriculol might be a less potent SA analog or might function upstream of SA to take more time than SA in induction of *OsWRKY45* gene expression. In addition, *OsWRKY45* was also the positive regulator between SA and JA pathway and JA exogenous application could also up-regulate *OsWRKY45* expression in rice (Mutuku *et al.*, 2015; Tamaoki *et al.*, 2013). This suggested that SA and JA had synergistic action in regulation of *OsWRKY45* and also explained the fact that wounding (JA pathway) could also effectively induce *OsWRKY45* gene expression and that SA could enhance wounding-induced *OsWRKY45*.

Salicylic acid glucosyltransferase (*OsSGT1*) was shown to be strongly induced by SA,

pyriuclol and wounding (Fig. 9B). *OsSGT* is the enzyme catalyzing salicylic acid into SA O-beta-glucoside (SAG) that acts as the storage form of salicylic acid in the vacuole (Dempsey *et al.*, 2011). The accumulation of SAG could be induced by salicylic acid in rice (Silverman *et al.*, 1995), by mechanical wounding in tobacco (Seto *et al.*, 2011), by tobacco mosaic virus in tobacco leaves (Enyedi and Raskin, 1993), by bacterial pathogen *Pseudomonas syringae* in *Arabidopsis thaliana* (Song, 2006) and by SA functional analogs, such as probenazole (PBZ) or 2, 6-dichloroisonicotinic acid in rice (Umemura *et al.*, 2009). This was consistent with the observation that SA and wounding could induce *OsSGT1* up-regulation in rice. Pyriculol's induction of *OsSGT1* expression further confirmed that pyriculol could activate SA-responsive genes. Moreover, both SA and pyriculol could enhance wounding-induced *OsSGT1* expression, indicating the synergism between SA and wounding (JA pathway), or pyriculol and wounding, in *OsSGT1* accumulation. One explanation for wounding-induced *SGT* expression was that wounding induced JA accumulation and one of the JA metabolites, hydroxyjasmonic acid could be glycosylated by *NtSGT* in tomato (Seto *et al.*, 2011).

By contrast, pyriculol-induced necrosis could be reduced by application of SA and this appeared to be contradictory with the finding that pyriculol activated SA-responsive genes. However, this could be explained by the function of SA as a pro-oxidant and an anti-oxidant. How could this be possible? On the one hand, SA could have the pro-oxidant role by promoting ROS accumulation at the early stage, since SA could mediate the inhibition of catalase and ascorbate peroxidase (two enzymes in decomposition of H_2O_2 to H_2O and O_2) (Chen *et al.*, 1993; Durner and Klessig, 1995). The early ROS accumulation by SA treatment could act as the signal for induction of SA responsive gene expression (e.g. *OsWRKY45* and *OsSGT1*) and later defence gene expression (*OsCPS2* and *OsCPS4* etc.). On the other hand, SA could also function as anti-oxidant. This was due to the the fact that SA could enhance

glutathione level and also the reducing power calculated by the ratio of GSH/GSSG (Mateo *et al.*, 2006). Thus, the anti-oxidant role of SA could explain that pyriculol-induced necrosis was inhibited by SA treatment.

Taken together, pyriculol could induce the expression of *OsWRKY45* and *OsSGT1* that functioned in the SA pathway. Also, the activation of these genes was frequently the early marker for chemically induced plant defence and was accompanied by induction of PRs at later stage (Shimono *et al.*, 2012; Hennig *et al.*, 1993; Song, 2006). Know-down of *OsWRKY45* and *OsSGT1* resulted in susceptibility to rice blast fungus attack and overexpression of *OsWRKY45* triggered strong defence responses in rice against rice blast fungus infection (Shimono *et al.*, 2012; Umemura *et al.*, 2009). Thus, it could be postulated that induction of *OsWRKY45* and *OsSGT1* by pyriculol and SA could lead to plant defence gene expression in rice.

4.4 Pyriculol and SA could enhance wounding-induced defence gene expression at late wounding stage

Firstly, both pyriculol and SA could induce and enhance wounding-induced defence-related genes, such as *OsCPS2* and *OsCPS4*. These two genes encoded the enzymes to form the precursors of diterpenoid phytoalexins, such as phytocassanes and momilactones, in rice (Miyamoto *et al.*, 2014). The induction of these two genes by pyriculol, SA and wounding could indicate that diterpenoid phytoalexin biosynthesis was activated in the SA and JA pathway. This was consistent with the reports that SA and JA were critical regulators in induction of diterpenoid phytoalexins in rice (Akagi *et al.*, 2014; Daw *et al.*, 2008; Rakwal *et al.*, 2014). However, it was revealed that JA was dispensable for the production of diterpenoid phytoalexins in rice (Riemann *et al.*, 2013; Miyamoto *et al.*, 2016). It was possible that the diterpenoid phytoalexin production was dependent on SA pathway, but this

required SA mutant-approach (such as NahG mutant which overexpresses bacterial salicylate hydroxylase to abolish SA accumulation) to verify. In addition, Pyriculol and SA could also enhance wounding-induced *OsCPS2* and *OsCPS4* expression, suggesting that SA or pyriculol act synergistically with JA in induction of diterpenoid phytoalexin biosynthesis gene expression.

Secondly, SA, pyriculol and wounding also induced *OsPR1a, OsPR1b* and *OsPBZ1* gene expression. Among these, *OsPR1a* and *OsPR1b* were in the category of PR1 proteins which were characterized to possess antifungal activities (Niderman *et al.*, 1995; Mitsuhara *et al.*, 2008), while *OsPBZ1* was in the category of PR10 protein family having ribonuclease-like activity (Huang *et al.*, 2016). The induction of these genes under wounding could also be enhanced by SA and pyriculol, indicating that these genes were synergistically induced in the JA and SA pathway.

SA and JA synergism in induction of defence genes in rice was not uncommon. More than half of transcriptome induced by JA was enhanced by SA functional analog BTH treatment in rice (Tamaoki *et al.*, 2013) and more than 65% of the genes were commonly regulated by SA and JA in rice (Garg *et al.*, 2012). This synergism between SA and JA was also described in other species, such as *Arabidopsis* and *Ginkgo biloba* (Liu *et al.*, 2016; Xu *et al.*, 2009). It was suggested that output of synergism or antagonism between SA and JA was concentration-dependent (Mur *et al.*, 2006).

However, in this study, antagonistic relationship between SA/pyriuclol and wounding at the early stage (0.5 h and 1 h) of wounding treatment was observed; however, the synergistic relationship between SA/pyriculol and wounding was found at the late stage of wounding treatment (24 h). This might indicate that synergism or antagonism between SA and JA was time-dependent. This might be associated with SA's biphasic redox dynamics in regulation of ROS accumulation in plants, which acts as signaling

component to affect other pathways (such as JA pathway) (Herrera-Vasquez *et al.*, 2015). This type of time-dependent antagonism and synergism was consistent with the previous reports in which SA and wounding-induced JA showed antagonism at the early wounding treatment, but not at 23 h after the wounding (Lee *et al.*, 2004).

4.5 Pyriculol could enhance rice defence in the presence of rice blast fungus infection

Before the inoculation using pyriculol or pyriculariol (the structural isomer of pyriculol) solution in combination with rice blast fungus spore suspension, the germination rate of spores under the treatment of different concentrations of pyriculariol was examined. Pyriculariol (at concentration of 0.64 mM) could completely inhibit spore germination instead of its solvent control (1% methanol), while pyriculol and pyriculariol did not inhibit spore germination at concentration below 160 μM (Fig. 11C-D). This was not first time reporting about pyriculol or pyriculariol's self-inhibiting property. The isolated compounds including pyriculol, epipyriculol and tenuazonic acid from the agar culture of *P. oryzae* could also inhibit its own spore germination (Kono *et al.*, 1991). In addition, there were other many pathogenic fungi that produced auto-inhibitors to prevent germination until the spores were dispersed or diluted out (Thines *et al.*, 2004). Why was pyriculol produced by the fungus to inhibit its own spore germination? One possible explanation is that pyriculol was produced from the hyphae in the liquid culture with no spores formed; therefore rice blast fungus does not care about any inhibitory effect on the spore germination in that case. The other possibility is that pyriculol is produced to repel other fungal competitors living in the same micro-environment. For this point, antimicrobial activity of pyriculol or pyriculariol against other fungi or other bacteria could be assessed in future.

What would happen if pyriculol or pyriculariol was artificially applied together with

spore suspension to infect rice plants? In terms of the symptoms, both pyriculol and pyriculariol could reduce the number and area of susceptible lesions. In other words, both of them could contribute to defence responses against rice blast fungus. For the defence gene expression, the results were consistent with the symptom quantification, namely pyriculol and pyriculariol could enhance defence gene expression induced by rice blast fungus infection. It was not surprising to observe this point, since pyriculol was confirmed to be able to induce SA pathway that was critical for rice blast fungus defence (Yang *et al.*, 2004; Jiang *et al.*, 2010). Even though pyriculol could inhibit JA biosynthesis and signaling during the early wounding responses, pyriculol and SA was found to synergize with JA in induction of defence response genes. This was also consistent with results in which SA and JA shared more than half of the defence system in rice, except that small part of signaling was antagonistic (Tamaoki *et al.*, 2013). Below is the model showing the possible mechanism of exogenous application of pyriculol and SA in repression of JA biosynthesis and signaling but enhancement of rice blast fungus defence (Fig. 18).

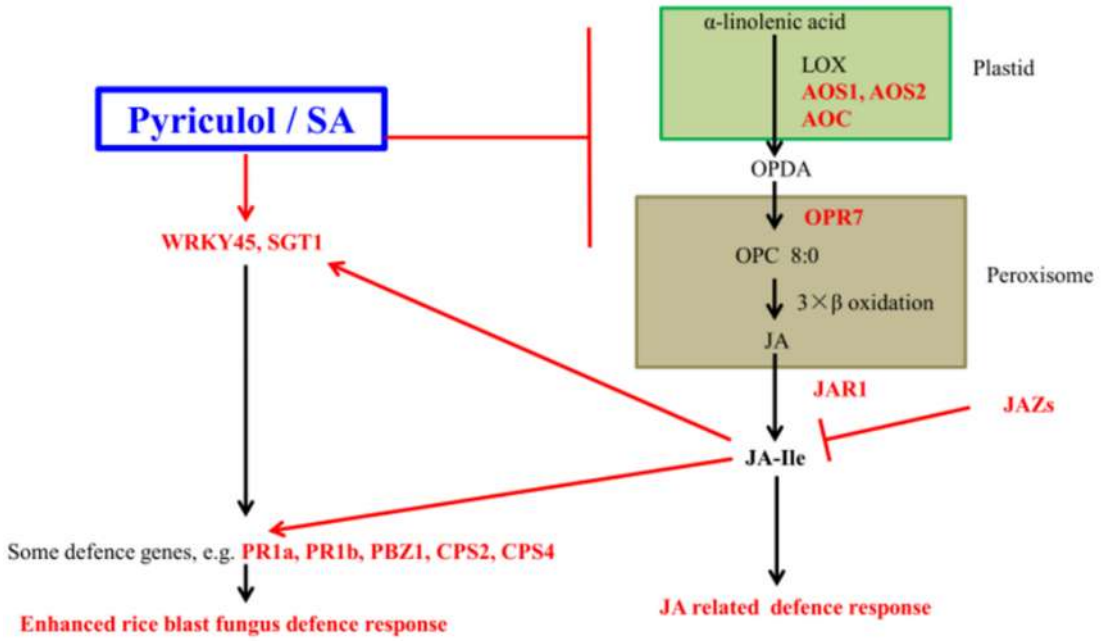


Fig. 18 The model summarising the mechanism of exogenous application of pyriculol and SA in suppression of JA biosynthesis and signaling, but improvement of rice blast fungus

defence. Wounding response induces JA biosynthesis that starts from clevage of α-linolenic acid from chloroplast membrane. Subsequently, α-linoleic acid is converted to be OPDA via three steps of enzymatic reaction by the enzymes LOX, AOS1 or AOS2, and AOC. OPDA is then reduced by the enzyme OPR7 to be OPC, followed by three steps of β oxidation to become JA. It needs be to activated by conjugation to the amino acid isoleucine to be JA-Ile. In the presence of JA-Ile, the repressors of JA signaling are degraded via 26S proteasome pathway. In contrast, Pyriculol and SA inhibits JA biosynthesis genes (such as *OsAOS1*, *OsAOS2*, *OsAOC* and *OsJAR1*) and also supresses JA signaling genes (JAZs) at the early wounding stage (0.5 h and 1 h after the wounding treatment), indicating an antagonistic relationship between pyriculol (or SA) and JA. However, at the later wounding stage (24 h after the wounding treatment), pyriculol and SA function synergistically with JA (wounding response) in induction of some defense-related genes (such as *OsSGT1, OsWKRY45, OsPR1a, OsPR1b, OsPBZ1, OsCPS2 and OsCPS4*), thereby triggering enhanced defence against rice blast fungus infection.s

4.6 Pyriculol was not involved in the infection process of rice blast fungus

As mentioned above, pyriculol might be manipulated by the fungus as SA mimicry to suppress JA pathway, thereby causing susceptibility to pathogen infection, since JA also plays important role rice blast fungus defence (Riemann *et al.*, 2013; Shimizu *et al.*, 2013; Wakuta *et al.*, 2011; Mei *et al.*, 2006). To test this possibility, pyriculol-related transgenic strains of rice blast fungus were used to infect 3 weeks old rice plants. In the initial screen of appropriate rice varieties, 8 rice varieties (4 Japonica and 4 Indica) were used. Unfortunately, none of the rice varieties showed significant difference in terms of dominant lesion type caused by infection of wild type strain MoWT, complementation strain MoC19OX1/OX1, deletion strains ΔMoPKS19 and ΔMoC19OX1, and over-expressor MoEF1::C19OX1 (Fig. 16A-P). This indicated that pyriculol was not involved in the infection process. However, there were some interesting points that two deletion mutants ΔMoC19tf1 and ΔMoC19tf2

showed reduced virulence as compared to the wild type strain on some rice varieties. ΔMoC19tf1 and ΔMoC19tf2 were both the knockout strains of the negative transcription factors regulating MoPKS19 enzyme (the key enzyme in pyriculol and pyriculariol biosynthesis) and had more accumulation of MoPKS19 in the absence of the negative regulators (Jacob *et al.*, 2017).

In the later symptom quantification on two selected rice varieties (Co39 and Maratelli), the results showed consistency with the previous screening results. For the expression analysis of the defence genes, wild type strain MoWT, complementation strain MoC19OX1/OX1, deletion strains ΔMoPKS19 and ΔMoC19OX1, and over-expressor MoEF1::C19OX1 showed no difference in induction of defence genes, such as *OsCPS4, OsPR1a* and *OsPBZ1*, suggesting that pyriculol was not required for the infection. Pyriculol was detected in the liquid culture of rice blast fungus, but it has not been detected in the infected plants. Also, identification of these transgenic strains at the level of pyriculol production was based on their growth in the liquid shaking culture. Thus, the explanation might be that pyriculol is only induced under some specific condition (e.g. rice extract medium), but not induced in infected plants (Jacob *et al.*, 2017). If pyriculol could not be induced in infected plants at any stage (the early biotrophic stage and the late necrotrophic stage) by the transgenic strains, MoWT, MoC19OX1/OX1, ΔMoPKS19, ΔMoC19OX1, and MoEF1::C19OX1 would be the same in the pathogenicity. Therefore, evidence of whether pyriculol was produced in infected plants would be required to conclude this point.

However, ΔMoC19tf1 was always inducing the highest defence gene expression on both rice varieties, indicating this strain was the most avirulent strains of all. Deletion of one negative transcription factor of MoPKS19 could in theory result in higher level of pyriculol production in the liquid culture. Based on above-mentioned explanation that pyriculol might not be induced at all in infected plants, ΔMoC19tf1 should have

been the same in pathogenicity as compared to the wild type strain MoWT. However, ΔMoC19tf1 was reduced in pathogenicity across almost all varieties, indicating that this transcription factor MoC19tf1 might have other important role in virulence independent of rice varieties. Given that polyketide synthases (PKSs) are quite abundant in the genome of rice blast fungus (Jacob *et al.*, 2017), MoC19tf1 might also regulate other PKSs which are potential effector proteins or enzymes catalyzing .the production of other virulent polyketides.

In terms of ΔMoC19tf2, it also showed slightly reduced virulence on the variety Maratelli by reducing the dominant lesion type from type 6 to type 5 as compared to the wild type strain. However, in the analysis of the defence genes, ΔMoC19tf2 induced the stronger defence gene expression when compared to that induced by wild type strain in Co39 instead of Maratelli. This inconsistency between symptoms and gene expression analysis suggested the following possibilities. Firstly, MoC19tf2 might be also a regulator playing a role in increasing virulence of rice blast fungus, but less prominent as MoC19tf1 to be shown on Co39 in the symptom quantification. Secondly, MoC19tf2 contributed to slight defence response in Maratelli from type 6 to type 5 (still in the category of susceptible lesions) and this slight increase in defence might take place at later infection stage (e.g. 4 dpi), but not enough to be visualized at 2 dpi in gene expression.

4.7 Conclusion

In order to examine the possible function of pyriculol in the infection process of rice blast fungus, purified pyriculol was assayed on detached leaf segments to find out how pyriculol-induced necrosis was regulated in the light and dark condition. Moreover, a comparative analysis of pyriculol and SA was conducted to investigate how pyriculol could affect JA and SA dependent gene expression and also how SA crosstalks with JA. This further gave rise to the question whether exogenous application of pyriculol together with rice blast fungus spores could augment the rice host's defence or susceptibility. At last, pyriculol's function in rice blast fungus infection was determined by the infection assay using different pyriculol-related transgenic strains of rice blast fungus. The conclusion was the following:

1. Pyriculol induced necrosis was light dependent. The necrosis induced by pyriculol in light condition could be inhibited by SA and DPI treatment, suggesting that pyriculol induced necrosis might require light to amplify the signal transmitted from NADPH oxidase (plasma membrane-localised) producing ROS.

2. Both pyriculol and SA could inhibit JA biosynthesis and signaling genes, but could promote SA dependent genes, indicating that pyriculol might function as SA mimicry to repress JA responsive gene expression.

3. The crosstalk between SA and JA was antagonistic in the early wounding stage in terms of SA's repression of JA biosynthesis and signaling gene expression and synergistic in the late wounding stage in output of some defence gene expression.

4. Exogenous application of pyriclol and pyriculariol enhanced rice blast fungus resistance and the expression of defence genes in the infection test, revealing that

pyriculol conferred resistance to the host plant when applied artificially.. However, no significant difference in the symptoms and defence gene expression between wild type strain, pyriculol-overexpressing strain and pyriculol-abolished strain were found in all the 8 rice varieties (4 Japonica and 4 Indica), demonstrating that pyriculol was not involved in the rice blast fungus infection process.

4.8 Outlook

In the results, pyriculol was shown to be able to induce necrotic lesions, which was strongly induced by light condition. This pyriculol-induced necrotic lesion was reminiscent of the necrosis occurring in hypersensitive response, namely programmed cell death (PCD). Could pyriuclol induced PCD? To confirm this, some events such as chromosomal DNA fragmentation, could be used as marker of PCD. If pyriculol could be confirmed to induce PCD, there should also be a corresponding R protein in the resistant rice varieties. The reason why pyriculol is not found in the infected plants was possibly that rice blast fungus did not want to be recognized by the rice host and therefore silenced pyriculol production during the infection process.

Pyriculol was able to inhibit JA biosynthesis and signaling as effectively as SA did and also induced SA signaling genes. Thus, pyriculol was assumed as a SA functional analog dependent on SA pathway. However, this could not be completely sure, if no SA signaling inhibitor (such as PAMD) or SA-deficient transgenic mutant (such as NahG overexpressing bacterial salicylate hydroxylase) was used to dissect this signaling (Jiang *et al.*, 2015; Yang *et al.*, 2004). Also, this could solve the question of whether pyriculol functioned upstream or downstream of SA pathway.

Moreover, it is still not clear whether pyriculol targeted JA biosynthesis genes or signaling genes (JAZs or JA responsive transcription factors). Quantification of JA at hormonal level would be needed to further confirm pyriucol's inhibitory effect on JA. It was known that SA could target JA responsive factor ORA59 to inhibit JA dependent gene expression in *Arabidopsis*, while in rice *OsNPR1* and *OsWRKY13* were reported as the key regulators between SA and JA antagonism (Li *et al.*, 2013; Van der Does *et al.*, 2013; Qiu *et al.*, 2007a). For future research, using the deletion mutant of *OsNPR1* or *OsWRKY13* would be a good approach to make this point clear.

Even though pyriculol was not involved in the infection process, deletion mutant of MoC19tf1 showed less virulence almost across all rice varieties tested. This transcription regulator MoC19tf1 probably also had other targets in the polyketide synthases (except MoPKS19) which might play important role avirulence or virulence in the infection process. It would be worth doing a screening of the transcript level of all polyketide synthases in the infected plants to identify which polyketide synthase was critical for the infection.

Exogenous application of pyriculol could enhance plant defence and also it could inhibit spore germination of rice blast fungus at the proper concentration. The question is that does pyriculol contribute to a broad spectrum of plant defence against bacterial pathogens or herbivores except for rice blast fungus? Does pyriculol have general antimicrobial activity toward to other fungi or bacteria?

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